Medicinal Chemistry of Anticancer Drugs

Medicinal Chemistry of Anticancer Drugs

Second Edition

By

Carmen Avendaño and J. Carlos Menéndez
Departamento de Química Orgánica y Farmacéutica,
Facultad de Farmacia,
Universidad Complutense,
Madrid, Spain

AMSTERDAM • BOSTON • HEIDELBERG • LONDON • NEW YORK • OXFORD
PARIS • SAN DIEGO • SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO

Elsevier
Radarweg 29, PO Box 211, 1000 AE Amsterdam, Netherlands
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK
225 Wyman Street, Waltham, MA 02451, USA

Notices
Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-444-62649-3

Foreword

Cancer can be considered a general term that covers over 200 malignancies different in their genetic basis, etiology, patterns of progression, and final clinical outcome. These pathogenic conditions are characterized by uncontrolled cellular proliferation and growth and, under some physiological conditions, spread to adjacent or remote organs and tissues.

We know now that cancer is a multifactorial disease, in which both environmental and genetic factors can transform normal cells into cancerous ones by altering the normal function of a wide spectrum of biological networks. The complexity of the underlying mechanisms of the biology of human cancer, in particular the multiple mutations that occur in oncogenes, tumor suppressor, or DNA repair genes, represents a major challenge in the identification and development of effective, durable, and safe cancer therapies.

The 15 chapters of "Medicinal Chemistry of Anticancer Drugs" provide a comprehensive overview of the different synthetic and biological approaches that have been attempted to interfere with cancer progression and, eventually, prevention (Chapter 15). The mechanisms of action of standard-of-care and novel anti-cancer drugs are broad and expand from the initial antimetabolites (Chapter 2), hormonal therapies (Chapter 3), radio- and photo-sensitizing agents (Chapter 4), DNA-interactive molecules (Chapters 5, 6, and 7), or tubulin inhibitors (Chapter 8) to the most recent-targeted therapeutic agents, which inhibit intracellular components of deregulated signal transduction, apoptotic, metastatic, or epigenetic processes (Chapters 9, 10, and 11).

One of the main issues faced by oncologists with the preceding agents is the development of drug resistance. To delay the onset of this problem, compounds that block some of the underlying mechanisms of resistance or are active against mutations in the kinase gene that abrogate drug binding have been identified and pursued in the clinic (Chapter 14). As shown in different parts of the book, combinations of drugs, including the use of alternative dosage regimes, are often required to maximize clinical benefit for the cancer patient.

In addition to chemotherapeutic agents, which for many years have been the backbone of numerous regimes for the treatment of solid and liquid tumors, a deeper understanding of the molecular events leading to tumor formation, invasion, angiogenesis, and metastasis and, more recently, the ability to boost the body's immune system have been exploited to advance new therapeutic modalities (e.g., humanized monoclonal antibodies, gene therapy, or antisense oligonucleotides; Chapter 12) or delivery systems (e.g. nanoparticles, liposomes, or polymer conjugates; Chapter 13) to achieve sustained responses and minimize systemic toxicity.

While many disappointments have been harvested in pre- and clinical settings, we start to witness the incremental improvements in survival achieved with the current armamentarium of anticancer drugs. Thus, recent epidemiological data provided by the American Cancer Society show that from 2006 to 2010, the death rate for all cancers combined decreased by 1.8% and 1.4% per year in men and women, respectively. These results are encouraging, provide hope to cancer patients and their families, and demonstrate that we are in the right path to achieve our ultimate objective: *to cure cancer*.

Carlos Garcia-Echeverria, PhD
VP, Global Head of Samall Molecule Drug Discovery
Sanofi

Preface

Chemistry, and medicinal chemistry in particular, is a very broad subject that bears a profound relationship with all phases of drug discovery, design, and development. The involvement of many facets of chemistry is needed for the translation of the knowledge of the cellular, molecular, and genetic basis of cancer into effective therapies. In the past decades, the boundaries between biology and chemistry have become increasingly diffuse because biology is close to becoming a chemical science. Indeed, it can be easily verified that in the past years many Nobel prizes in chemistry have been awarded for discoveries that are biological in their nature or applications. As our understanding of the basic chemistry of life increases, we begin to understand complex phenomena at molecular levels, and this level of understanding allows for the design of molecular entities that are selectively suited to interact with a given biological target because drug action is always a consequence of a chemical interaction.

As when the first edition of this book was planned, we believe that there is a clear need for an updated monograph of anticancer drugs from the point of view of medicinal chemistry. We have attempted to produce a concise but reasonably comprehensive treatment to fill the gap between the elementary medicinal chemistry textbooks and the primary literature and help readers to achieve a deeper understanding of the molecular basis of the action of antitumor drugs. Because of the huge number of agents that show *in vitro* antitumor activity, we had to make some difficult decisions on the inclusion or exclusion of certain topics and, with some exceptions related to recently validated targets, we have limited our discussion to agents that have been approved or at least have entered clinical trials. The organization of the book is based on targets and mechanisms of action using the main mechanism of action of each drug as the criterion, although some decisions taken in this regard might be debatable. We have purposefully excluded the discussion of antitumor drug synthesis, not because we believe that it is not pertaining in a book devoted to medicinal chemistry but because it would have required a full volume in itself to do some justice to the achievements made in this area.

In the past years, anticancer therapy has continued to be a very active field of research and, in addition to the large number of validated targets and new drugs that have been developed, a huge amount of knowledge has been generated, mainly based on genomic data and the understanding of cancer as a multifactorial disease. Modern anticancer drug research has become increasingly focused on signal transduction therapy, and many of the validated targets are transduction-related macromolecules. The development of specific monoclonal antibodies targeted at tumor antigens has undergone very fast growth. Drugs that interact with proteolytic enzymes involved in the proteasome machinery and in angiogenesis and metastasis, as well as drugs targeting epigenome alterations, also have much current relevance. Active immunotherapy (therapeutic vaccines), immunomodulators of the tumor environment, and nanotechnology approaches are innovative therapies for personalized treatments, which are expected to have a major clinical impact in the treatment of cancer.

The second edition of this book contains many new features. We have undertaken a thorough update of the text to include new drugs that have been introduced in recent years. In each chapter, we emphasize the basis of drug discovery and design, analyzing the problems found in their development, and updating the information on their clinical applications. We now include drug trade names in the belief that they will make the book more practical. We hope that the introduction of color in the figures and a large number of three-dimensional structures of drug–target complexes generated from Protein Data

Bank files leads to a clearer explanation of many aspects of the mechanisms discussed in the text and generally improves the reader's experience.

All chapters have been thoroughly rewritten and updated with discussion of many new targets and drugs, which has required the creation of a separate chapter for epigenetic cancer therapy (Chapter 8). A much-expanded treatment of targeted anticancer therapy by small molecules is provided in Chapters 10 and 11, and drugs acting on a large number of new targets are discussed. These include hepatocyte growth factor receptor (HGFR), fibroblast growth factor receptor (FGFR), anaplastic lymphoma kinase (ALK), JAK-STAT and PRL pathways, tropomyosin receptor kinase (Trk), Bruton's tyrosine kinase (BTK), checkpoint kinases (CHKs), Pim kinases, transforming growth factor-β (TGF-β)-Smad, NEDD 8 activating enzyme (NAE), anaphase-promoting complex (APC), aminopeptidase N, cathepsin, and integrins. New sections are devoted to several topics of current interest in this area, including drugs targeting cancer stem cells acting on the wingless/β-catenin (Wnt//β-Cat), Notch, and Hedgehog signaling pathways and drugs that interfere with oncogenic protein–protein interactions, with special emphasis on anticancer drugs acting on apoptotic signaling pathways. Inhibitors of kinases involved in anaerobic glycolysis are also discussed. Another new chapter dealing with biological cancer therapy has been introduced (Chapter 12), comprising topics such as monoclonal antibodies, cancer immunotherapy, cancer vaccines, cancer gene therapy, and antisense oligonucleotides. Chapter 13, devoted to methods for the specific delivery of anticancer drugs to tumors, has been much expanded, and approximately half of this material is now devoted to nanotechnologies. The chapter devoted to anticancer drug resistance (Chapter 14) now includes a discussion of cellular adhesion molecules and SPARC protein as mechanisms of resistance. Cancer chemoprevention (Chapter 15) has also been updated and expanded.

We expect that this book will be useful to undergraduate and postgraduate students of medicinal chemistry and their instructors, in courses related to pharmacy, chemistry, or the health sciences, and should also have some appeal for students of pharmacology or biochemistry courses. We also hope that the inclusion of a large number of references to the review and primary literature will also make the book useful for researchers and practitioners of health professions.

Carmen Avendaño
José Carlos Menéndez
Madrid, January 2015

The image bank and other information could be found in the web site: http://booksite.elsevier.com/9780444626493/

Abbreviations

ACAT	Acyl-coenzyme A: cholesterol acyl transferase
ACL	ATP-citrate lyase
ADC	Antibody–drug conjugate
ADCC	Antibody-mediated cellular cytotoxicity
ADEPT	Antibody-directed enzyme prodrug therapy
ADI-PEG20	PEG-recombinant arginine deiminase
ADP	Adenosine diphosphate
AEBS	Antiestrogen binding site
AF	Activating function
AI	Aromatase inhibitor
AICARFT	Aminoimidazolecarboxamide ribonucleotide formyltransferase
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
ALDH	Aldehyde dehydrogenase
ALK	Anaplastic lymphoma kinase
ALK5 (TβR-I)	Activine-like kinase 5
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
AMP	Adenylic acid
AOL	Amine oxidase-like
AP-1	Activator protein 1
APC	Antigen-presenting cell
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex/cyclosome
APL	Acute promyelocytic leukemia
APN (CD13)	Aminopeptidase N
AR	Androgen receptor
ARCON	Accelerated radiotherapy with carbogen and nicotinamide
5ARI	5α-Reductase inhibitor
ASC carrier	Alanine, serine, and cysteine carrier
ASO	Antisense oligonucleotide
ATC	Anaplastic thyroid cancer
ATM	Ataxia telangiectasia mutated
TP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid (tretinoin)
BCL2	B-cell lymphoma protein 2
BCR	Breakpoint cluster region
BER	Base excision repair
BET	Bromodomain and extra-terminal
BFC	Bifunctional chelate
BIR	Baculovirus IAP repeat
BLMs	Bleomycins
BNCT	Boron neutron capture therapy
BPA	Boronophenylalanine
BR	Binding region
BRCA	“Berkeley California” genes

BSH	Sodium borocaptate
BTK	Bruton's tyrosine kinase
BTZ	Benzotriazinyl
CAC	Cancer-associated cachexia
CaM	Calmodulin
CAM	Cell adhesion molecule
CBP	Cyclic AMP response element-binding protein
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDL	Cullin-dependent ligase
CDP	Cytidine diphosphate
CHK-1	Checkpoint kinase 1
CKI	CDK inhibitor
c-Met (HGFR)	Mesenchymal–epithelial transition factor
CML	Chronic myeloid leukemia
CNUs	1-(2-Chloroethyl)-1-nitrosoureas
CR	Cisplatin resistant
CRD	Cysteine-rich domain
CRPC	Castration-resistant prostate cancer
CS	Cisplatin sensitive
CSC	Cancer stem cell
CSCC	Cholesterol side chain cleavage enzyme
CSNK1G3	Casein kinase 1, gamma 3
CtBP2	C-terminal binding protein 2
CTGF	Connective tissue growth factor
CTL	Cytotoxic T lymphocyte
Cts-L	Cathepsin L
cyt C	Cytochrome *c*
CYP	Cytochrome P
Dapk	Death-associated protein kinase
DASI	Dual aromatase–sulfatase inhibitor
DAUF	Daunoform
dFdC	Gemcitabine (difluorodeoxycytidine)
dG	Deoxyguanosine
DG	Diacylglycerol
2-DG	2-Deoxy-D-glucose
dGTP	Deoxyguanosine triphosphate
DHEA	Dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHFU	Dihydrofluorouracil
DHT	5α-Dihydrotestosterone
DISC	Death-inducing signaling complex
DKK1	Dickkopf-1
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
DNR	Daunomycin
DNRol	Daunorubicinol
DOS	Diversity oriented synthesis
DOX	Doxorubicin (adriamycin)
DOXF	Doxoform

DOXol	Doxorubicinol
DPD	Dihydropyrimidine dehydrogenase
DR	Death receptor
DSB	Double-strand break
DTD (NQO1)	DT-diaphorase
dTDP	Deoxythymidine diphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
E_1	Estrone
E_1S	Estrone sulfate
E_2	Estradiol
E_2-1,2-Q	Estradiol-1,2-quinone
E_2-2,3-Q	Estradiol-2,3-quinone
E_2-3,4-Q	Estradiol-3,4-quinone
EBRT	External beam radiotherapy
EBV	Epstein–Barr virus
EGFR (HER-1)	Epidermal growth factor receptor
EML4	Echinoderm microtubule-associated protein-like 4
EMT	Epithelial–mesenchymal transdifferentiation
ENL	Erythema nodosum leprosum
EpCAM	Epithelial cell adhesion molecule
EPR	Enhanced permeability and retention
ER	Estrogen receptor
ER	Endoplasmic reticulum
ERE	Estrogen response element
FADD	Fas-associated protein with death domain
FapyAde	Formamidopyrimidine adenine derivative
FapyGua	Formamidopyrimidine guanine derivative
Fas	Fatty acid synthetase
FasL (Apo-1)	Fas ligand
FBS	Fragment-based screening
FdUMP	5-Fluoro-2′-deoxyuridine-monophosphate
5-FdUTP	5-Fluoro-2′-deoxyuridine-triphosphate
FGAR	Formylglycinamide ribonucleotide
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGPS	Folylpolyglutamate synthetase
FLT-3 (CD135)	Fms-like tyrosine kinase 3
FmdC	Fluoromethylenedeoxycytidine (tezacitabine)
5-FP	5-Fluoro-2-pyrimidinone
FPGS	Folylpolyglutamate synthetase
FPP	Farnesyl pyrophosphate
FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
5-FU	5-Fluorouracil
5-FUdR	5-Fluorouracil deoxyribonucleoside (floxuridine)
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GAR	Glycinamide ribonucleotide
GARFT	Ribonucleotide formyltransferase
GBM	Glioblastoma multiforme
GCSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GGTase	Geranylgeranyl transferase
GIST	Gastrointestinal stromal tumor
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GLDC	Glycine decarboxylase
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GMP	Guanylic acid
Gn	Gonadotropin
GnRH (LHRH)	Gonadotropin-releasing hormone
Grb	Growth factor receptor bound
GS	γ-Secretase
GSI	γ-Secretase inhibitor
GST-π	Glutathione-*S*-transferase of the π class
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HDBC	Hormone-dependent breast cancer
HER-1 (EGFR)	Human epidermal growth factor receptor 1
HER-2 (ERBB2)	Human epidermal growth factor receptor 2
HGFR (c-Met)	Hepatocyte growth factor receptor
HGPRT	Hypoxantine guanine phosphoribosyl transferase
HIF	Hypoxia-inducible factor
HK	Hexokinase
HLA	Human leukocyte antigen
HMT	Histone methyltransferase
HMTA	Hexamethylenetetramine
HPMA	*N*-(2-hydroxypropylmethacrylamide)
HPV	Human papillomavirus
3β-HSD/isom	3β-Hydroxyesteroid dehydrogenase/isomerase
HSP	Heat shock protein
hTR	Human telomerase RNA component
HTS	High-throughput screening
IAPs	Inhibitors of apoptotic signals
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor
IκB (IKK)	Inhibitory protein of κB family
ImiD	Immunomodulatory drug
IMP	Inosinic acid
IP_3	Inositol triphosphate
IPMK	Inositol polyphosphate multikinase
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
IORT	Intraoperative radiotherapy
IRP-1	Iron regulatory protein 1
JAK	Janus kinase
JNK	Jun *N*-terminal kinase
KLH	Keyhole limpet hemocyanin
KMT	Lysine methyltransferase

LDH-A	Lactate dehydrogenase A
LEF	Lymphoid enhancer factor
LH	Luteinizing hormone
LH-RF	Luteinizing hormone-releasing factor
LRP	Lipoprotein receptor-related protein
LSD (KDM)	Lysine-specific demethylase
mAb	Monoclonal antibody
MAO	Monoaminooxidase
MAOP	Methyl 5-aminolevulinate
MAPK (ERK)	Mitogen-activated protein kinase (extracellular signals regulated kinase)
MAPKK (MEK)	MAPK kinase (MAP/ERK kinase)
MBD	Methyl-binding protein
MCL	Mantle cell lymphoma
Mcl-1	Induced myeloid leukemia cell differentiation protein
M_1dG adducts	Pyrimidopurine derivatives of dG
MDR	Multidrug resistance
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
MELC	Murine erythroleukemia cell
MetAp	Methionine aminopeptidase
MFR (α-FR)	Membrane folate receptor
MGB	Minor groove binder
MHC	Major histocompatibility complex
MKP1	JNK-MAPK phosphatase 1
MM	Multiple myeloma
MPE	Malignant pleural effusion
mRCC	Metastatic renal cell carcinoma
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MTA	Microtubule targeting agent
mtDNA	Mitochondrial DNA
MTH1	MutT homolog 1
mTOR	Mammalian target of rapamycin
MUTYH	Adenine/2-hydroxyadenine DNA glycosylase that excises adenine opposite 8-oxoG
NAE	NEDD8-activating enzyme
N-BP	Nitrogen-containing biphosphonate
NCS	Neocarzinostatin
NET	Neuroendocrine tumor
NF-κB	Nuclear transcription factor κB
NMC	NUT midline carcinoma
NPY	Neuropeptide Y
NQO2	Quinone oxidoreductase 2
Nrf2	Nuclear-related factor 2
NRR	Negative regulatory region
NSCLC	Non-small cell lung cancer
NTR	Nitroreductase
NUT	Nuclear protein in testis
OGG1	8-Oxoguanine glycosylase
2-OHE2	2-Hydroxyestradiol
8-OHGua	8-Hydroxyguanine
5′-OH-Hyd	5′-Hydroxyhydantoin

5-OH-MeUra	5-(Hydroxymethyl)uracyl
OPRT	Orotic phosphoribosytransferase
8-oxo-dGTP	2′-Deoxy-8-oxoguanosine triphosphate
OXPHOS	Oxidative phosphorylation
PALA	*N*-phosphonoacetyl-L-aspartate
PAP	Prostatic acid phosphatase
PBR	Phosphate-binding region
PCa	Prostate cancer
PCD	Programmed cell death
PDAC	Pancreatic ductal adenocarcinoma
PDD	Photodynamic diagnosis
PD-ECGF	Platelet-derived endothelial cell growth factor
PDEPT	Polymer-directed enzyme prodrug therapy
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDLIM2	PDZ-LIM domain-containing protein 2
PDPK1	Phosphoinositide-dependent protein kinase-1
PDT	Photodynamic therapy of cancer
PEG	Polyethyleneglycol
PEG-PGA	PEGylated glutaminase
PET	Positron emission tomography
PFK	Phosphofructokinase
PGA	Polyglutamic acid
PHGDH	Phosphoglycerate dehydrogenase
PI	Proteasome inhibitor
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol(4,5)-diphosphate
PIP3	Phosphatidylinositol(3,4,5)-trisphosphate
PK	Pyruvate kinase
PKC	Protein kinase C
PLC	Phosphoplipase C
PLK-1	Polo-like kinase 1
PLP	Pyridoxal phosphate
p38 MAPK	P38 mitogen-activated protein kinase
PML	Promyelocite leukemia protein
PNA	Peptide nucleic acid
PNP	Purine nucleoside phosphorylase
PPAR	Peroxysome proliferator-activating receptor
PPI	Protein–protein interactions
PPIM	PPI modulator
PPP	Pentose phosphate pathway
PPRE	Peroxisome proliferator hormone response element
pRb	Retinoblastoma
PRL	Polypeptide hormone prolactin
PRLR	Polypeptide hormone prolactin receptor
PRPP	Phosphoribosyl pyrophosphate
PS	Photosensitizers
PTEN	Phosphatase and tensin homolog
PTK	Protein kinase
PUFA	Polyunsaturated fatty acids
RAP	Radiation-activated prodrug
RAR	Retinoic acid receptor

RARE	Retinoic acid response element
RES	Reticuloendothelial system
RFC	Reduced folate carrier
RIT	Radioisotope therapy
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase (equivalent to NDPR)
ROS	Reactive oxygen species
ROS1	C-ros oncogene 1
RPTK	Receptor protein kinase
RXR	Retinoid X receptor
SAC	Spindle assembly checkpoint
SARM	Selective androgen receptor modulator
SCFR (c-Kit, CD117)	Stem cell growth factor receptor
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SERM	Selective estrogen receptor modulator
sFRP	Secreted Frizzled-related glycoprotein
SGK1	Serum glucocorticoid-regulated kinase 1
SHMT	Serine hydroxymethyltransferase
SIRT	Silent information regulator
SLL	Small lymphocytic lymphoma
SMA	Styrene maleic acid
Smac	Second mitochondria-derived activator of caspase
Smo	Smoothended
SOS	Son of Sevenless
Sp1	Specificity protein 1
SPARC	Secreted protein acidic and rich in cysteine
SRIF	Somatostatin (somatotropin release-inhibiting factor)
SRP	Signal recognition particle
SST	Somatostatin
SSTR	Somatostatin receptor
STAT	Signal transducer and activator of transcription
STS	Steroid sulfatase
TACA	Tumor-associated carbohydrate antigen
TCF	Tumor cell factor
TCR	T-cell receptor
TDDP	*trans*-diaminedichloroplatinum(II)
Tdp	Tyrosyl-DNA-phosphodiesterase
TEM	Triethylenemelamine
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor (tumor growth factor)
THF	Tetrahydrofolate
ThyGly	Thymine glycols
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TMP	Thymidylate (thymidine monophosphate)
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
Top1	Topoisomerase I
Top2	Topoisomerase II
Topo	Topoisomerase
TP	Thymidine phosphorylase

TPMT	Thiopurine methyltransferase
TPZ	Tirapazamine
TR (TERC)	Telomerase RNA
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor
Treg	Regulatory T cell
TS	Thymidylate synthase
TSC	*trans*-sodium crocetinate
TSP1	Trombospondin-1
TSR	Thrombospondin type 1 repeat
TWEAK	TNF-like weak inducer of apoptosis
UAE (E1)	Ubiquitin-activating enzyme
UBC (E2)	Ubiquitin-conjugating enzyme
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate
VDEPT	Virus-directed enzyme prodrug therapy
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL gene	Von Hippel–Lindau (E3 ubiquitin protein ligase)
VTPT	Vascular targeted photodynamic therapy
WAT	White adipose tissue
WBRT	Whole-brain radiation therapy
XIAP	X-linked inhibitor of apoptosis protein
XMP	Xanthylic (or xantosinic) acid

CHAPTER

GENERAL ASPECTS OF CANCER CHEMOTHERAPY

1

CONTENTS

1. INTRODUCTION: SOME GENERAL COMMENTS ABOUT CANCER

Cancer is a collective term used to describe a group of different diseases that are characterized by the loss of control of cell growth and division, leading to a primary tumor that invades and destroys adjacent tissues. It may also spread to other regions of the body through a process known as metastasis, which is the cause of 90% of cancer deaths. Cancer remains one of the most difficult diseases to treat and is responsible for approximately 14.5% of all deaths worldwide. This incidence is increasing due to the aging of the population in most countries, including those under development. Indeed, against a widely held belief, more than two-thirds of all cancer deaths occur in low- and middle-income countries, and the estimated increase in cancer incidence by 2030, compared with 2008, will be greater in low- (82%) and lower-middle-income countries (70%) compared with the upper-middle- (58%) and high-income countries (40%).[1]

The creation in late 1971 of the U.S. National Cancer Program led by the National Cancer Institute (NCI) had as its most important consequence that the amount of basic science implied in these studies permitted the initial understanding of cancer development. Cancer has been redefined throughout the years,[2] and currently comprehensive views exist of how most cancers arise and function at the genetic and biochemical level. However, the cure of cancer continues to be a daunting objective[3] because of the high mutation potential of tumor cells and the original heterogeneity in genetic alterations of

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00001-6

tumors—properties that permit the relapse of patients following initial treatment success, which creates a pressing need for alternative agents that could be used as later lines of therapy. In fact, drug resistance is still a major problem in oncology and affects old therapies, new targeted drugs, and personalized cancer treatments.

2 TUMORIGENESIS AND ONCOGENES: PHARMACOGENOMICS

Tumorigenesis is a multistep process whose steps reflect genetic alterations including small-scale changes in DNA sequences, such as point mutations; larger scale chromosomal aberrations, such as translocations, deletions, and amplifications; and changes that affect the chromatin structure and are associated with dysfunctional epigenetic control, such as aberrant methylation of DNA or acetylation of histones.[4] Any of these genetic alterations confers one or another type of growth advantage that drives the progressive transformation of normal cells into highly malignant cancer cells. Hanahan and Weinberg reported six hallmarks or biological capabilities acquired during the multistep development of human tumors: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.[5] Later conceptual progress added new hallmarks to this list: evading immune destruction, deregulating cellular energetics (reprogramming of energy metabolism), genomic instability and mutation, and tumor-promoted inflammation (Figure 1.1).

Furthermore, cancer is not only a cell disease but also a tissular disease in which the normal relationships between epithelial cells and their underlying stromal cells are altered.[6] In fact, tumors contain recruited cells that contribute to the acquisition of the previously mentioned hallmark traits by creating an adequate tumor microenvironment.[7]

Although cancer is not a contagious disease, infectious agents such as viruses can contribute to its origin. Most oncogenes were identified by using retroviruses, and the first evidence of the tumorigenic potential of some genes derived from studies on malignant diseases caused by them. The term *oncogene* was introduced in the mid-1960s to denote special parts of the genetic material of certain viruses

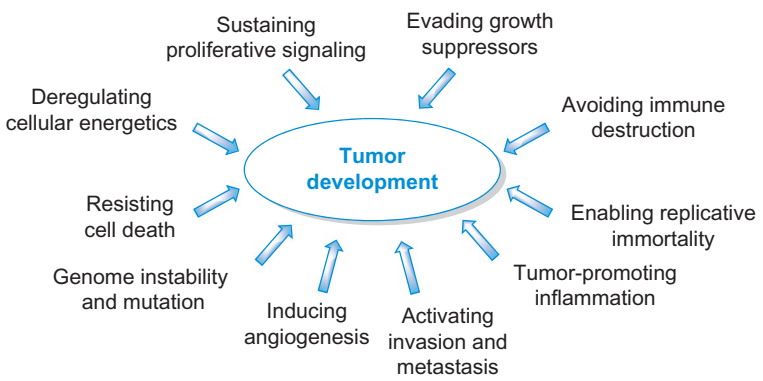


FIGURE 1.1

The tumorigenesis process.

that, as it was believed, could direct the transformation of a normal cell into a tumor cell. The favorite theory of the time was that virus-mediated cell-to-cell transmittance of oncogenes was the origin of all forms of cancer. This view was later proven to be incorrect because, from the standpoint of cancer development, the crucial issue is the comparison between oncogenes in normal cells (proto-oncogenes) and in tumor cells. Oncogenes are identified by the use of three-letter abbreviations; in addition, cellular and viral oncogenes are sometimes distinguished by *c-* and *v-* prefixes, respectively (e.g., *c-src* and *v-src*).

The first oncogene to be identified was *v-src*, discovered in 1970 as a component of a cancer-causing virus in chickens known as the Rous sarcoma virus. This is a member of the retroviruses family, characterized because their RNA genetic material is transcribed into DNA by the enzyme reverse transcriptase. This reverse transcription permits the integration of the genetic material of retroviruses into the chromosomal DNA in the cells. However, retroviruses play a relatively limited role in the development of cancer under natural conditions, with human T-lymphotropic virus type 1 (HTLV-1) the only known example in humans in which a retrovirus infection contributes to the origin of a cancer. Other kinds of viruses with DNA as their genetic material can also contribute to the development of tumors in humans, although other factors in addition to the virus infection are required for the cancer to develop. Certain types of papillomaviruses play a role in the development of cervical cancer in the genital tract, whereas Epstein–Barr virus is an important factor in the development of Burkitt's lymphoma in Africa and nasopharyngeal cancer in Asia.

In 1975, Bishop and Varmus demonstrated the true origin of the *v-src* oncogene by its detection in different species throughout the animal kingdom. It controls cell growth and division, and in humans it is involved in a variety of cancers, such as colon, liver, lung, breast, and pancreatic cancer.[8] Accordingly, this oncogene is not a true viral gene but, rather, a cellular gene picked up by the virus during its replication in cells.

In 1982, the first human oncogene, currently known as *H-RAS*, was cloned and characterized from the T24 bladder carcinoma.[9] Approaches to the true genetic complexity of cancer evolved as a result of the Human Genome Project (1988–2003), which led to the knowledge that among a total of approximately 25,000 human genes, mutations of approximately 200 are able to promote abnormal growth and cell division as well as evasion of programmed death, leading to cancer. Nevertheless, the regulation of growth and division of cells is much more complex than originally believed. Cellular oncogene products with different properties act in different positions in elaborate signal systems to transmit signals from one cell to another or within a single cell.

Several oncogene products function as receptors in the cytoplasmic membrane of the cells and catalyze the phosphorylation of the amino acid tyrosine. There are two groups of oncogene products with phosphokinase activity: tyrosine/phosphokinases, which lack receptor function and are located on the inside of the cytoplasmic membrane, and serine/threonine phosphokinases, which are found in the cytoplasm. Thus, oncogene products function as links in signal chains stretching from the surface of the cell to the genetic material in the nucleus. In the cytoplasm, there is one more group of oncogene products, such as Ras, that are related to the important cellular signal factors known as G proteins. Finally, several oncogene products, such as Myc, Myb, Fos, and ErbA, are located in the nucleus of the cell and direct the transcription of DNA into RNA, playing a critical role in the selection of proteins to be synthesized by the cell.

In the development of a tumor, a normal cellular oncogene may be hyperactive or an oncogene product may be altered so that it can no longer be regulated in a normal way. Oncogenes with point

mutations may cause alterations in the amino acid composition of the gene product, and they have been observed in many tumors. A well-known example of such a modification is the exchange of amino acid 12 from glycine to valine in the Ras gene product. The mutation may be more extensive, leading to the absence of part of the protein (deletion). Repeated copying of a normal oncogene can lead to its amplification in the chromosome and consequently to increased amounts of the oncogene product. The same effect can be seen when there is a reciprocal exchange of segments between chromosomes (translocation). Thus, the normal *myc* gene on chromosome 8 has been translocated to chromosome 14 in many patients with Burkitt's lymphoma, a form of non-Hodgkin's lymphoma in which cancer starts in the B cells of the immune system. Chromosome translocations occur in many different tumors.

Mutated genes that encode protein components of signal transduction pathways enable external signals such as growth and survival factors to move from the cell surface receptors to key promoter–enhancer regions along the 24 human chromosomes, where they turn up the expression of genes needed for cell growth and division and evasion of programmed cell death (apoptosis). The latter event is very important and underlies the ever-growing resistance of late-stage aggressive cancer cells to radio- and chemotherapeutic therapies. Among the multiple molecular pathways that bring about cell growth and proliferation, each with their own specific surface receptors, cytoplasmic transducers and promoters as well as enhancers of gene expression, exists much potential cross talk, which allows new DNA mutations to create new pathways to cancer when preexisting ones are blocked by a given treatment.

In 1984, Mak, a pioneer in developing genetically engineered mice known as "knockout mice" because one or more of their genes have been inactivated, demonstrated the inhibitory effect on T cells of a protein called cytotoxic T-lymphocyte antigen 4 (CTLA-4), also known as CD152 (cluster of differentiation 152). This protein is an inhibitory co-receptor that interferes with T-cell activation and proliferation.[10] This landmark discovery was an important breakthrough in understanding the human immune system, pioneering further work in the genetics of immunology that has had a direct impact on the development of personalized cancer medicine. In recent years, clinical researchers have developed techniques for re-engineering the T-cell receptor gene to target certain cancers. Such treatments, although still in the experimental stage, have yielded dramatic results in some patients, especially those with leukemia and melanoma, in part because T cells are capable of being better targeted than surgery, radiation, chemotherapy, or hormonal therapy. Those findings led to the development of ipilimumab (Yervoy®), which blocks CTLA-4 and enables T cells to proliferate and destroy certain cancer cells.[11] The editors of the journal *Science* chose cancer immunotherapy, a strategy that harnesses the body's immune system to combat tumors, as the scientific breakthrough of the year for 2013.[12]

Pharmacogenomic studies first focused on inherited genetic variants of the germline DNA, but they have been extended to somatic alterations of DNA in a tumor. These studies allow the establishment of a relationship between a drug response and the patient's genetic alterations, maximizing the chance of treatment success and minimizing the risk of toxicity. Genomic markers may be predictive, identifying whether a patient will respond or not to a drug, or prognostic, predicting the clinical course of a given cancer irrespective of treatment. Because cancer is a disease of the genome, each cancer cell may harbor many genomic alterations that differ in different tumor types, even within the same tumor in the same patient.

The impact of variations in the human genome depends on their nature and on their location. These variations may be single nucleotide polymorphisms (SNPs), variations in copy numbers, and chromosomal rearrangements (inversions and translocations). The function of proteins is altered most when nucleotide mutation alters their amino acid sequence as a consequence of nonsynonymous variations

occurring in the gene coding regions (exons). Synonymous variations, which do not alter the amino acid sequence, were thought to be silent, but they can also influence mRNA splicing, mRNA stability, and protein conformation and function.

Changes in drug response often involve germline variations that affect the pharmacokinetics of an anticancer drug by reducing the expression or activity of coded enzymes. For instance, patients with acute lymphoblastic leukemia treated with 6-mercaptopurine and who have a homozygous deficiency in thiopurine *S*-methyltransferase enzyme activity have an extreme sensitivity to this drug as a result of the accumulation of higher cellular concentrations of thioguanine nucleotides. Consequently, they have an increased risk of myelosuppression and require a substantial dose reduction.[13] However, most drug response phenotypes respond to variations in multiple genes encoding proteins that are involved in drug absorption, transport, metabolism, elimination, and mechanism of action. The aggregate effect of multiple polymorphisms or alleles that are closely linked, known as a haplotype, is frequently inherited together and, fortunately, it can be considered as a functional unit that may be represented by a marker SNP. This property allows for large sections of the genome to be studied using relatively fewer marker SNPs. Unlike germline variations, somatic mutations are not present in normal cells and are not inheritable, and they can functionally be divided into driver and passenger. Most of them are temporary and do not contribute to cancer development, but driver mutations confer growth or survival advantages in cancer cells. When they are located in oncogenes, the cancer cells become "addicted" to their function, and the oncogenes may be the target of the therapy.[14]

The success of imatinib in the treatment of chronic myeloid leukemia paved the way for the development of treatments targeting genomic aberrations in solid tumors, an approach that has been especially effective in gastrointestinal stromal tumors, breast cancer, colorectal cancer, non-small cell lung cancer, and melanoma.[15] The International Cancer Genome Consortium, the Cancer Genome Atlas, and the Cancer Genome Project have afforded comprehensive genomic information on several cancer types and have identified genomic aberrations that are potentially targetable or associated with drug resistance, thus enabling a personalized approach to cancer therapy.[16]

3 EARLY DIAGNOSIS OF CANCER AND ITS THERAPEUTIC RELEVANCE

The high potential for mutation of tumor cells limits the usefulness of tissue biopsy as a standard prognostic procedure for cancer because, due to the genetic diversity within a single solid tumor, cells from one end may differ from those at the other and only some mutations are shared throughout the whole mass. Accordingly, a biopsy could miss mutations that might radically change the diagnosis and prognosis of a patient, and although it can provide data about specific mutations that might make a tumor vulnerable to targeted therapies, that information may become inaccurate as the cancer evolves.[17] For an early diagnosis, prognosis, and epidemiology of cancer, it is necessary to detect specific biomarkers that, ideally, should be collected from biofluids such as blood or serum. Several genetic, epigenetic, proteomic, glycomic, and imaging biomarkers are currently used for cancer diagnosis and therapeutic monitoring, including AFP (liver cancer), Bcr-Abl (chronic myeloid leukemia), BRCA1/BRCA2 (breast/ovarian cancer), BRAF V600E (melanoma/colorectal cancer), CA-125 (ovarian cancer), CA19-9 (pancreatic cancer), CEA (colorectal cancer), EGFR (non-small cell lung carcinoma), HER-2 (breast cancer), KIT (gastrointestinal stromal tumor), PSA (prostate cancer), and S100 (melanoma).[18] Although proteins are used in the clinic to diagnose illnesses and

monitor people undergoing treatment, many of those used as cancer biomarkers are inaccurate. For example, prostate-specific antigen (PSA) can give false positives because this antigen can be elevated in blood for other reasons.

Circulating DNA (ctDNA) in human blood, first reported in the blood of cancer patients in 1977, might perform better than proteins as a biomarker because it bears mutations that are hallmarks of cancer.[19] Circulating tumor DNA is composed of genome fragments that are released when cancer cells die and float freely through the bloodstream, and it could be an excellent cancer biomarker. Unfortunately, ctDNA is not yet ready for a leading role in the clinic, mainly because the most sensitive techniques for its detection require some knowledge about which mutations to search for, and this is a laborious task that must be performed for each individual patient. One alternative is to use exome sequencing, which does not require a previous knowledge about the cancer but is prohibitively expensive. A focused approach to the therapy of lung cancer that would permit keeping costs low has been developed. This approach is based on the identification of a small fraction of the genome (0.004%) that is repeatedly mutated in these cancers. Because almost all patients with lung cancer have at least one mutation in these regions, these mutations may be found by sequencing this small fraction 10,000 times over. The method should work in almost every cancer, except in the case of brain cancers, in which the blood–brain barrier stops tumor DNA from reaching the bloodstream. Unfortunately, the potential of ctDNA as a cancer-screening tool is limited to advanced forms of cancer, which discharge relatively high levels of DNA, but it does not perform well for detecting early cancer forms.[20] It is likely that molecular characterization of a given cancer will lead to the identification of different subsets of cancer disease with a different natural history, sensitivity, and resistance to treatment. In this task, efforts to develop, validate, and implement predictive biomarkers in clinical trials and eventually in routine care are important.

Despite the current emphasis on the early diagnosis of cancer, statistical data demonstrate that advances in this field have not led to a proportional decline in later stage disease.[21] Emphasis on early diagnosis of cancer may lead to overdiagnosis—that is, the detection of tumors that if left unattended would not become clinically apparent or cause death. To minimize overdiagnosis of cancer, some oncologists have proposed a change in terminology, with the term “cancer” reserved only for lesions with a reasonable likelihood of lethal progression if left untreated.

4 A BRIEF HISTORY OF CANCER CHEMOTHERAPY

In addition to biological knowledge, chemistry has had varying roles in the discovery and development of anticancer drugs since the beginning of cancer therapies.[22]

Modern cancer chemotherapy has its origin in the development of nitrogen mustards as chemical weapons. Since those early years, synthetic chemistry has been extensively used to modify drug leads, especially those of natural origin, and to solve the problem of the often scarce supply of anticancer natural products by developing semisynthetic or fully synthetic strategies.

The first cytotoxic agents, most of which are still used in the clinic, were discovered through different approaches, although their mechanism was unknown. The synthesis of folate analogs was undertaken following the observation that folic acid stimulates the proliferation of acute lymphoblastic leukemia (ALL) cells, which led to the discovery of methotrexate, the first drug that induced remission in children with ALL. It is interesting to note that the development of resistance induced by old drugs such as nitrogen mustards and methotrexate was apparent since the earliest studies.

The discovery of cisplatin in the 1960s is a classic case of serendipity, when studies on the effect of an electric current on the growth of *Escherichia coli* showed that the inhibition of cell growth was not due to the electric current but, rather, to the production of a platinum complex in the electrodes. Two important anticancer drugs, doxorubicin and paclitaxel, were discovered in the screening of natural product extracts in mouse leukemia models. A more targeted approach to cancer chemotherapy was developed after the early discovery of the strong relationship between estrogens and some breast cancers.[23] The recognition that breast and prostate cancers are subject to hormonal regulation led to the introduction of antihormones that directly or indirectly target the estrogen or androgen receptors. This knowledge also led to the approval of the estrogen receptor modulator tamoxifen (Novaldex®) for cancer chemoprevention in 1998.[24,25]

Since the 1950s, the biological activities of many antitumor drug leads have been discovered through *in vitro* screening programs promoted by the NCI by using a range of cancer cell lines. In this early period, transplantable rodent tumor models characterized by a high growth rate were used for *in vivo* screening. Later, human tumor xenografts, based on transplantation of human tumor tissue into immune-tolerant animals, also became important tools for selecting antitumor drugs because these models allowed simulating a chemotherapeutic effect under conditions closer to humans. In the late 1970s and early 1980s, the role of chemotherapy was extended to preoperative and postoperative adjuvants, radiosensitizers to enhance radiation effects, and supportive therapy to increase the tolerance of the organism toward toxicity.[26] We have progressed in a few years from a lack of targets to having too many, as shown by the Cancer Gene Census, which catalogs those genes for which mutations have been causally implicated in cancer.[27] To use this information to design better drugs, improved methods for validation of these new targets are needed. In this respect, the use of high-throughput RNAi methods and genetically modified mouse models are very valuable, although removal of the target is not necessarily equivalent to its inhibition by a small molecule.

The rationale for the use of conventional cytotoxic agents as antitumor drugs was based on the notion that rapidly proliferating and dividing cells are more sensitive to these compounds that are normal cells.[28] However, as the interactions of these agents with DNA were better defined, new compounds targeting particular base sequences that may inhibit transcription factors in a more specific manner were studied. DNA was considered a molecular receptor capable of molecular recognition and triggering of response elements,[29] and the binding properties of the DNA ligands were rationalized on the basis of their structural and electronic complementarity with the functional groups present in the major and minor grooves of particular DNA sequences, which are mainly recognized by specific hydrogen bonds.[30] However, although DNA continues to be a target for anticancer chemotherapy, more recent efforts have been directed at discovering antitumor drugs specifically suited to target molecular aberrations that are specific to tumor cells.[31] This new generation of specific antitumor agents, or anticancer targeted drugs, is based on advances in molecular biology that occurred by the late 1980s, providing greatly increased understanding of regulatory and signaling networks that control fundamental cellular processes such as vascularization, cell growth and proliferation. It was then known that many of these signaling networks are enhanced in tumor cells in response to activated oncogenes.

The beginning of the twenty-first century was marked by the development of targeted therapeutics in the fight against cancer. Today, conventional chemotherapy is frequently replaced by monoclonal antibodies, kinase inhibitors, and cell differentiation or immunomodulatory agents. After the approval of trastuzumab (Herceptin®), other HER2-targeting agents, such as the small molecule lapatinib (Tykerb®) and the antibody pertuzumab (Perjeta®), were developed. Metastatic melanoma treatment

has experienced a marked revolution with the introduction of the antibody ipilimumab (Yervoy®) and the small molecule vemurafenib (Zelboraf®), which are directed against the mutated kinase bRaf V600E. The dual SRC and Abl kinase inhibitor bosutinib (Bosulif®) has improved the treatment of previously treated Philadelphia chromosome-positive chronic myeloid leukemia patients, and crizotinib (Xalkori®) is changing the management of ALK-positive lung cancers. The multikinase inhibitor regorafenib (Stivarga®) and the vascular endothelial growth factor-directed recombinant fusion protein aflibercept (Zaltrap®) are useful for metastatic colon cancer, and ruxolitinib (Jakafi®) and axitinib (Inlyta®) are used for myelofibrosis and for renal cell carcinoma, respectively. The advent of immunoconjugates in which antibodies are linked to toxins or radioisotopes has opened a new horizon for antibody-based targeted therapeutics. One example is the antibody–drug conjugate brentuximab vedotin (Adcetris®), which was approved in 2011 for the treatment of relapsed or refractory Hodgkin's lymphoma. Trastuzumab emtansine (T-DM1, Kadcyla®), approved in 2013, is another immunoconjugate for patients with metastatic breast cancer.

5 GENERAL COMMENTS ABOUT ANTICANCER DRUG DISCOVERY

Cancer therapy is based on local interventions such as surgery and radiotherapy, which are quite successful when viable, and on systemic chemotherapy. Approximately 50% of cancer patients are not cured by systemic chemotherapy and obtain only a prolonged survival.

Many cancer chemotherapeutic drugs currently in clinical use try to kill malignant tumor cells by inhibiting some of the mechanisms involved in cellular division. Accordingly, the antitumor compounds developed through this approach are cytostatic or cytotoxic to every dividing cell, including normal cells, and for this reason these drugs are nonspecific. However, the explosion in knowledge in tumor biology during the past decades has paved the way for specific, targeted anticancer drugs.[32] Success with the new molecularly targeted approach was demonstrated by the approval by the U.S. Food and Drug Administration (FDA) of a number of innovative drugs, both antibodies and small molecules, since the introduction of trastuzumab (Herceptin®) in 1998 as part of a treatment regimen containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant treatment of women with node-positive, HER-2-overexpressing breast cancer.[33] Trastuzumab is a humanized monoclonal antibody that targets the extracellular region of the HER-2 receptor, leading to its internalization and degradation. The introduction in 2001 of the tyrosine kinase inhibitor imatinib (Glivec®) as a highly effective drug for patients with Philadelphia chromosome-positive chronic myeloid leukemia and gastrointestinal stromal tumors[34] was proof of the concept of effective drug development based on the knowledge of tumor biology.[35] These anticancer drugs are signal transduction inhibitors that differ from compounds developed during the cytotoxic era because they target the precise molecular mechanisms responsible for the initiation and progression of cancer. Anti-oncogene drugs have had positive results and even cured some cancers, such as lung cancers with EGFR mutations, breast cancer with mutations in HER2, or, more recently, melanoma with b-RAF mutations. Unfortunately, currently known drugs cannot replace the function of tumor suppressor genes, whose mutations are more predominant that those that activate oncogenes.

Targeted therapies may use small molecule drugs or other macromolecular structures, such as monoclonal antibodies, to bind antigens that are preferentially or exclusively present on tumor cells. Other approaches try to develop compounds that interfere with gene expression in order to suppress the

production of damaged proteins involved in carcinogenesis. In the antisense oligonucleotides approach, the mRNA translation is interfered by inhibiting the translation of the information at the ribosome, whereas in the anti-gene therapy, a direct binding to the DNA double strand inhibits transcription.[36]

The knowledge of the three-dimensional (3D) structure of these new target macromolecules, which are normally proteins, by using X-ray crystallography, permits the rational design of small molecules that mimic the stereochemical features of the macromolecule functional domains. The principal steps in structure-based drug design using X-ray techniques are summarized in Figure 1.2.

In the absence of a 3D structure of a target protein, homology criteria may be applied by using the experimental structure of similar proteins, which is especially useful in the case of individual subfamilies. The knowledge of the 3D structure of a target also permits to design and generate virtual libraries of potential drug molecules to be used for *in silico* screening.

Many targets have different subtypes and functions, which makes finding therapeutically interesting inhibitors difficult. For instance, because matrix metalloproteases (MMPs) are involved in the cleavage of some bioactive molecules besides of extracelular matrix proteins, elimination of some of them in *knockout* animals—especially MMP-3, −8, and −9—has led to the development and metastasis of tumors. For this reason, only specific MMPs must be selected as anticancer targets. An example among ligands with multiple functions is transforming growth factor-β (TGF-β). This cytokine received that name based on its ability to induce fibroblast malignancy and favor metastasis by avoiding the immune system action in the last steps of a cancer, but it has been compared to the main character in "The strange case of Dr. Jekyll and Mr. Hyde" because it may also eliminate tumors in early

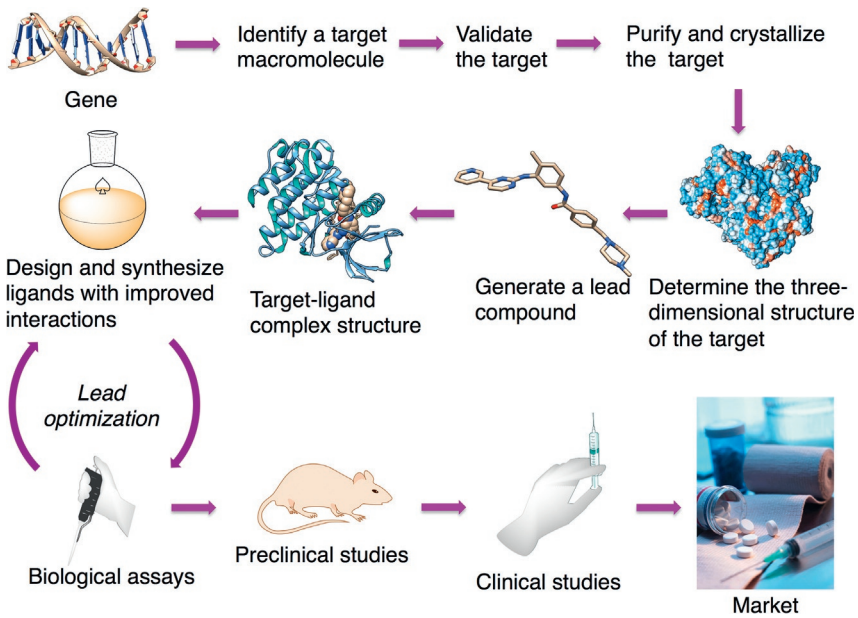


FIGURE 1.2

Structure-based drug design.

development. For this reason, it is necessary to select those patients for whom TGF-β inhibition is therapeutically useful.

Progress in the development of potential drug molecules is often problematic because it is difficult to convert hits into "druggable" compounds—that is, into molecules with adequate pharmaceutical properties. To this end, it is necessary to know the chemical properties of a lead compound, especially solubility and reactivity, because these properties are relevant for cellular uptake and metabolism in order to transform a lead compound into a real drug. The "druggability" of a drug candidate describes its adequate absorption, distribution, metabolism, and excretion (ADME) properties. In this task, the individualized knowledge of important metabolic enzymes, such as cytochrome P450 CYP3A4, permits improvement of the effectiveness and patient tolerance for antitumor compounds. A preliminary knowledge of ADME properties may be gained by using *in silico* techniques, although an experienced chemist can provide accurate insights into this picture by simple inspection of a given structure. The chemical properties of a drug candidate also govern its proposed formulation. In connection with ADME properties, the nonspecific biodistribution of anticancer drugs throughout the body,[37] requiring the administration of a large total dose to achieve high local concentrations in a tumor, is a major problem in cancer chemotherapy. Drug targeting aims at preferent drug accumulation in the target cells, independently of the method and route of drug administration.[38] One approach to improve the selectivity of cytotoxic compounds is the use of prodrugs that are selectively activated in tumor tissues, taking advantage of some unique aspects of tumor physiology such as selective enzyme expression, hypoxia, and low extracellular pH. More sophisticated tumor-specific delivery techniques allow the selective activation of prodrugs by exogenous enzymes (gene-directed and antibody-directed enzyme prodrug therapy) and the increased permeability of vascular endothelium in tumors (enhanced permeability and retention effect, EPR) permits that nanoparticles loaded with an antitumor drug can extravasate and accumulate inside the interstitial space, where the drug can be released as a result of normal carrier degradation.[39] In this discussion of contributions to the development of antitumor agents, it has to be mentioned that chemistry has also made possible important advances in prodrug development and in related targeted approaches, such as antibody-coupled drugs or photoactive agents.

Another major issue in cancer chemotherapy is acquired drug resistance, which is often developed by cancer cells after an initially effective treatment. Furthermore, following the development of a resistance mechanism in response to a single drug, cells can display cross-resistance to other structural and mechanistically unrelated drugs, a phenomenon known as multidrug resistance (MDR), in which ATP-dependent transporters have a significant role.[40] Resistance problems were observed during the early stages of cancer chemotherapy in the very first patient treated with a nitrogen mustard in 1942.

An additional problem in the development of anticancer drugs is the large gap from promising findings in preclinical *in vitro* and *in vivo* models to the results of clinical trials. Conventional anticancer drug screening is typically performed in the absence of accessory cells of the tumor microenvironment, and this preclinical drug testing may overestimate potential clinical activity, explaining at least in part the gap between preclinical and clinical efficacy in cancers.[41] Although a large number of clinical trials are in progress and new results are continuously being published, a statistically significant benefit is observed for very few of them.[42] In this regard, it has been claimed that to increase the efficacy of anticancer clinical trials, it is necessary to develop and use more clinically relevant cancer models. With the help of advanced engineering techniques, the development of complex 3D *in vitro* cancer

models may provide a better opportunity to understand crucial cancer mechanisms and to develop new clinical therapies.[43]

Genome-based medicine has permitted the development of personalized treatments in which effective targeted therapies may be suitable only for small subgroups of patients.[44] DNA microarray technology permits the study of alterations in the transcriptional level of entire genomes, and it may become an important tool for predicting the chemosensitivity of tumors before treatment. Pharmacogenetics, which focuses on intersubject variation in therapeutic drug effects and toxicity depending on polymorphisms, is also particularly interesting in oncology because anticancer drugs usually have a narrow margin of safety, and the parameters generally used to adjust the dose of chemotherapeutic agents (weight or body surface area) are not sufficient to overcome differences in drug disposition.[45]

Cancer stem cells (CSCs) have similar characteristics to normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer. They persist in tumors as a distinct population and cause relapse and metastasis, giving rise to new tumors. Conventional chemotherapies kill differentiated or differentiating cells that form the bulk of the tumor, but a population of CSCs can remain untouched, causing a relapse of the disease. Therefore, the development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for patients with metastatic disease. Cancer treatments targeting CSCs are discussed in Chapter 11, Section 7.

In parallel with these scientific developments, the cost of cancer drugs has increased exponentially. A controversial example is Provenge®, an autologous vaccine designed to stimulate the immune response to prostate cancer by targeting prostatic acid phosphatase and that costs $93,000 per treatment (2010 data). It is likely that we are witnessing a "bubble" based more on goodwill and hope than on results, and many researches think that there is an obvious need for a change of paradigm.[46]

6 COMBINATION THERAPY AND PERSONALIZED ANTICANCER TREATMENTS

Combination chemotherapies have been a mainstay in the treatment of disseminated malignancies for almost 60 years, but even the most successful regimens fail to cure many patients. Part of this failure is due to the absence of mechanistic information about how drugs interact to promote combination effects.[47]

It is now evident that the Ehrlich's magic bullet concept cannot be generally applied to cancer because it is a multifactorial disease and also a network problem. For this reason, the design of therapies should not focus on individual targets within a single pathway but, rather, on dysregulated cellular networks as a whole, giving place to combinatorial personalized therapies as the rational approach to overcome the failure of single drugs in complex diseases such as cancer, diabetes, and schizophrenia.[48] The strength of network biology lies in the multidimensional data that can be computationally integrated and used to identify specific and reliable therapeutic network targets to construct models of cellular decision-making processes. In this respect, in addition to protein networks, the cellular microenvironment is very important.[49]

The shift from single drug targeted therapy to combinatorial personalized therapies in cancer introduces a new challenge if we consider the whole arsenal of targeted therapies as a treatment option

for every patient. New methodologies are needed to optimize the design of combinatorial therapies to achieve the best response rates with minimal toxicity because this decision requires a transition from the one-drug/one-biomarker approach to global strategies that simultaneously assign markers to a catalog of drugs.

Classically, the drug mechanism of action refers to the description of a specific biochemical event, although in recent years, a series of drug-induced molecular/phenotypic measurements in an experimental system afford "signature"-based predictions. When these measurements arise from many drugs, the corresponding data provide multivariate signatures that fingerprint the drugs according to their relative signature similarity, but these molecular/phenotypic signatures have not been adapted to the examination of multidrug combinations. For instance, by examining the response to a diverse selection of chemotherapeutics of cells expressing short hairpin RNAs (shRNAs), which are sequences of RNA used to silence target gene expression via RNA interference (RNAi),[50] it is possible to generate a functional shRNA signature that permits the accurate grouping of these drugs into established biochemical modes of action and the prediction of mechanisms of action for molecules that are poorly characterized.[51] However, when two drugs are combined, one agent may simply reinforce the action of another or, alternatively, their combined effects may be distinct from either individual compound. Correspondingly, the shRNA signature of a combination drug would either resemble that of an individual drug or exhibit distinct genetic dependencies. In the latter case, the combined signature may be an average of individual drugs to mimic a compound not present in the combination or to adopt a novel signature. Some strategies to optimize the design of combinatorial therapies in order to achieve the best response rates with minimal toxicity have been proposed.[52]

Personalized treatments with targeted therapies designed to treat cancers carrying specific molecular alterations have been proposed as the next battle in the war against cancer. The use of clinical biomarkers to identify patients who are more likely to benefit from a given target therapy makes it possible to match a given treatment with specific patient characteristics.[53] At this point, problems associated with rapidly mutating targets and the development of drug resistances appear. For instance, in the context of breast cancer, the level of the receptor tyrosine kinase HER2/*neu* is used to select the monoclonal antibody trastuzumab (Herceptin®) as an adjuvant therapy,[54] but some patients who initially respond to the targeted therapy regress subsequently due to the occurrence of secondary molecular alterations such as the expression of the protein p95HER-2, a truncated form of the HER2 receptor lacking the extracellular domain to bind the antibody. In the context of melanoma, vemurafenib (Zelboraf®) is useful in patients with the b-Raf (V600E) mutation, where the valine-600 residue of this protein is replaced by glutamic acid.[55] However, most effectively treated patients regress after approximately 1 year due to compensatory pathways, such as receptor tyrosine kinases or N-Ras upregulation or dimerization of aberrantly spliced b-Raf (V600E).[56] In contrast to melanoma patients, colon cancer patients harboring the same b-Raf (V600E) mutation show a very limited response to this drug—a difference that can be understood by considering that treatment with vemurafenib induces feedback activation of EGFR, leading to stimulated cell growth. This adverse effect counteracts the advantages of b-Raf inhibition, especially in colon cancer, in which EGFR levels are higher than those in melamoma.[57]

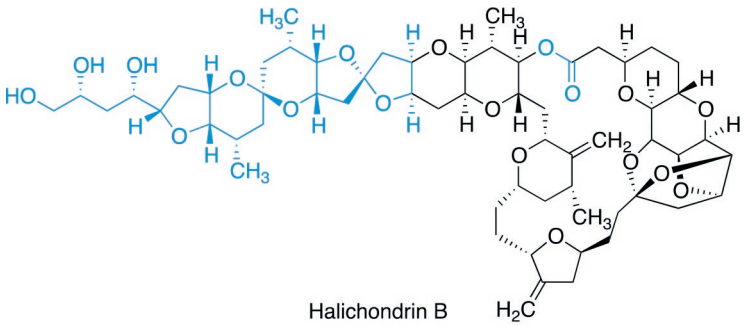
The development of monoclonal antibodies as anticancer agents has been remarkable in the past few years, but many antibody-based approaches have serious limitations because they are ineffective on target antigen-negative tumor cells, which may be preexistent in the lesion or raised through antigen shedding, masking, or therapy-induced downregulation. Other advanced strategies, such as the use of

recombinant fusion proteins in which a tumor-selective antibody fragment is fused to sTRAIL or sFasL ligands of death receptors, also fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in death receptor or caspase apoptosis pathways. In these cases, to have apoptosis induction with minimal effects on normal cells, the combinatorial use of various pro-apoptotic agents working along different or complementary apoptotic signaling routes is necessary. The strategies to achieve a longer efficacy for anticancer treatments rely on the identification of specific cancer-related aberrancies in each patient, and they require the development of reliable, cost-effective, and high-throughput diagnostic tools. In this respect, laser-capture microscopy and DNA microarray technology permit the obtention of large quantities of gene expression data from individual cancer cells, although it is still difficult to extract meaningful information from these data and to connect them to tumor-specific phenomena or drug information.

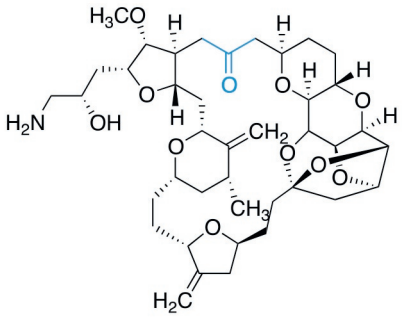
7 NATURAL PRODUCTS IN CANCER CHEMOTHERAPY

Since the beginning of chemotherapy, plants, microorganisms, and, more recently, marine organisms of various types have traditionally represented a main source of cytotoxic anticancer agents.[58] Nature is a source of potential chemotherapeutic agents and also of lead compounds that have provided the basis and inspiration for the semisynthesis or total synthesis of effective new drugs. The discovery of several effective anticancer agents from plants may be attributed, directly or indirectly, to a history of use of the relevant plant in traditional medicine. From the mechanistic standpoint, microtubules are a very frequent target of cytotoxic natural products.

A large number of drugs in clinical use as anticancer drugs are of natural product origin, and it has been estimated that approximately 80% of new chemical entities with small-molecule structures introduced during the period from 1950 to 2010 in this field were natural products or were natural-product inspired (small molecules, in turn, represent 77% of the total).[59] Despite this statistic, pharmaceutical companies have neglected the development of potential natural drug candidates. The main reason for this reluctance lies primarily in supply problems, which make necessary the development of synthetic routes often long and difficult to scale up because of the structural complexity of natural products. It is becoming increasingly apparent, however, that the unguided production of vast libraries of compounds is unlikely to result in the identification of new drugs, whereas natural products have in general several functional groups that are located in a precise 3D position, providing specific interactions with target molecules. It is often assumed that secondary metabolites have been optimized through evolution and that, consequently, they may be considered as highly advanced lead compounds in which further optimization of activity is difficult.[60] Nevertheless, in many cases, some parts of the complex structure of a natural product act only as a framework to position determined atoms, and simpler analogs may be developed without considerable loss of activity. For this reason, structural modification of natural products is often directed to find a possible simplest portion that maintains most of the biological activity—that is, its pharmacophoric unit. One example of this approach is the discovery of the antitumor agent eribulin (E-7389) in the development of synthetic strategies to obtain halichondrin B. Studies revealed that deletion of a large portion of this natural product and the replacement of the unstable lactone by a ketone function did not significantly affect its antimitotic properties (see Chapter 9, Section 2.1.2).[61] Eribulin was approved to treat several cancers and is under clinical trials for other types.



Halichondrin B



Eribulin

Although combinatorial chemistry, diversity-oriented synthesis, and high-throughput screening (HTS) of large compound libraries are important technologies in the discovery of bioactive molecules, the role of natural sources in providing new cytotoxics continues to be relevant.[62,63] Indeed, the notion that the use of natural-product templates combined with chemical modifications leading to more selective analogs has a better chance of success than combinatorial approaches is gaining acceptance. In other words, it appears that, at least in the anticancer field, "nature has already carried out the combinatorial chemistry" and all we have to do is refine the structures.[64] These ideas have led to an increased interest in natural products as drug candidates.[65]

8 A BRIEF COMMENT ABOUT CANCER NANOTECHNOLOGY

Nanotechnology is a field of applied science that covers a broad range of topics in which matter is controlled on a scale of 1–100 nm. Its application to cancer chemotherapy includes the use of nanovectors for the targeted delivery of antitumor compounds and imaging contrast agents, aiming at increasing the efficacy per dose of therapeutic or imaging contrast formulations.[66]

Liposomes, which are the simplest forms of nanovectors, use the EPR effect to increase drug concentration at tumor sites, and they were first applied to anthracyclines in order to avoid their cardiotoxicity. The refinement of liposomes and their application in cancer chemotherapy is still an active field of research, although other novel drug-delivery modalities have appeared.[67,68] In general, a nanovector has a core constituent material, a therapeutic and/or imaging payload, and biological surface

modifiers to enhance biodistribution and tumor targeting. Among several types of nanoparticles directed to enhance the properties of magnetic resonance imaging (MRI) contrast agents,[69] dendrimers, which are self-assembling polymers, have been used in mouse models of breast cancer to study the lymphatic drainage by MRI.[70]

Beyond nanovectors, several nanotechnologies are realistic candidates for the precise patterning of biological molecules, including DNA microarrays and surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectroscopy.[71]

Microarrays are devices used for molecular diagnostics, genotyping, and biomarking. They are single-stranded DNA probes that are prepared through a sequential procedure that implies selective ultraviolet deprotection of hydroxyl groups. With the ability to control the molecular depositions of polynucleotides in a nanometer range, the information density might be packed in nanoarrays directed at nucleic acids[72] or at the detection of proteomic profiles.[73]

9 SUMMARY OF FDA-APPROVED ANTICANCER DRUGS

Tables 1.1–1.9 summarize the main drugs approved by the FDA for use as anticancer agents according to the chapter in which they first appear.[74] The preponderance in recent years of targeted approaches to cancer treatment over cytotoxicity-based chemotherapy is readily appreciated. Orphan drug designations, drug combinations, and adjuvants in cancer therapy have been excluded.

Table 1.1 FDA-Approved Anticancer Drugs Described in Chapter 2

Drug	Mechanism of Action	Approval Date (First Indication)
6-Mercaptopurine (Purinethol®)	Purine biosynthesis inhibitor	1953
Methotrexate	Dihydrofolate reductase inhibition	1962
5-Fluorouracil (5-FU)	Thymidylate synthase inhibitor	1966
Tegafur (Ftorafur®)	Thymidylate synthase inhibitor	1967
Hydroxyurea (Hydrea®)	Ribonucleotide reductase inhibitor	1967
Cytarabine (Ara-C, Cytosar U®)	DNA synthesis inhibitor	1969
Floxuridine (FUDR)	Thymidylate synthase inhibitor	1970
L-Asparaginase (Elspar®)	Hydrolysis of circulating L-asparagine	1978
Pentostatin (Nipent®)	Adenosine deaminase inhibitor	1991
Fludarabine (Fludara®)	DNA synthesis inhibitor	1991
Cladribine (Litak®)	DNA synthesis inhibitor	1992
Trimetrexate (Neutrexin®)	DHFR inhibitor	1994
Gemcitabine (Gemzar®)	DNA synthesis inhibitor	1996
Capecitabine (Xeloda®)	DNA synthesis inhibitor	1998
Raltitrexed (Tomudex®)	Thymidilate synthase inhibitor	1998
Azacitidine (Vidaza®)	DNA synthesis inhibitor	2004
Clofarabine (Clolar®)	DNA synthesis inhibitor	2004
Pemetrexed (Alimta®)	Thymidilate synthase and dihydrofolate reductase inhibitor	2004

Continued

Table 1.1 FDA-Approved Anticancer Drugs Described in Chapter 2—cont'd

Drug	Mechanism of Action	Approval Date (First Indication)
Eniluracil	DNA synthesis inhibitor	2005
Nelarabine (Arranon®)	DNA synthesis inhibitor	2005
Pegaspargase (Oncaspar®)	Hydrolysis of circulating L-asparagine	2006
Fludarabine (Fludara®)	DNA synthesis inhibitor	2008
Pralatrexate (Folotyn®)	Dihydrofolate reductase inhibitor	2009
Erwinia chrysanthemi asparaginase (Erwinaze®)	Hydrolysis of circulating L-asparagine	2011

Table 1.2 FDA-Approved Anticancer Drugs Described in Chapter 3

Drug	Mechanism of Action	Approval Date (First Indication)
Medroxyprogesterone (Provera®)	Gestagen receptor agonist	1959
Testolactone (Teslac®)	Steroidal aromatase inhibitor	1970
Mitotane (Lysodren®)	Glucocorticoid biosynthesis inhibitor	1970
Megestrol acetate (Megace®)	Gestagen receptor agonist	1971
Tamoxifen (Nolvadex®)	Antiestrogen	1977
Aminoglutethimide (Cytadren®)	Aromatase inhibitor	1981
Leuprorelin (Lupron®)	GnRH (LHRH) agonist	1985
Lupron Depot®		1996
Flutamide (Eulexin®, Drogenil®)	Antiandrogen	1989
Goserelin (Zoladex®)	GnRH (LHRH) agonist	1989
Finasteride (Proscar®)	5α-Reductase inhibitor	1992
Bicalutamide (Casodex®)	Antiandrogen	1995
Anastrozole (Arimidex®)	Aromatase inhibitor	1995
Goserelin acetate (Zoladex®)	GnRH (LHRH) agonist	1996
Nilutamide (Nilandron®)	Antiandrogen	1996
Letrozole (Femara®)	Aromatase inhibitor	1997
Octeotride (Sandostatin®)	Somatostatin analog	1998
Exemestane (Aromasin®)	Aromatase inhibitor	1999
Alitretinoin (Panretin®)	Retinoid	1999
Bexarotene (Targretin®)	Retinoid	1999
Triptorelin (Trelstar®)	GnRH (LHRH) agonist	2000
Fulvestrant (Faslodex®)	Antiestrogen	2002
Dutasteride (Avodart®)	5α-Reductase inhibitor	2002
Abarelix (Plenaxis®)	GnRH (LHRH) antagonist	2003
Histrelin (Vantas®)	GnRH (LHRH) agonist	2004
Raloxifene (Evista®)	Antiestrogen	2007
Degarelix (Firmagon®)	GnRH (LHRH) antagonist	2008
Abiraterone (Zytiga®)	CYP17A1 inhibitor	2011
Enzalutamide (Xtandi®)	Antiandrogen	2012
Pasireotide (Signifor®)	Somatostatin analog	2012

Table 1.3 FDA-Approved Anticancer Drugs Described in Chapter 4

Drug	Mechanism of Action	Approval Date (First Indication)
Actinomycin D (Cosmege®)	ROS generation	1964
Bleomycin (Blenoxane®)	ROS generation	1973
Doxorubicin (Adriamycin®)	ROS generation	1974
Daunomycin (Cerubidine®)	ROS generation	1979
Idarubicin (Idamycin®)	ROS generation	1990
Valrubicin (Valstar®)	ROS generation	1998
Porfimer sodium oligomer (Photophrin®)	Photodynamic therapy of cancer	1995
Epirubicin (Ellence®)	ROS generation	1999
Methoxsalen (Uvadex®)	Non-porphirin photosensitizers	1999
^{90}Y-ibritumomab tiuxetan (Zevalin®)	Radiolabeled monoclonal antibody	2002
^{111}In-capromab pendetide (ProstaScintas®)	Radiolabeled monoclonal antibody	2002
^{131}I-tositumomab (Bexxar®)	Radiolabeled monoclonal antibody	2003
ALA (Levulan®)	Photosensitizer	2004
Amifostine (Ethiol®)	Radioprotector	2008
Alpharadin (Xofigo®, $^{233}RaCl_2$)	Radiopharmaceutical	2013

Table 1.4 FDA-Approved Anticancer Drugs Described in Chapters 5 and 6

Drug	Mechanism of Action	Approval Date (First Indication)
Mechlorethamine (Mustargen®)	DNA alkylation	1949
Triethylenemelamine (Tetramine®)	DNA alkylation	1953
Busulfan (Myleran®)	DNA alkylation	1954
Chlorambucil (Leukeran®)	DNA alkylation	1957
Cyclophosphamide (Cytoxan®)	DNA alkylation	1959
Thiotepa (Thioplex®)	DNA alkylation	1959
Melphalan (L-PAM, Alkeran®)	DNA alkylation	1959
Uracil mustard	DNA alkylation	1962
Pipobroman (Vercyte®)	Unknown	1966
Procarbazine (Matulane®)	DNA alkylation	1969
Mitomycin C (Mutamycin®)	Minor groove alkylation	1974
Dacarbazine (DTIC-Dome®)	DNA alkylation	1975
Lomustine (CCNU, CeeNU®)	DNA alkylation	1976
Carmustine (BiCNU®)	DNA alkylation	1977
Cisplatin (Platinol®)	DNA complexation	1978
Streptozotocin (Zanosar®)	DNA alkylation	1982
Ifosfamide (Mitoxana®)	DNA alkylation	1988
Carboplatin (Paraplatin®)	DNA complexation	1989
Altretamine (Hexalen®)	DNA alkylation	1990
Melphalan (Alkeran®)	DNA alkylation	1993
Busulfan (Myleran®)	DNA alkylation	1999
Temozolomide (Temodal®, Temodar®)	DNA alkylation	1999
Oxaliplatin (Eloxatin®)	DNA complexation	2002
Bendamustine (Ribomustin®)	DNA alkylation	2008

Table 1.5 FDA-Approved Anticancer Drugs Described in Chapters 7 and 8

Drug	Mechanism of Action	Approval Date (First Indication)
Actinomycin D (Cosmege®)	DNA intercalation	1964
Doxorubicin (DOX, Adriamycin®)	Topoisomerase II inhibition	1974
Daunomycin (DNR, Cerubidine®)	Topoisomerase II inhibition	1979
Etoposide (VP-16-213)	Topoisomerase II inhibition	1983
Mitoxantrone (Novantrone®)	Topoisomerase II inhibition	1988
Idarubicin (Idamycin®)	Topoisomerase II inhibition	1990
Teniposide (Vumon®)	Topoisomerase II inhibition	1992
Topotecan (Hycamtin®)	Topoisomerase I inhibition	1996
Irinotecan (Camptosar®)	Topoisomerase I inhibition	1996
Valrubicin (Valstar®)	Topoisomerase II inhibition	1998
Epirubicin (Ellence®)	Topoisomerase II inhibition	1999
Mithramycin A (Mithracin®)	Histone methyltransferase inhibitor	1970
5-Azacitidine (Vidaza®)	DNA methyltransferase inhibitor	2004
Decitabine (Dacogen®)	DNA methyltransferase inhibitor	2006
Vorinostat (SAHA, Zolinza®)	Histone deacetylase inhibitor	2006
Romidepsin (FK-228, Istodax®)	Histone deacetylase inhibitor	2009
Belinostat (PDX-101, Beleodaq®)	Histone deacetylase inhibitor	2014

Table 1.6 FDA-Approved Anticancer Drugs Described in Chapter 9

Drug	Mechanism of Action	Approval Date (First Indication)
Vincristine (Oncovin®)	Microtubule polymerization inhibitor	1963
Vinblastine (Velban®)	Microtubule polymerization inhibitor	1965
Estramustine (Estracyt®, Emcyt®)	Microtubule polymerization inhibitor	1981
Paclitaxel (Taxol®)	Microtubule-stabilizing agent	1992
Vinorelbine tartrate (Navelbine®)	Microtubule polymerization inhibitor	1994
Docetaxel (Taxotere®)	Microtubule-stabilizing agent	1996
Ixabepilone (Ixempra®)	Microtubule-stabilizing agent	2007
Eribulin mesylate (Halaven®)	Microtubule polymerization inhibitor	2010
Cabazitaxel (Jevtana®)	Microtubule-stabilizing agent	2010

Table 1.7 FDA-Approved Anticancer Drugs Described in Chapters 10 and 11

Drug	Mechanism of Action	Approval Date (First Indication)
Trastuzumab (Herceptin®)	HER-2 inhibitor	1998
Arsenic trioxide (Trisenox®)	Apoptosis induction	2000
Imatinib mesylate (Glivec®)	Bcr-Abl inhibitor	2001

Table 1.7 FDA-Approved Anticancer Drugs Described in Chapters 10 and 11—cont'd

Drug	Mechanism of Action	Approval Date (First Indication)
Gefitinib (Iressa®)	EGFR inhibitor	2003
Erlotinib (Tarceva®)	EGFR inhibitor	2004
Cetuximab (IMC-C225, Erbitux®)	EGFR inhibitor	2004
Bevacizumab (Avastin®)	VEGF inhibitor	2004
Sorafenib (Nexavar®)	Multikinase inhibitor	2005
Dasatinib (Sprycel®)	Bcr-Abl and Src inhibitor	2006
Sunitinib (Sutent®)	VEGFR inhibitor	2006
Panitumumab (Vectibix®)	EGFR inhibitor	2006
Nilotinib (Tasigna®)	Bcr-Abl inhibitor	2007
Temsirolimus (Torisel®)	mTOR inhibitor	2007
Lapatinib (Tyverb®)	EGFR and HER-2 inhibitor	2007
Plerixafor (Mozobil®)	CXCR4 inhibition	2008
Pazopanib (Votrient®)	VEGFR inhibitor	2009
Everolimus (Afinitor®)	mTOR inhibitor	2009
Vemurafenib (Zelboraf®)	Raf inhibitor	2011
Crizotinib (PF-02341066, Xalkori®)	ALK and c-Met inhibitor	2011
Vandetanib (Caprelsa®)	VEGFR and EGFR inhibitor	2011
Ruxolitinib (Jakavi®)	JAK inhibitor	2011
Bosutinib (SKI-606, Bosulif®)	Bcr-Abl and Src inhibitor	2012
Cabozantinib (Cometriq®)	Inhibitor of VEGFR and related receptors	2012
Ponatinib (AP24534, Iclusig®)	Bcr-Abl inhibitor	2012
Regorafenib (Stivarga®)	Raf inhibitor	2012
Omacetaxine mepesuccinate (Synribo®)	Inhibitor of protein synthesis	2012
Pertuzumab (2C4, Perjeta®)	HER-2 inhibitor	2012
Axitinib (Inlyta®)	Inhibitor of VEGFR and related receptors	2012
Dabrafenib (Tafinlar®)	Raf inhibitor	2013
Trametinib (Mekinist®)	MEK inhibitor	2013
Afatinib (Gilotrif®)	EGFR inhibitor	2013
Ibrutinib (PCI-32765, Imbruvica®)	Bruton's tyrosine kinase inhibitor	2013
Idelalisib (Zydelig®)	PI3K inhibitor	2014
Ceritinib (Zykadia®)	ALK inhibitor	2014
Bortezomib (Velcade®)	Proteasome inhibitor	2003
Thalidomide (Thalomid®)	Angiogenesis inhibition	2006
Lenalidomide (Revlimid®)	Angiogenesis inhibition	2006
Carfilzomib (Krypolis®)	Proteasome inhibitor	2012
Vismodegib (GDC-0449, Erivedge®)	Smo receptor inhibitor	2012
Pomalidomide (CC- 4047, Imnovid®)	Angiogenesis inhibition	2013
Lenvatinib (Lenvima®)	VEGFR-2 inhibitor	2015

Table 1.8 FDA-Approved Anticancer Drugs Described in Chapter 12

Drug	Mechanism of Action	Approval Date (First Indication)
Aldesleukin (Proleukin®)	Modified human IL-2	1992
Rituximab (Rituxan®)	Anti-CD20	1997
Recombinant interferon α-2b (Intron A®)	Immunotherapeutic agent	1997
Denileukin diftitox (Ontak®)	IL-2 receptor antagonist	1999
Alemtuzumab (Campath®)	Anti-CD-52	2001
^{131}I-tositumomab (Bexxar®)	Anti-CD20 with radioactive iodine	2003
Imiquimod (Aldara®, Zyclara®)	Agonist of toll-like receptors 7 and 8	2004
Gardasil®	Cancer vaccine	2006
Plerixafor (Mozobil®)	Chemokine receptor-4 (CXCR4) antagonist	2008
Cervarix®	Human papillomavirus vaccine	2009
Ofatumumab (Arzerra®)	Anti-CD20	2009
Sipuleucel-T (Provenge®)	Autologous vaccine	2010
Denosumab (Prolia®)	Anti-RankL	2010
Brentuximab vedotin	Anti-CD30	2011
Ipilimumab (Yervoy®)	Anti-CTLA-4	2011
Catumaxomab (Removab®)	Anti-EpCAM, CD3, and FcγRs	2011
Ziv-aflibercept (Zaltrap®)	VEGF inhibitor	2011
Obinutuzumab (Gazyva®)	Anti-CD20	2013
Ramucirumab (Cyramza®)	VEGFR-2	2014
Pembrolizumab (Keytruda®)	PD-1 receptor antibody	2014

Table 1.9 FDA-Approved Anticancer Drugs Described in Chapters 13–15

Drug	Mechanism of Action	Approval Date (First Indication)
Pegaspargase (Oncaspar®)	Hydrolysis of circulating L-asparagine	1994
Liposomal doxorubicin (Doxil®)	ROS generation, topoisomerase II inhibition	1995
Liposomal daunorubicin (DaunoXome®)	ROS generation, topoisomerase II inhibition	1996
Liposomal cytarabine (Depocyt®)	Inhibition of DNA strand elongation	1999
Gemtuzumab ozogamicin (Mylotarg®)	Radical-induced DNA strand scission	2000
Pegfilgrastim (Neulasta®)	Granulocyte colony-stimulating factor (G-CSF)	2002
Albumin-bound paclitaxel (Abraxane®)	Microtubule stabilization	2005
Ibritumomab tiuxetan (^{90}Y) (Zevalin®)	Ionizing radiation	2009
Genexol-PM®	Microtubule stabilization	2010
Brentuximab vedotin (Adcetris®)	(Antibody–drug conjugate)	2011
Marqibo®	(Liposomal vincristine)	2012
Oxorubicin liposome (LipoDox®)	(Liposomal doxorubicin)	2013
Trastuzumab-DM1, ado-trastuzumab	Microtubule depolymerization	2013
Ado-trastuzumab emtansine (Kadcyla®)	Microtubule depolymerization	2013
Tamoxifen (Nolvadex®, Valodex®)	Selective estrogen receptor modulator (SERM)	1998

REFERENCES

1 Alwan A, editor. *Global status report on noncommunicable diseases 2010*. Geneva: World Health Organization; 2011.
2 Mukherjee S. *The emperor of all maladies: a biography of cancer*. New York: Simon & Schuster; 2010.
3 Watson J. *Open Biol* 2013;**3**:120144.
4 Nelson SM, Ferguson LR, Denny WA. *Cell Chromosome* 2004;**3**:2.
5 Hanahan G, Weinberg RA. *Cell* 2000;**100**:57.
6 Bissell MJ, Radisky D. *Nature Rev Cancer* 2001;**1**:46.
7 Hanahan G, Weinberg RA. *Cell* 2011;**144**:646.
8 Lebowitz P. *J Clin Oncol* 1983;**1**:657.
9 (a) Shih Ch, Weinberg RA. *Cell* 1982;**29**:161; (b) Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M. *Nature* 1982;**298**:343.
10 (a) Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. *Nature* 1984;**308**:145; (b) Williams AF. *Nature* 1984;**308**:108.
11 (a) Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. *N Engl J Med* 2010;**363**:711; (b) Robert C, Thomas L, Bondarenko I, O'Day S, Garbe C, Lebbe C, et al. *N Engl J Med* 2011;**364**:2517.
12 Couzin-Frankel J. *Science* 2013;**342**:1432.
13 Relling MV, Hancock M, Rivera GK, Sandlund JT, Ribeiro RC, Krynetski EY, et al. *J Natl Cancer Inst* 1999;**91**:2001.
14 Innocenti F, Schilsky RL. *Dis Model Mech* 2009;**2**:426.
15 Yong WP, Soo R, Innocenti F. In: Neidle S, editor. *Cancer drug design and discovery*. 2nd ed. New York: Academic Press; 2014.
16 The Cancer Genome Atlas Network. *Nature* 2012;**490**:61.
17 Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. *N Engl J Med* 2012;**366**:883.
18 Yong E. *Nature* 2014;**511**:524.
19 Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. *Cancer Epidemiol Biomarkers Prev* 1994;**3**:67.
20 Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. *Nat Med* 2014;**20**:548.
21 Esserman LJ, Thompson Jr IM, Reid B. *JAMA* 2013;**310**:797.
22 Neidle S, Thurston DE. *Nature Rev Cancer* 2005;**5**:285.
23 Lathrop AE, Loeb L. *J Cancer Res* 1916;**1**:1.
24 Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. *J Natl Cancer Inst* 1998;**90**:1371.
25 Goss PE, Ingle JN, Alés-Martínez JE, Cheung AM, Chlebowski RT, Wactawski-Wende J, et al. *N Engl J Med* 2011;**364**:2381.
26 Eckhardt S. *Curr Med Chem Anticancer Agents* 2002;**2**:419.
27 http://www.sanger.ac.uk/genetics/CGP/Census.
28 Marchini S, Broggini M. *Curr Med Chem Anticancer Agents* 2004;**4**:247.
29 Hurley LH. *Nature Rev Cancer* 2002;**2**:188.
30 Gago F. *Methods* 1998;**14**:277.
31 Longley DB, Harkin DP, Johnston PG. *Nature Rev Cancer* 2003;**3**:330.
32 Bradbury RH, editor. *Top Med Chem* 2007;**1**.
33 Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. *Semin Oncol* 1999;**26**:60.
34 Capdeville R, Buchdunger E, Zimmermann J, Matter A. *Nature Rev Drug Discov* 2002;**1**:493.
35 Atkins JH, Gershell LJ. *Nature Rev Drug Discov* 2002;**1**:491.

36 Segota E, Bukowski RM. *Cleveland Clin J Med* 2004;**71**:551.
37 Nygren P, Larsson R. *J Internal Med* 2003;**253**:46.
38 Torchilin VP, Eur J. *Pharm Sci* 2000;**11**:81.
39 Jain RK. *Cancer Metastasis Rev* 1987;**6**:559.
40 Gottesman MM, Fojo T, Bates SE. *Nature Rev Cancer* 2002;**2**:48.
41 Hanahan D, Coussens LM. *Cancer Cell* 2012;**3**:309.
42 Nygren P, Larsson R. *J Internal Med* 2003;**253**:46.
43 Wang Ch, Tang Z, Zhao Y, Yao R, Li L, Sun W. *Biofabrication* 2014;**6**:022001.
44 Díaz LA, Saurabh S. *Nature Rev Drug Dis* 2005;**4**:375.
45 Toffoli G, Cecchin E, Corona G, Boiocchi M. *Curr Med Chem Anticancer Agents* 2003;**3**:225.
46 Schwartz L, Summa M, Steyaert JM, Guais-Vergne A, Baronzio GF. *Conference Papers in Medicine* 2013; Article ID 827686, http://dx.doi.org/10.1155/2013/827686.
47 Pritchard JR, Bruno PM, Gilbert LA, Capron KL, Lauffenburger DA, Hemann MT. *Proc Natl Acad Sci U S A* 2013;**110**:E170.
48 Pawson T, Linding R. *FEBS Lett* 2008;**582**:1266.
49 Cox T, Releer JT. *Tumor Microenviron Ther* 2012;**1**:14.
50 Wang Z, Rao DD, Senzer N, Nemunaitis J. *Pharm Res* 2011;**28**:2983.
51 Jiang H, Pritchard JR, Williams RT, Lauffenburger DA, Hemann MT. *Nat Chem Biol* 2011;**7**:92.
52 See, for instanceVázquez A. *BMC Systems Biol* 2013;**7**:31.
53 Trusheim MR, Berndt ER, Douglas FL. *Nature Rev Drug Discov* 2007;**6**:287.
54 Burstein HJ. *N Engl J Med* 2005;**353**:1652.
55 Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. *N Engl J Med* 2011;**364**:2507.
56 (a) Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. *Nature* 2010;**468**:973; (b) Poulikakos PI, Persaud Y, Janakiraman M, Kong X, Ng C, Moriceau G, et al. *Nature* 2011;**480**:387.
57 Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. *Nature* 2012;**483**:100.
58 Butler MS. *Nat Prod Rep* 2005;**22**:162.
59 Newman DJ, Cragg GM. *J Nat Prod* 2012;**75**:311.
60 Cozzi P, Mongelli N, Suarato A. *Curr Med Chem Anticancer Agents* 2004;**4**:93.
61 Zheng W, Seletsky BM, Palme MH, Lydon PJ, Singer LA, Chase CE, et al. *Bioorg Med Chem Lett* 2004;**14**:5551.
62 Cragg GM, Grothaus PG, Newman DJ. *Chem Rev* 2009;**109**:3012.
63 Cozzi P, Mongelli N, Suarato A. *Curr Med Anticancer Agents* 2004;**4**:93.
64 Mann J. *Nature Rev Cancer* 2002;**2**:143.
65 Paterson I, Anderson E. *Science* 2005;**310**:451.
66 Ferrari M. *Nature Rev Cancer* 2005;**5**:161.
67 Couvrer P, Vauthier C. *Pharm Res* 2006;**23**:1417.
68 Nishiyama N, Kataoka K. *Pharmacol Ther* 2006;**112**:630.
69 Kircher MF, Mahmood U, King RS, Weissleder R, Josephson L. *Cancer Res* 2003;**63**:8122.
70 Kobayashi H, Choyke PL, Brechbiel MW, Waldmann TA. *J Natl Cancer Inst* 2004;**96**:703.
71 Vorderwülbecke S, Cleverley S, Weinberger SR, Wiesner A. *Nat Methods* 2005;**2**:393.
72 Demers LM, Ginger DS, Park S-J, Li Z, Chung S-W, Mirkin CA. *Science* 2002;**296**:1836.
73 Brickbauer A, Klenerman D. *J Am Chem Soc* 2004;**126**:6508.
74 A reference Web page for FDA-approved drugs, classified by therapeutic area: http://www.centerwatch.com/drug-information/fda-approved-drugs/therapeutic-area/12/oncology/?mp=HealthLinks.

CHAPTER

ANTIMETABOLITES THAT INTERFERE WITH NUCLEIC ACID BIOSYNTHESIS

2

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00002-8

1 INTRODUCTION

Antimetabolites can be defined as analogs of naturally occurring compounds that interfere with their formation or utilization, thus inhibiting essential metabolic routes. Although the enzymes inhibited by antimetabolites are also present in normal cells, some selectivity toward cancer cells is possible due to their faster division rates.

Most antimetabolites interfere with nucleic acid synthesis, and for this reason we study in this chapter the antitumor compounds that hamper the production of DNA or RNA by a variety of mechanisms, including the following:

1. Competition for binding sites of enzymes that participate in essential biosynthetic processes
2. Incorporation into nucleic acids, which inhibits their normal function and triggers the apoptosis process

Because of this mode of action, most antimetabolites have high cell cycle specificity.

A brief outline of DNA biosynthesis is given in Figure 2.1, including the main steps where antimetabolite drugs discussed in this chapter exert their action.

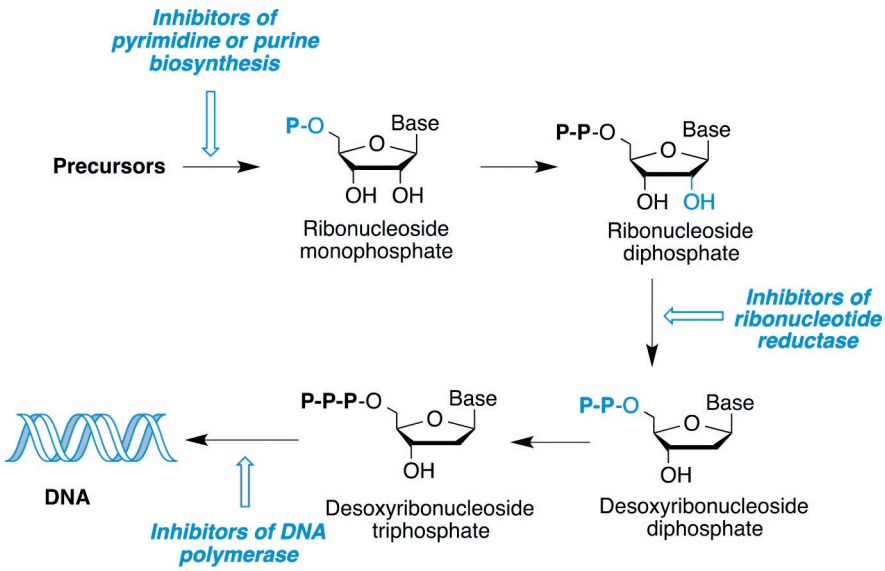


FIGURE 2.1

Types of anticancer drugs that interfere with DNA biosynthesis.

Although clinically useful antimetabolites ultimately inhibit DNA (and sometimes RNA) synthesis, their site of action may be separated many steps away from these reactions. Specific interference with the *de novo* nucleic acid pathways in cancer cells is probably not possible because tumoral and normal cells use the same biosynthetic routes. Nevertheless, some antimetabolites are remarkably effective against some human cancers and are still one of the bases of cancer chemotherapy.

2 INHIBITORS OF THE BIOSYNTHESIS OF URIDYLIC ACID

The biosynthesis of pyrimidine nucleotides starts with the construction of the heterocyclic system by carbamoylation of aspartate followed by cyclization to dihydroorotate. Its dehydrogenation gives orotate, which then reacts with phosphoribosyl pyrophosphate (PRPP) to give orotidylate. Finally, uridylic acid (uridine monophosphate (UMP)) is generated by decarboxylation (Figure 2.2). UMP is the precursor to other pyrimidine nucleotides, after its conversion to the corresponding nucleoside triphosphate (UTP).

Among the many compounds known to inhibit reactions of this pathway, we mention only *N*-phosphonoacetyl-L-aspartate (PALA), an inhibitor of aspartate transcarbamoylase that acts as a

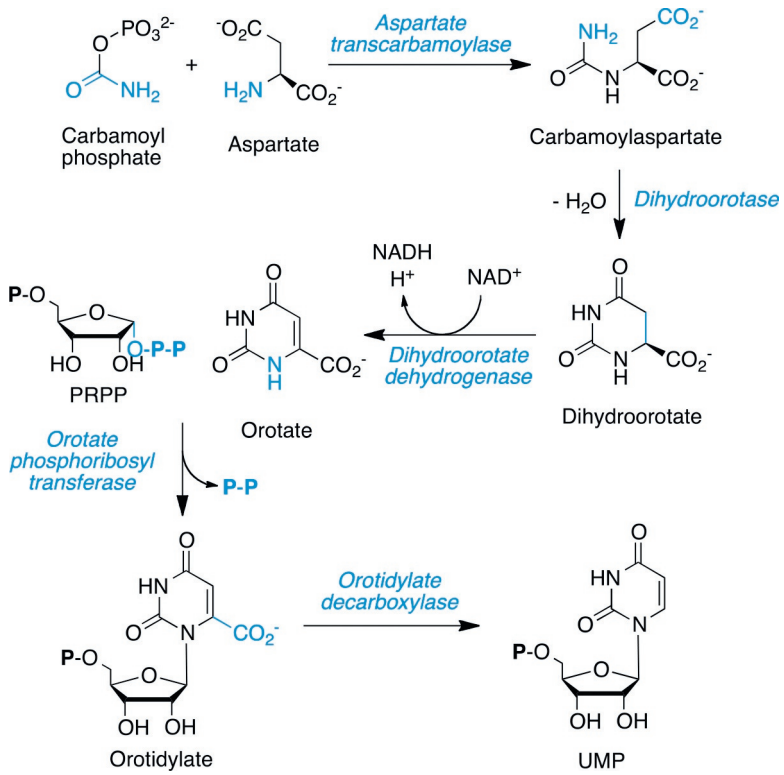


FIGURE 2.2

Biosynthesis of pyrimidine nucleotides.

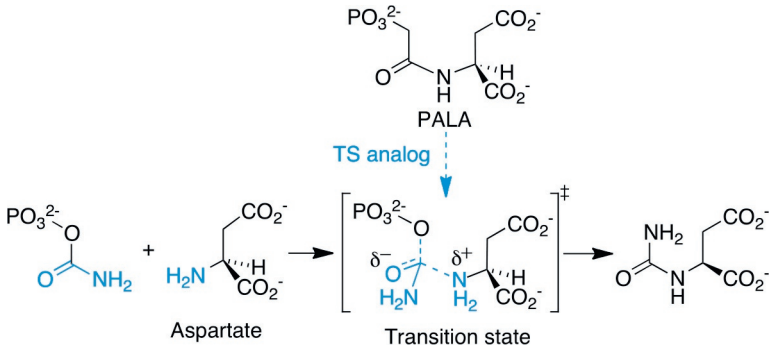


FIGURE 2.3

Inhibition of aspartate transcarbamoylase by PALA.

transition state analog (Figure 2.3). This compound has undergone some clinical trials, normally in combination with 5-fluorouracil, another pyrimidine antimetabolite.[1]

3 INHIBITORS OF RIBONUCLEOTIDE REDUCTASE

The biosynthesis of 2′-deoxyribonucleotides, the immediate precursors of DNA, involves the replacement of the 2′-OH group by a hydrogen atom (Figure 2.4). This reaction takes place on ribonucleoside-5′-diphosphates and is catalyzed by the enzyme ribonucleotide reductase (RNR), also known as nucleoside diphosphate reductase (NDPR). RNR is the rate-determining enzyme in the supply of deoxynucleotides, and its substrates are ADP, GDP, CDP, and UDP. Deoxythymidine diphosphate (dTDP) is synthesized by another enzyme (thymidylate kinase) from deoxythymidine monophosphate (dTMP). Ribonucleotide reductase thus plays a central role in cell growth and proliferation by ensuring a balanced supply of nucleotide precursors for DNA synthesis, and it has been identified as an important target for hematologic malignancies.[2]

3.1 STRUCTURE AND CATALYTIC CYCLE OF RIBONUCLEOTIDE REDUCTASE

The most extensively studied ribonucleotide reductase is that from *Escherichia coli*, which is considered as a suitable prototype for the mammalian enzyme. In eukaryotes, ribonucleotide reductase has two subunits, with each containing a dinuclear iron center that generates an essential stable tyrosyl

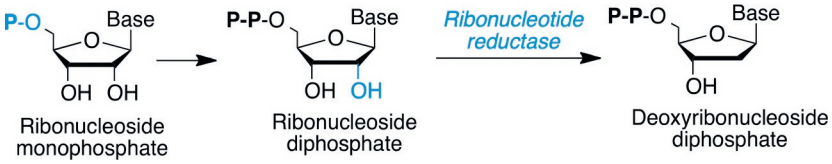


FIGURE 2.4

Biosynthesis of 2-deoxyribonucleotides.

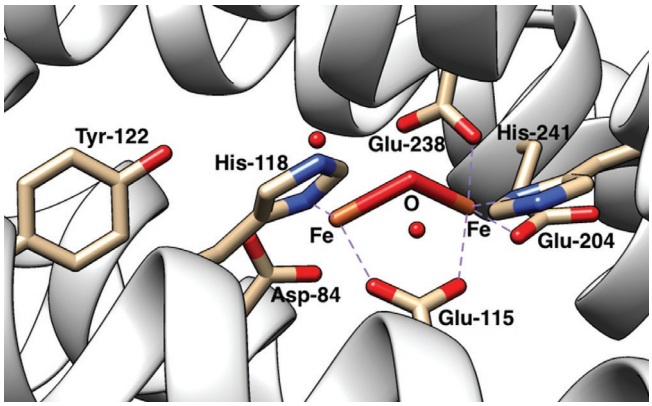


FIGURE 2.5

Structure of the iron binding site in the ribonucleotide reductase R2 subunit from *E. coli*, generated from Protein Data Bank reference 1AV8 and displayed with Chimera 1.8.1.

radical by one electron oxidation of a nearby tyrosine (Tyr-122) deeply buried inside the protein, in a highly hydrophobic environment. The Fe cations are coordinated to a number of residues (Asp-84, His-118, His-241, Glu-115, Glu-204, and Glu-238) and two molecules of water (Figure 2.5).

The enzyme generates and stabilizes a tyrosyl radical through a redox process that transforms the initial Fe(II) complex into a binuclear oxo-bridged Fe(III) complex. A high-resolution X-ray diffraction study has shown that the first iron atom is pentacoordinate, although it maintains an octahedral structure, and the second one is hexacoordinate (Figure 2.6).[3]

Although the Tyr-122 radical triggers the reductive process, it is too far away from the catalytic site. Therefore, it must generate a second radical in the vicinity of the substrate, probably a thiyl radical from Cys-439. The cysteine radical then abstracts the $C_{3'}$–H atom of the nucleoside diphosphate substrate and generates the anion-radical **2.1**, with prior or simultaneous deprotonation of the $C_{3'}$–OH group by the Glu-441 residue of the enzyme. Two cysteine residues, probably Cys-225 and Cys-462, form the redox-active sulfhydryl pair responsible for the reduction of this radical. Thus, protonation of the $C_{2'}$–OH and subsequent elimination of a molecule of water yields a cation that is stabilized by migration of the unpaired electron from C-3′ to C-2′ to give **2.2**. The Cys-462 mercapto group transfers a proton and one electron to this radical to give **2.3**, with concomitant formation of a disulfide anion radical, which then transfers one electron to the carbonyl group in **2.3**, leading to **2.4**. Radical **2.4** is transformed into **2.5** by a mechanism reverse to the one that produced **2.1**, and the active center of the enzyme is finally regenerated by reduction of the newly formed disulfide unit by thioredoxin, an ubiquitous protein that has a pair of proximal cysteine residues, which reacts with the oxidized form of ribonucleotide reductase via disulfide exchange (Figure 2.7).[4]

It is interesting to note that the enzymatic reaction of ribonucleotide reductase is initiated by the formation of a radical (species **2.2**), even though the reactions leading to reductive elimination of the $C_{2'}$–OH group are ionic. The reason for this type of mechanism may be the stabilization of **2.2** through the effect of the radical at C-3 on the intermediate carbocation formed at C-2, as shown by the following resonance structures:

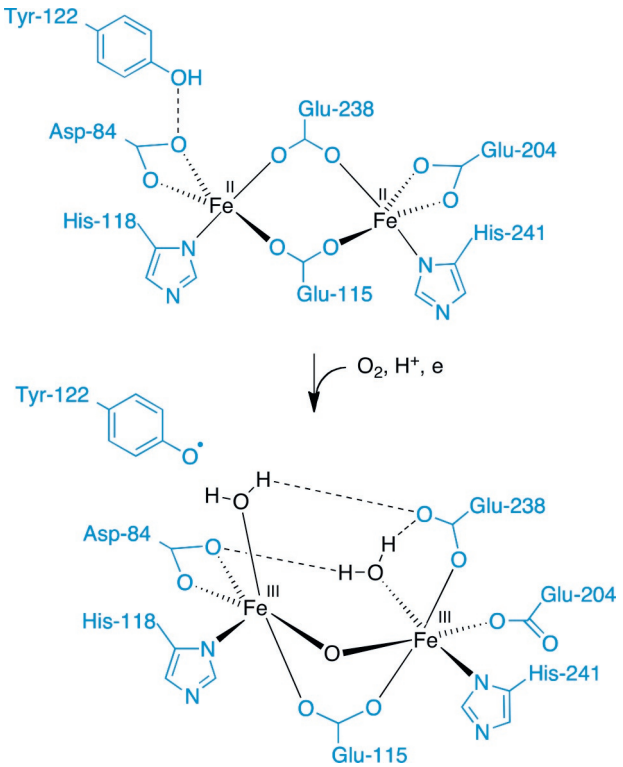
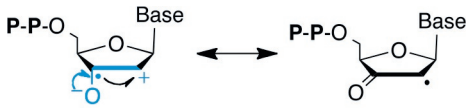


FIGURE 2.6

Generation of a tyrosyl radical in the active site of ribonucleotide reductase.



2.2

3.2 GALLIUM SALTS AND COMPLEXES

Gallium ions can inhibit DNA synthesis through substitution of Ga^{3+} for Fe^{3+} in the M_2 subunit of ribonucleotide reductase. Furthermore, their synergy with hydroxyurea has been demonstrated.[5] Ga^{3+} is usually administered as its nitrate salt or as gallium maltolate, a complex formed by a Ga^{3+} cation coordinated and three maltolate ligands, derived from 2-methyl-3-hydroxy-4-pyrone (maltol). Clinical studies have shown gallium nitrate to have significant antitumor activity against non-Hodgkin's lymphoma and bladder cancer,[6] but only 60% of patients show a positive response due to resistance problems associated with decreased Ga uptake and other mechanisms. It shows renal toxicity because it tends to

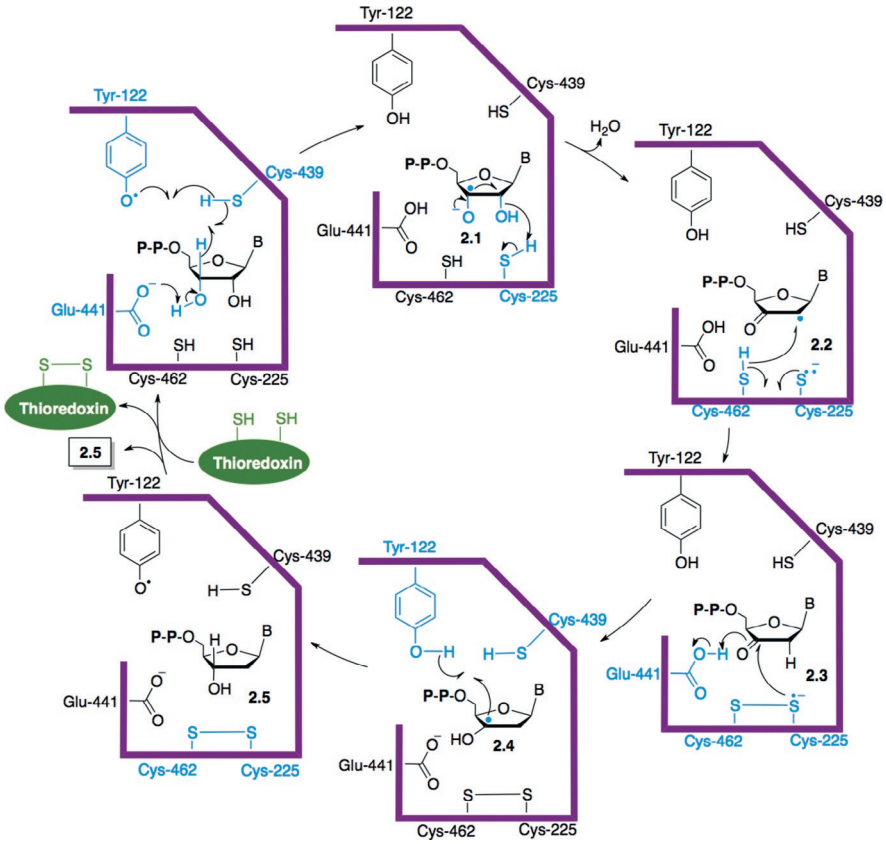
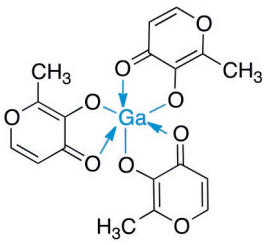


FIGURE 2.7

Catalytic cycle of ribonucleotide reductase.

form gallate anion ($Ga(OH)_4^-$) in blood, which is rapidly excreted in the urine. Gallium maltolate has the advantage of a lower renal toxicity, which is probably due to the fact that it becomes nearly entirely protein-bound in plasma. This drug has been tested in phase II clinical studies in patients with metastatic prostate cancer and refractory multiple myeloma. Interestingly, it has shown very good potential against metastasis, which has been attributed to its good transport into all kinds of cells by transferrin.[7]



Gallium maltolate

3.3 RADICAL SCAVENGERS

The best known inhibitor of ribonucleotide reductase is hydroxyurea (Hydrea®, Droxia®).[8] After oral administration, this compound is well absorbed and transported into cells, where it quenches the tyrosyl radical at the active site of ribonucleotide reductase, inactivating the enzyme (Figure 2.8).[9]

Nitric oxide, an important cell signaling molecule involved in many physiological processes, is one of the metabolic products of hydroxyurea, and its formation may contribute to the antitumor effect of the latter. In fact, nitric oxide is known to inhibit ribonucleotide reductase by itself, probably because it contains an unpaired electron and therefore it is able to quench the Tyr radical.[10] The mechanisms involved in the metabolic transformation of hydroxyurea into nitric oxide are multiple[11] and involve three-electron reduction processes. As an example, the mechanism of the peroxidase-mediated formation of nitric oxide from dismutation of the hydroxyurea radical to generate a nitroso derivative followed by hydrolysis of the latter is shown in Figure 2.9.

Hydroxyurea is primarily used in the management of myeloproliferative disorders, such as chronic granulocytic leukemia, polycythemia vera, and essential thrombocytosis, and is sometimes combined with other antitumor drugs such as the tyrosine kinase inhibitor imatinib.[12] Other applications of hydroxyurea include its use as a radiosensitizer and in AIDS therapy, in combination with didanosine. Hydroxyurea is also useful in the treatment of sickle cell anemia[13] because it eases the pain of the patients. This has been attributed to the previously mentioned generation of nitric oxide, a potent vasodilator.[14]

Thiosemicarbazones, represented by triapine, are another important class of inhibitors of ribonucleotide reductase. Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP) is a very strong iron chelator, and the iron chelate is probably the active species that quenches the active site

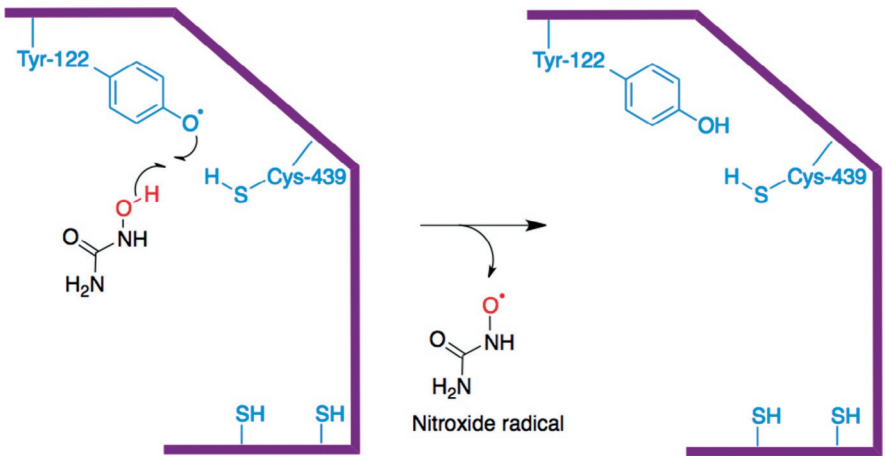


FIGURE 2.8

Mechanism of RNR inhibition by hydroxyurea.

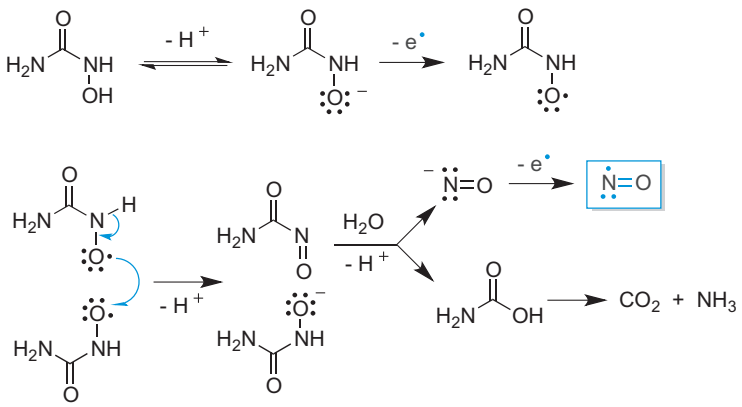
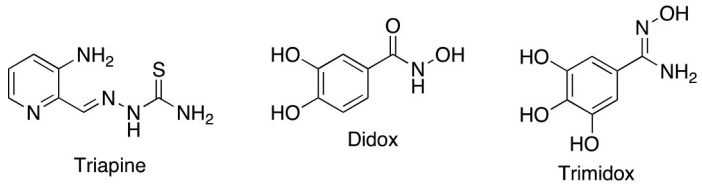


FIGURE 2.9

Mechanism of the peroxidase-mediated formation of nitric oxide from hydroxyurea.

tyrosyl radical of ribonucleotide reductase. 3-AP is a broad-spectrum anticancer agent[15] that has undergone phase I and II clinical studies for a variety of cancers, including solid tumors,[16] metastatic breast cancer,[17] and, in combination with cisplatin, locally advanced cervical cancer.[18]

Hydroxamic acid derivatives such as didox and trimidox are also RNR inhibitors. Didox, which is one of the most potent known inhibitors of the enzyme, has been recommended as a free radical scavenger to be used in combination with doxorubicin in order to lower its cardiotoxicity while enhancing its anticancer activity.[19] Trimidox was initially considered as an anticancer agent,[20] but it is employed mainly as an antibacterial agent for veterinary use.

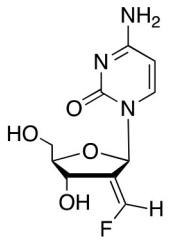


Triapine Didox Trimidox

3.4 SUBSTRATE ANALOGS AS RIBONUCLEOTIDE REDUCTASE INHIBITORS

Ribonucleotide reductase substrate analogs are normally modified at C-2′, which is the position that undergoes reduction in the natural substrate. Many of these compounds bind covalently to the enzyme.

Tezacitabine (FmdC) is a nucleoside prodrug that shows a dual mechanism of action. Following intracellular phosphorylation, the tezacitabine diphosphate irreversibly inhibits ribonucleotide reductase, whereas the tezacitabine triphosphate can be incorporated into DNA during replication or repair, resulting in DNA chain termination.[21]

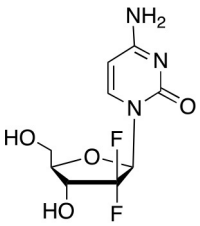


Tezacitabine (FMdC)

The mechanism of RNR irreversible inhibition by tezacitabine (2.6) starts by its conversion into tezacitabine diphosphate **2.7**, which, similarly to the natural substrate of the reaction, undergoes H-3′ abstraction by Cys-439, leading to a radical that is stabilized by delocalization onto the adjacent C=C double bond (structure **2.8**). The subsequent evolution of this radical has been studied through theoretical calculations, and the mechanistic pathway that has been proposed to be the major one is summarized in Figure 2.10.[22] The main steps involved are abstraction of the Cys-225 mercapto hydrogen by **2.8** to generate **2.9**, followed by attack from the Cys-439 mercapto group (probably assisted by deprotonation by Glu-441) and concomitant displacement of HF to give **2.10**. Final abstraction of a hydrogen atom by the Cys-225 radical leads to the proposed final reaction product, the stabilized radical **2.11**.

After initially promising clinical data, analysis of the data from a phase II trial in patients with gastroesophageal cancer prompted the decision to discontinue further development of tezacitabine.

Gemcitabine (dFdC) is another nucleoside prodrug that has DNA polymerase inhibition as its primary mechanism of action (see Section 8.1), but it also has some activity as an RNR inhibitor.



Gemcitabine
(dFdC)

The mechanism of the interaction of gemcitabine diphosphate (**2.12**) with the active site of RNR is very similar to that of the natural substrate, and it deviates from the natural course only after the formation of the bisulfide bridge, as suggested by theoretical calculations.[23] The first steps are the abstraction of the 3′-OH proton by the Glu-441 residue and the abstraction of the 3′-H atom by the radical sulfur of Cys-439, leading to anion radical **2.13**. Protonation of the α-fluorine atom by the Cys-225 thiol group facilitates the elimination of a molecule of HF and the formation of radical **2.14**, where the unpaired electron is stabilized by the neighboring carbonyl groups and fluorine atom. Transfer

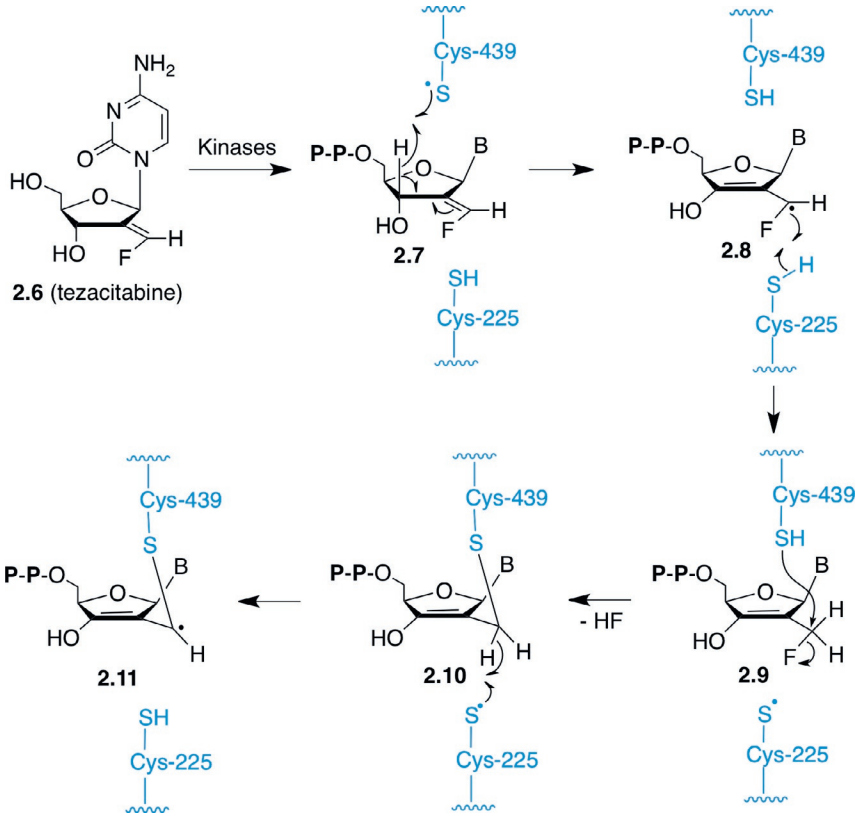


FIGURE 2.10

Mechanism of RNR inhibition by tezacitabine.

of one electron from the Cys-225 anion gives enolate **2.15**, which is protonated by Cys-462 to generate the neutral species **2.16**. The formation of a bisulfide bond and simultaneous transfer of a proton from Glu-441 back to the 3′-O atom leads to radical **2.17**, which upon elimination of HF generates the C-2 radical **2.18**. Abstraction of a hydrogen atom from the mercapto group of Cys-439 gives the sulfur radical **2.19**. Although in the natural substrate, Cys-439 does not reach the α face of the ribose ring, the conditions in this case are different because **2.19** cannot be stabilized by hydrogen bonds with the active site residues Glu-441 (which is charged) or Cys-225 and Cys-462, which are oxidized to a disulfide. This fact, together with possible interactions with the eliminated HF molecules, allows some degree of deviation of the position of the inhibitor, making it possible for Cys-439 to reach the 4′-H atom and allowing the generation of the stable radical **2.20** (Figure 2.11). This prevents the reaction of Cys-439 with Tyr-122 and hence the regeneration of the essential tyrosine radical (see also the transformation of **2.4** into **2.5** in Figure 2.7).

The stability of **2.20** is due to the captodative effect of the tetrahydrofuranone oxygen atoms because the unpaired electron is adjacent to both an electron-withdrawing and an electron-releasing group (Figure 2.12).

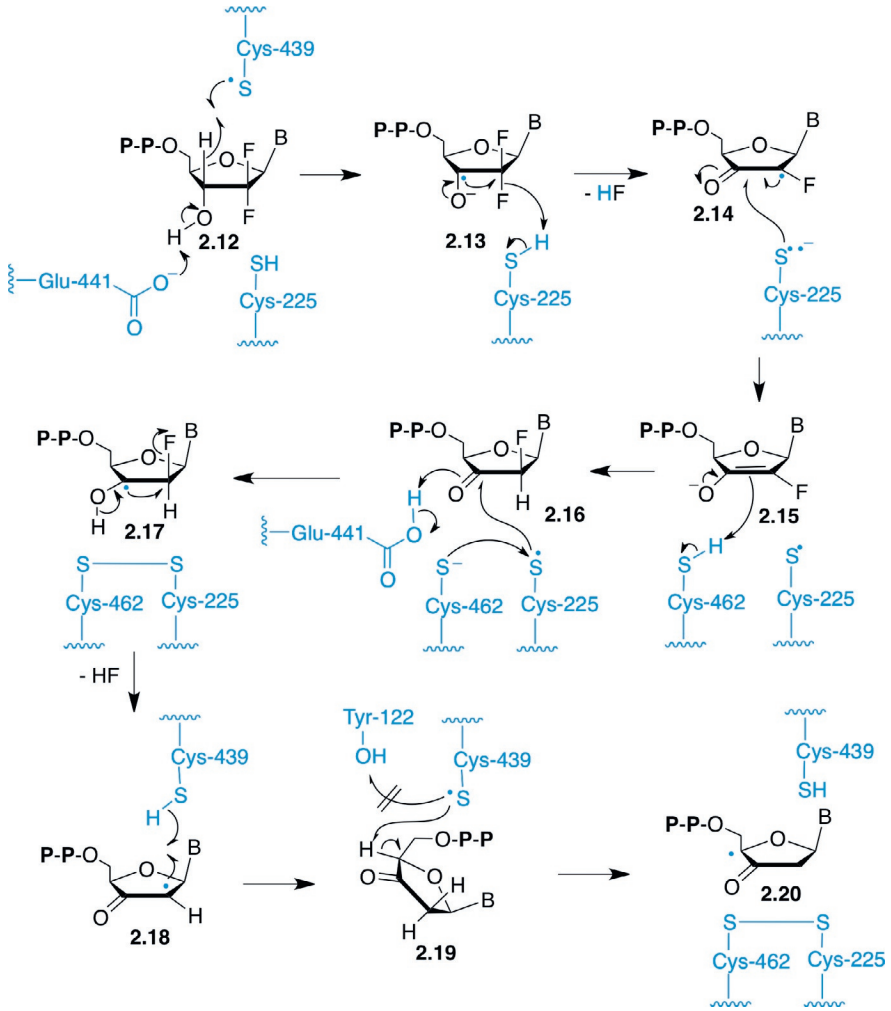


FIGURE 2.11

Mechanism of RNR inhibition by gemcitabine.

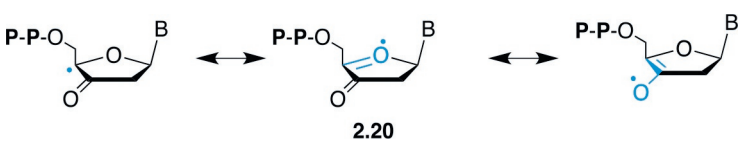


FIGURE 2.12

Captodative stabilization of radical **2.20**.

3.5 ALLOSTERIC INHIBITION OF RIBONUCLEOTIDE REDUCTASE VIA INHIBITION OF PURINE NUCLEOSIDE PHOSPHORYLASE

Therapeutically significant inhibition of RNR can also be achieved through a feedback mechanism by accumulation of deoxyguanosine triphosphate (dGTP) as a consequence of the inhibition of purine nucleoside phosphorylase (PNP), an enzyme that has a key role in purine catabolism, known as the "salvage pathway." This enzyme, also known as PNPase, catalyzes the phosphorolysis of the N-ribosidic bonds of purine nucleosides and deoxynucleosides to form purine and α-D-phosphorylated ribosyl products. This inhibition leads to increased blood levels of one of its substrates, deoxyguanosine (dG), which is specifically transported and phosphorylated by T-cell deoxynucleoside kinases. This process leads to pathologically elevated levels of dGTP in these cells, which results in allosteric RNR inhibition that results in apoptosis of T lymphocytes and B lymphocytes (Figure 2.13). PNP is

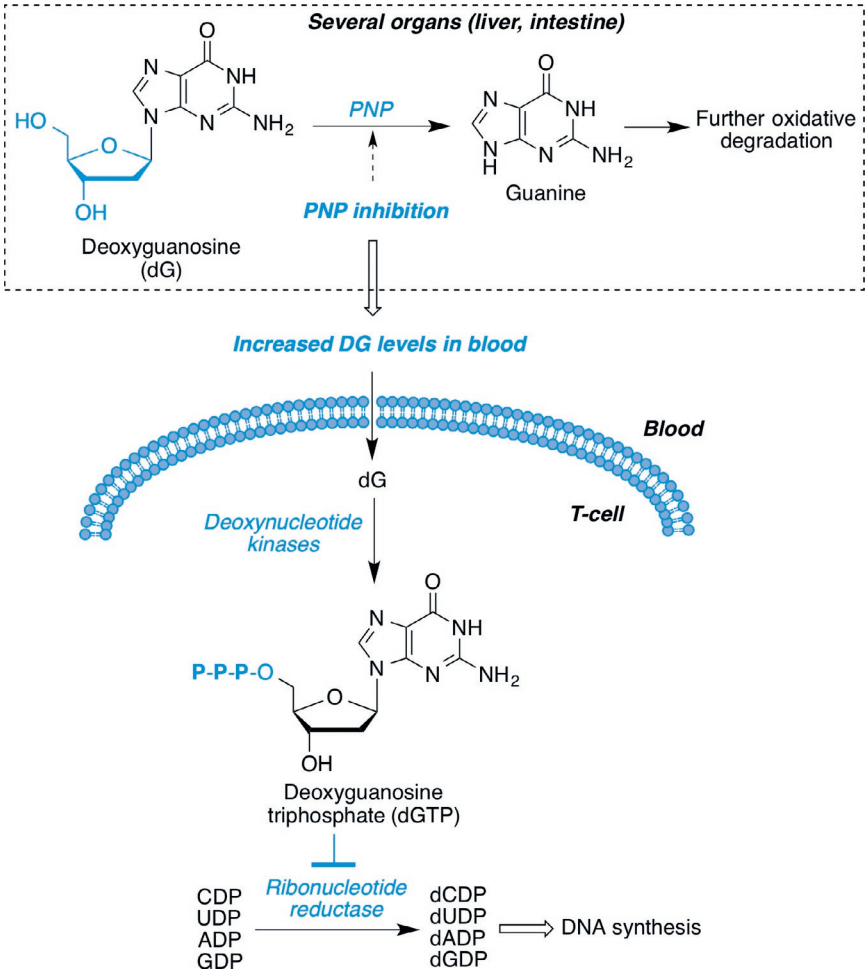


FIGURE 2.13

Feedback inhibition of ribonucleotide reductase.

thus a suitable target for inhibitor development aiming at T-cell immune response modulation, and specifically in the treatment of relapsed or refractory T-cell lymphoblastic lymphoma, acute leukemia, and T-cell prolymphocytic leukemia.[24]

Forodesine (immucillin H) is a 9-deazanucleoside with a pyrrolidine ring replacing the ribose tetrahydrofuran. It behaves as a very potent inhibitor of purine nucleoside phosphorylase because of the analogy of its protonated form with the structure of the transition state, which has oxacarbenium ion character with partial positive charge near C-1′. Forodesine has an NH group at N-7, and its charge distribution resembles that of the transition state when N-4′ is protonated to the corresponding cation (Figure 2.14).[25,26] This compound is orally bioavailable and is being developed as an anticancer agent. It has undergone phase II clinical trials in patients with a variety of B-cell and T-cell leukemias.[27] Orphan drug designation has been granted by both the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) for the use of forodesine for several indications.

Peldesine (BCX-34) is a structurally related purine nucleoside phosphorylase inhibitor. It has been clinically studied in cream formulation as topical therapy for patch and plaque-phase cutaneous T-cell lymphoma, but it was not significantly better than the control as therapy.[28]

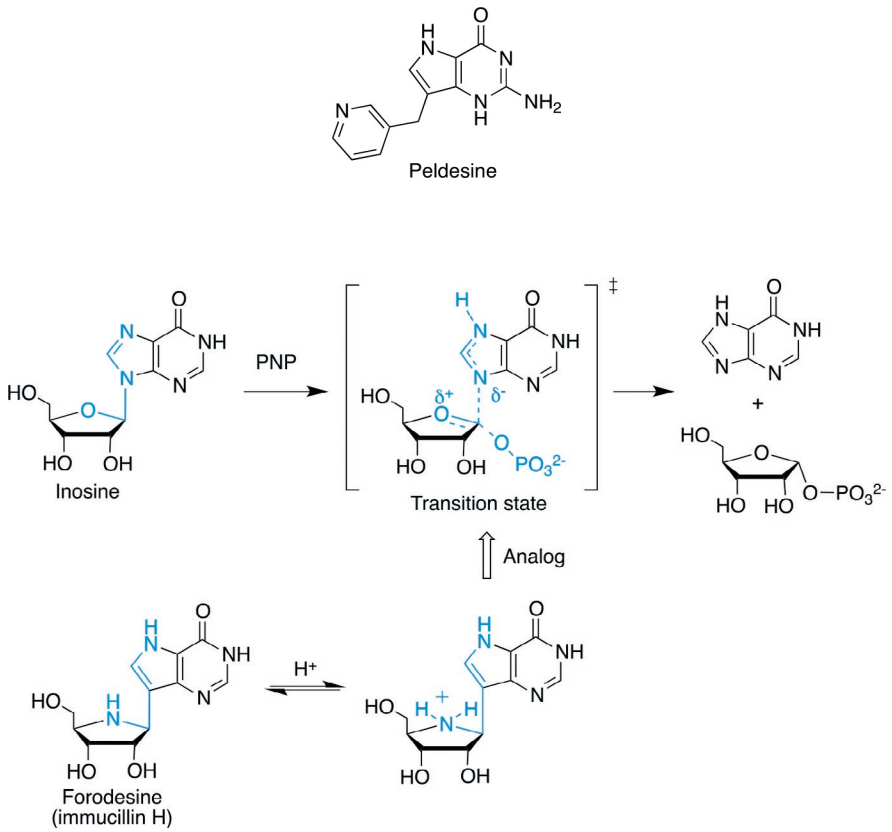


FIGURE 2.14

Mechanism of PNP inhibition by immunocillin H (forodesine).

Some clinically relevant deoxyadenosine derivatives acting primarily as inhibitors of DNA polymerases are also allosteric inhibitors of RNR after their conversion into the corresponding 5′-triphosphates, as discussed in Section 8. Therefore, these compounds have a dual action that has been described as "self-potentiation."

4 INHIBITORS OF THE BIOSYNTHESIS OF THYMIDILIC ACID

4.1 THYMIDYLATE SYNTHASE

Thymidylate synthase (TS) catalyzes the conversion of dUMP to thymidylate (TMP) in a reductive methylation that involves the transfer of a carbon atom from the cofactor 5,10-methylenetetrahydrofolate to the 5 position of the pyrimidine ring. Although methylation of uracil is apparently a small structural change, the extra lipophillicity and bulk associated with the methyl group is essential for the proper discrimination of thymine from the other three bases present in DNA chains by transcription factors, repressors, enhancers, and other DNA-binding proteins. This methylation process, which is the only *de novo* source of thymidilate, is part of the so-called thymidylate cycle (Figure 2.15), in which two other enzymes take part, namely serine hydroxymethyl transferase (SHMT) and dihydrofolate reductase (DHFR). SHMT catalyzes the formation of 5,10-methylenetetrahydrofolate from tetrahydrofolate (THF), coupled

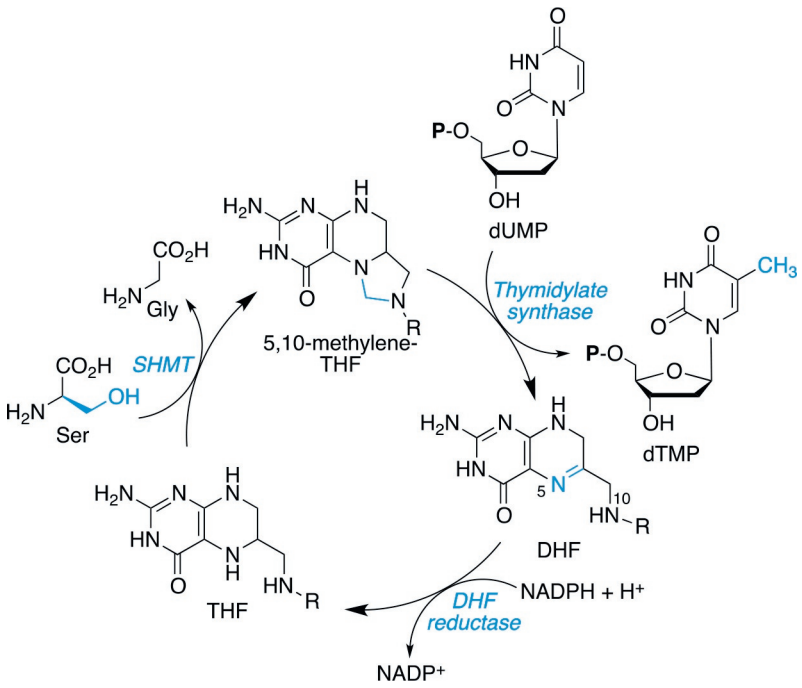


FIGURE 2.15

The thymidylate cycle.

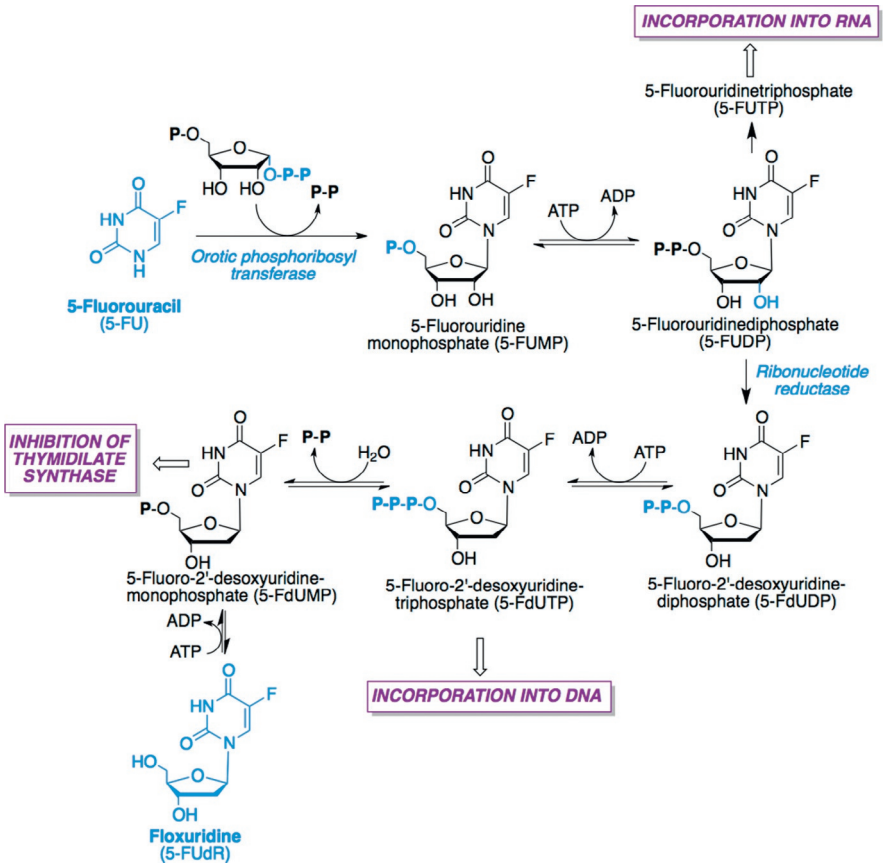


FIGURE 2.16

Antitumor species from the metabolism of 5-fluorouracil and floxuridine.

with the conversion of serine into glycine, with pyridoxal phosphate (PLP) as a cofactor. In the reaction catalyzed by TS, the 5,10-methylenetetrahydrofolate thus formed donates its methylene group to dUMP, being transformed into dihydrofolate (DHF) by a mechanism that is discussed below (see Figure 2.16). DHFR finally closes the cycle by reducing DHF to THF.

4.2 5-FLUOROURACIL AND FLOXURIDINE

The main inhibitors of thymidylate synthase are 5-fluorouracil (5-FU) and its deoxynucleoside floxuridine (5-FUdR), and these fluoropyrimidines represent the most widely prescribed class of anticancer drugs worldwide.[29] In particular, 5-FU is widely used in the treatment of cancers of the aerodigestive tract, breast, head, and neck and especially in colorectal cancers in combination therapies with oxaliplatin and irinotecan.[30,31] Administered as a cream, it is also useful for the treatment of some skin

cancers. 5-FUwas developed in the 1950s following the observation that rat hepatomas utilized uracil at a higher rate than normal tissues, which suggested that uracil metabolism could be a relevant antitumor target. Floxuridine is employed in the treatment of colorectal cancer metastatic to the liver. Due to its nucleoside structure, it has a very poor oral bioavailability and is administered in intra-arterial injection.

5-FUis a prodrug that enters the cell using the same facilitated transport mechanism as uracil and is activated to 5-fluoro-2′-deoxyuridine monophosphate (5-FdUMP) through the complex pathway summarized in Figure 2.16. Floxuridine requires a much simpler bioactivation, consisting of its monophosphorylation. Besides TS inhibition, an additional mechanism that explains the cytotoxic effect of these drugs is based on the misincorporation of their nucleotide and deoxynucleotide triphosphates to RNA and DNA, respectively.

The catalytic cycle of TS involves a two-stage process. Initially, dUMP binds to its recognition site and induces a conformational change that opens an adjacent binding site for the cofactor (5,10-CH_2-THF). A cysteine residue in the active site then covalently binds to the unsaturated carbonyl system in dUMP via a Michael addition (Figure 2.17).

The methyleneiminium cation **2.21**, generated from the cofactor, is attacked by the C-5 enolate **2.22**, arising from the reaction between the Cys residue and dUMP, to yield the covalent ternary complex **2.23**. An enzyme-catalyzed abstraction of the acidic H-5 proton promotes a β-elimination reaction of a molecule of tetrahydrofolate (**2.24**) and generates the methylene intermediate **2.25**. The last step of the sequence involves reduction of **2.25** by hydride transfer from **2.24**, leading to TMP and DHF (Figure 2.18). The overall reaction involves the oxidation of 5,10-methylenetetrahydrofolate to

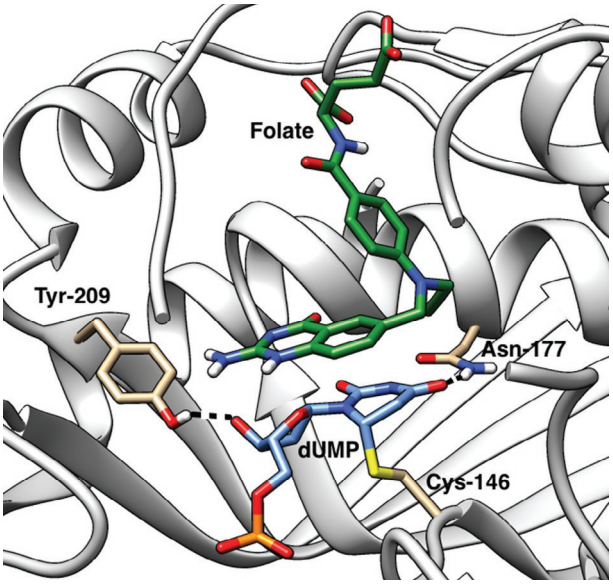


FIGURE 2.17

Structure of the ternary complex formed by *E. coli* thymidylate synthase, dUMP, and the folic acid analog CB3717, shown as a cofactor surrogate. Generated from Protein Data Bank reference 4KNZ and displayed with Chimera 1.8.1.

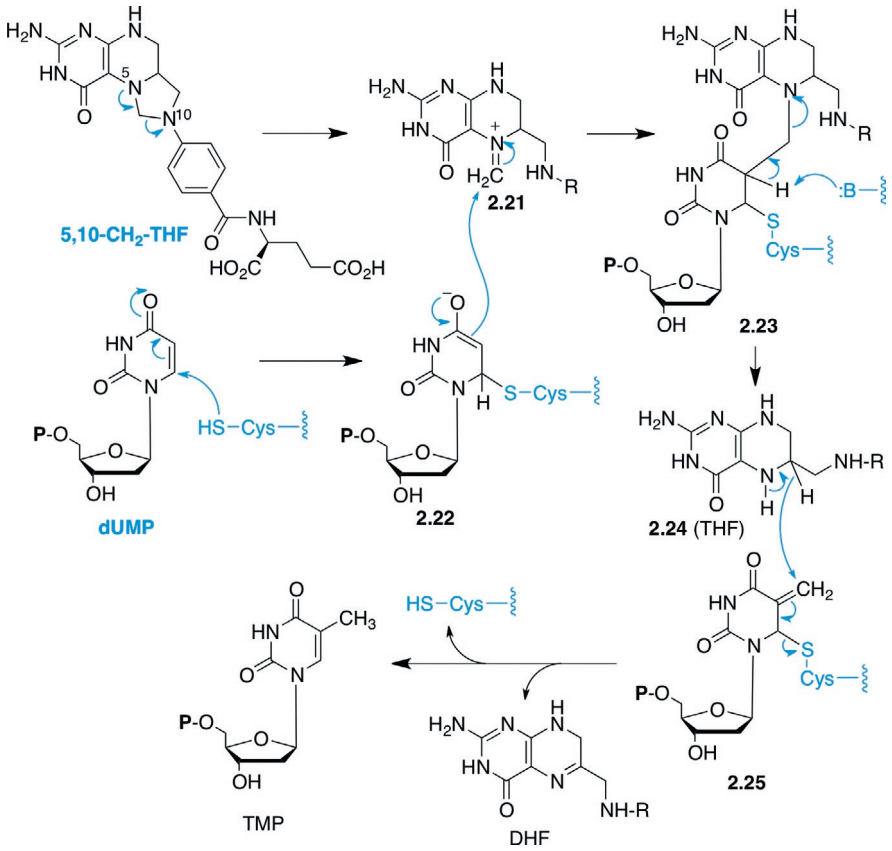


FIGURE 2.18

Mechanism of dUMP methylation by thymidylate synthase.

DHF, which must then be recycled by reduction by DHFR and subsequent methylenation, as discussed in Section 5.

Fluorine (1.47 Å) and hydrogen (1.20 Å) have very similar Van der Waals radii, and this allows 5-FdUMP to bind to TS in the same site and with the same affinity as dUMP. The strong electron-withdrawing effect of the fluorine atom increases the electrophilicity of the unsaturated carbonyl system and facilitates the formation of **2.26**, but the final β-elimination reaction is not possible due to the presence of the fluorine atom at C-5 and therefore the ternary complex **2.27** is stable (Figure 2.19). Because of the need for an activation step by nucleophilic attack of a cysteine residue of TS prior to enzyme inhibition, 5-FdUMP can be considered as a suicide inhibitor.

Thymidylate synthase inhibition leads to depletion of TMP and hence of dTTP, which induces alterations in the levels of other deoxynucleotides through various feedback mechanisms. These imbalances result in an alteration of the dATP/TTP ratio, among others, which disrupts DNA synthesis and repair and leads to the so-called thymineless death.[32] A cytotoxicity mechanism alternative to TS inhibition is based on the generation of 5-fluoro-2′-deoxyuridine triphosphate (5-FdUTP), which acts as a

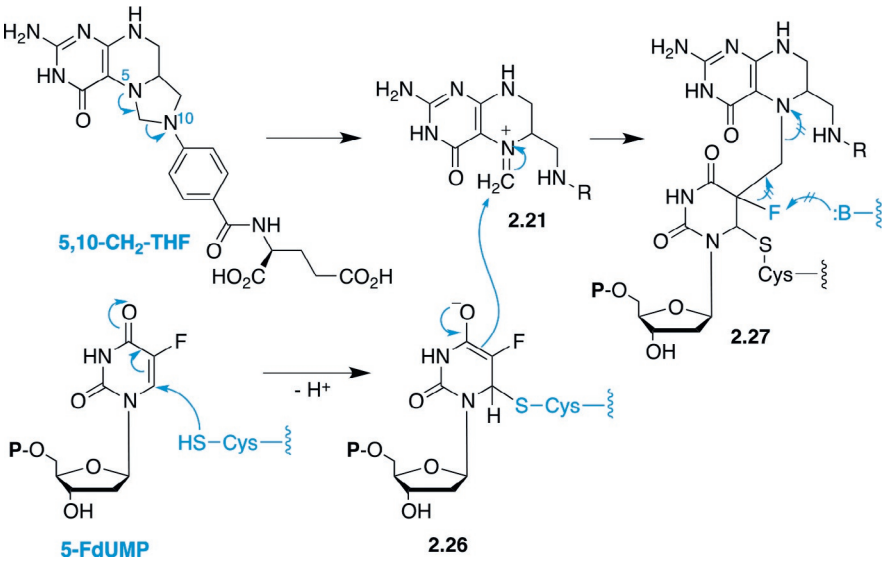


FIGURE 2.19

Mechanism of TS inhibition by 5-FdUMP, the 5-FU active metabolite.

false substrate of DNA polymerase and is misincorporated into DNA. As a consequence of the accumulation of dUMP after TS inhibition, dUTP can also be generated and incorporated into DNA. Ultimately, this change halts DNA synthesis and promotes DNA fragmentation by repair enzymes. Similarly, transformation of 5-FUDP, a metabolite of 5-fluorouracil, into the corresponding triphosphate allows the misincorporation of fluoronucleotides into RNA, leading to profound effects on cell metabolism and viability. Both TS inhibition and misincorporation of 5-FU metabolites in DNA result in the stabilization of p53, a tumor suppressor that maintains DNA integrity by activating genes that arrest cell cycle in response to DNA damage or trigger apoptosis (Figure 2.20). *In vitro* studies have proven that loss of p53 function is associated with a reduced sensitivity to 5-FU.[33]

The clinical efficacy of 5-FU may be decreased by several mechanisms, the first of which is diminished incorporation of 5-FUTP into RNA as a consequence of competition from high intracellular levels of UTP. On the other hand, the formation of the ternary TS–FdUMP–CH_2-THF complex induces TS expression due to the inhibition of a negative feedback mechanism whereby TS binds to its own mRNA and inhibits the translation process. The ternary complex is not able to bind to this mRNA, leading to increased expression of TS and constituting a possible resistance mechanism. Finally, TS inhibition leads to an increase in intracellular dUMP pools, which eventually compete with 5-FdUMP for binding with TS.

4.3 5-FLUOROURACIL PRODRUGS

5-FU requires intravenous administration, and a number of oral prodrugs have been designed to circumvent this limitation.[34] One of them is tegafur (Ftorafur®), which is completely absorbed in the gastrointestinal tract and metabolized to 5-FU through two major pathways. The first involves microsomal

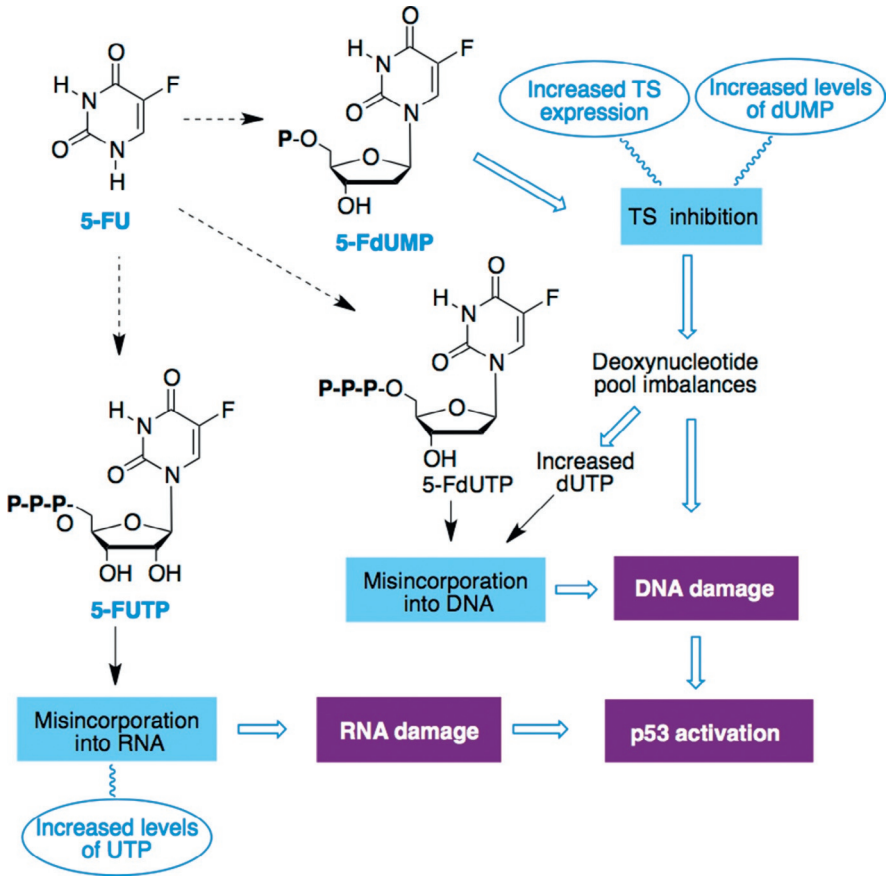


FIGURE 2.20

Mechanisms of the antitumor action and clinical efficacy decrease of 5-FU.

hydroxylation of the C-5′ position of the tetrahydrofuran moiety by cytochrome P450, followed by spontaneous decomposition to 5-FU and succinic aldehyde. A hydrolytic pathway due to cytosolic hydrolases (pyrimidine nucleoside phosphorylase) is also possible, giving 5-FU and 2-tetrahydrofuryl phosphate (Figure 2.21). Tegafur was introduced in the clinic in 1967 and showed a significant antitumor response. However, due to severe digestive and cardiac toxicities, it was soon replaced by its combination with several other enzyme inhibitors, especially UFT and S-1 (see Sections 4.4.1 and 4.4.3).

The levels of the hydrolytic enzyme thymidine phosphorylase (TP) are significantly higher in several solid tumors, such as colorectal, breast, and kidney cancers, compared to normal tissues. This led to assay doxifluridine (5′-deoxy-5-fluorouridine) as a 5-FU prodrug, but this compound showed gastrointestinal toxicity (diarrhea) after oral administration due to release of 5-FU by intestinal pyrimidine nucleoside phosphorylase (Figure 2.22). It is worth mentioning that because TP is identical to platelet-derived endothelial cell growth factor (PD-ECGF) and is believed to have angiogenic properties, it is considered as a cancer target.[35]

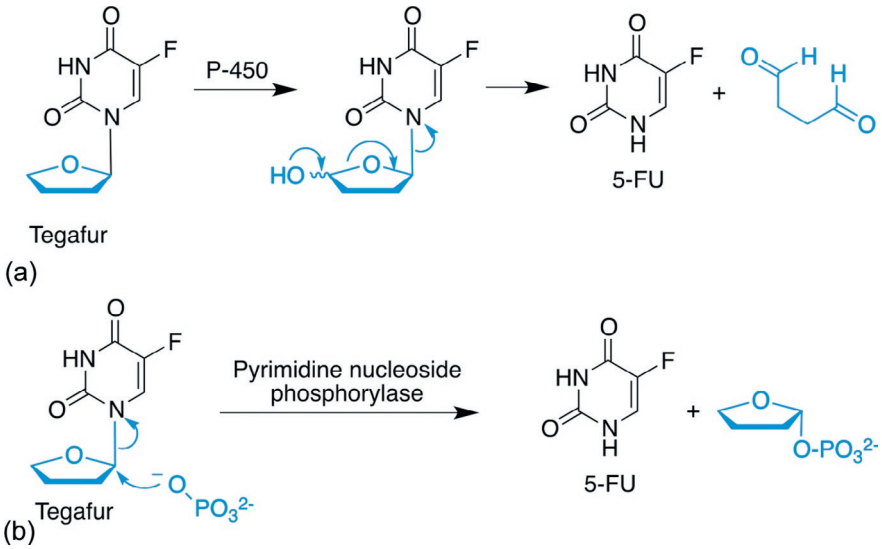


FIGURE 2.21

Bioactivation of tegafur.

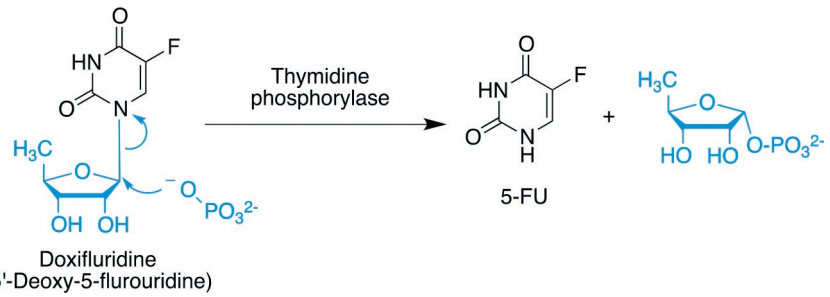


FIGURE 2.22

Bioactivation of doxifluridine.

Efforts to circumvent the problem of gastric toxicity led to the development of capecitabine (Xeloda®) as a multiple prodrug, designed for specific activation in tumor cells by a three-enzyme cascade process. Due to the increased lipophilicity associated with the presence of the pentyloxycarbonyl chain, this prodrug is rapidly absorbed unaltered after oral administration and metabolized by carboxylesterase in the liver to 5′-deoxy-5-fluorocytidine. Subsequent activation steps include deamination by cytidine deaminase and finally transformation into 5-FU by thymidine phosphorylase (Figure 2.23). The last step takes place up to 10 times more efficiently in cancer cells than in normal cells, leading to selective delivery of 5-FU into the tumors. In fact, pharmacokinetic data indicate a low systemic exposure to 5-FU and intratumor concentrations of this compound higher than those achieved by administration of equitoxic doses of 5-FU. Capecitabine is indicated as first-line treatment of patients

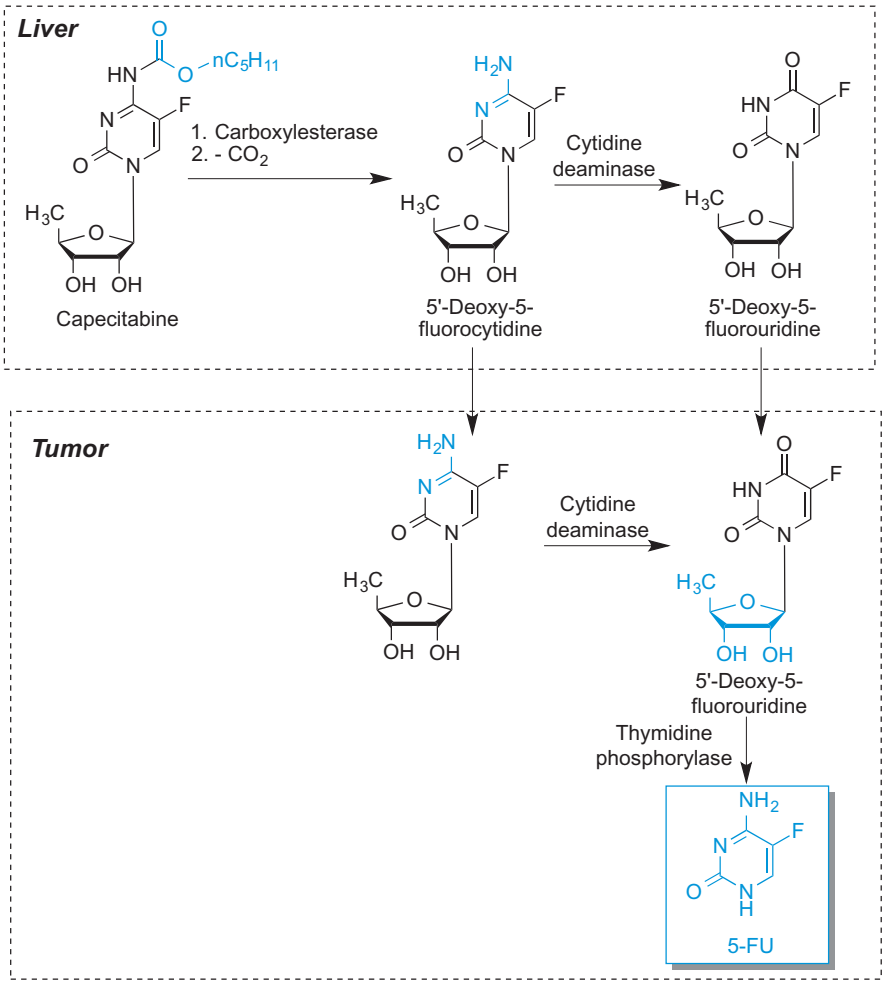


FIGURE 2.23

Bioactivation of capecitabine.

with metastatic colorectal carcinoma when treatment with fluoropyrimidine therapy alone is preferred and also in combination chemotherapy. Several anticancer drugs, such as paclitaxel, docetaxel, and cyclophosphamide, enhance the level of thymidine phosphorylase, facilitating the generation of 5-FU from capecitabine. The combination of capecitabine and some of these drugs (e.g., docetaxel) underwent clinical trials for the treatment of patients with metastatic breast cancer after failure of prior anthracycline-containing chemotherapy.[36] Capecitabine and docetaxel is a chemotherapy treatment used to treat breast cancer and the combination with platinum-based therapy (with or without epirubicin), was approved by the EMA for the first-line treatment of advanced stomach cancer. A suicide gene therapy (see Chapter 12, Section 5.2) by using the TP gene has been proposed to treat the mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) syndrome. These patients show

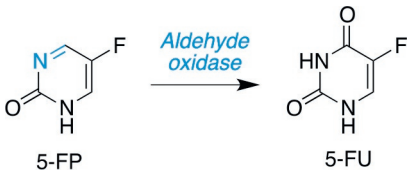


FIGURE 2.24

Bioactivation of 5-FP.

a TP deficiency due to a genetic defect, and tumors originated from the gene-modified cells could be selectively targeted by capecitabine.[37]

Another prodrug of 5-FU is 5-fluoro-2-pyrimidinone (5-FP), which is activated by hepatic aldehyde oxidase after oral or intravenous administration (Figure 2.24). A phase I clinical study proved that this compound is suitable for oral outpatient therapy.[38]

4.4 MODULATION OF 5-FLUOROURACIL ACTIVITY

Major efforts have been made to modulate the activity of 5-FU. These efforts have focused on the following aspects: (1) decreasing its degradation, (2) enhancing its potency as a thymidylate synthase inhibitor, and (3) increasing its activation.

4.4.1 Decreased Degradation of 5-FU

More than 80% of administered drug is degraded in the liver by dihydropyrimidine dehydrogenase (DPD), which reduces the pyrimidine double bond of 5-FU to give dihydrofluorouracil (DHFU).[39] This metabolite is inactive because it cannot give the initial Michael addition with the nucleophilic site of the active center in TS (Figure 2.25).

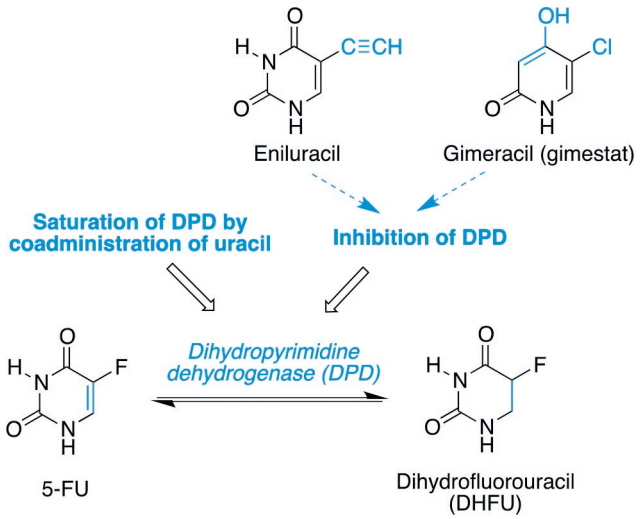


FIGURE 2.25

Drug combination approaches leading to a decreased degradation of 5-FU.

Three different approaches have been developed to improve the biostability of 5-FU:

1. The first consists of the co-administration of a large amount of uracil, which saturates the DPD enzyme because uracil is its natural substrate; for instance, the formulation known as UFT uses a 4:1 ratio of uracil and the 5-FU prodrug tegafur.[38] The combination of UFT with leucovorin calcium was called Orzel®.[40]
2. An alternative is the coadministration of 5-FU with DPD inhibitors, such as 5-chloro-2,4-dihydroxypyridine (CDHP; gimeracil, gimestat) and eniluracil (5-ethynyluracil),[41] as well as the use of the UFT combination plus DPD inhibitors.[42] In 2005, eniluracil received FDA orphan drug designation for its use in combination with fluoropyrimidines in the treatment of hepatocellular cancer. Although the eniluracil–5-FU combination (in a 10:1 ratio) tended to produce less antitumor activity than the control therapy in two phase III trials, it was subsequently discovered that the dose and schedule used might not have been optimal because high eniluracil–5-FU ratios decrease antitumor activity.
3. Finally, emitefur (BOF-A2) is an orally active drug that was designed as a mutual prodrug of 5-FU and a DPD inhibitor, namely 5-cyano-6-dihydroxypyridin-2(1*H*)-one. Two consecutive hydrolytic steps liberate the DPD-inhibiting fragment, and a third hydrolysis, followed by an oxidative activation involving the loss of two molecules of acetaldehyde, furnish 5-FU avoiding high peaks of this drug and decreasing the formation of toxic metabolites (Figure 2.26). Emitefur entered clinical trials for colorectal cancer,[43] but later studies showed typical fluorouracil-related toxicities, with some patients experiencing more severe toxicity, and its development was discontinued.

4.4.2 Enhancement of the Inhibition of Thymidylate Synthase by 5-FU

The action of TS requires the presence of 5,10-methylenetetrahydrofolate, and for this reason the coadministration of precursors of this cofactor increases the cytotoxicity of 5-FU in many cancer cell lines. For instance, the combination of 5-FU or tegafur with leucovorin (5-formyl-THF) gave superior response rates compared to those of the single agents, and particularly the use of leucovorin to modulate the uracil–tegafur combination leads to a three-component combination called Orzel® that has been proposed as first-line chemotherapy of colorectal cancer.[44] Another important combination is Folfirinox®, which contains leucovorin and 5-FU together with irinotecan and oxaliplatin and is used to treat metastatic pancreatic cancer.

Leucovorin enters the cell via the reduced folate carrier and is metabolized to 5,10-methylene-THF, without requiring the participation of DHFR, by cyclization to 5,10-methenyl-THF followed by NADP-mediated reduction of the iminium function (Figure 2.27). The International Nonproprietary Names (INN) name for leucovorin is folinic acid, but this may cause some confusion because in biochemistry it is often employed as a collective name, comprising 5-formyl-THF but also other related compounds.

4.4.3 Enhancement of 5-FU Activation

It has been proposed that pretreatment with methotrexate, an antifolate agent, enhances the activity of 5-FU[45] because methotrexate inhibits the biosynthesis of tetrahydrofolic acid (THF), which is necessary for some steps of purine biosynthesis (see Section 6). This leads to accumulation of phosphoribosyl pyrophosphate, essential for the activation of 5-FU, even though the levels of the TS cofactor should also be diminished (Figure 2.28). Clinically, this combination has not always shown increased antitumor activity.[46] On the other hand, several phase II studies have shown a modest clinical benefit of 5-FU modulation utilizing methotrexate and leucovorin in patients with metastatic colorectal cancer.[47]

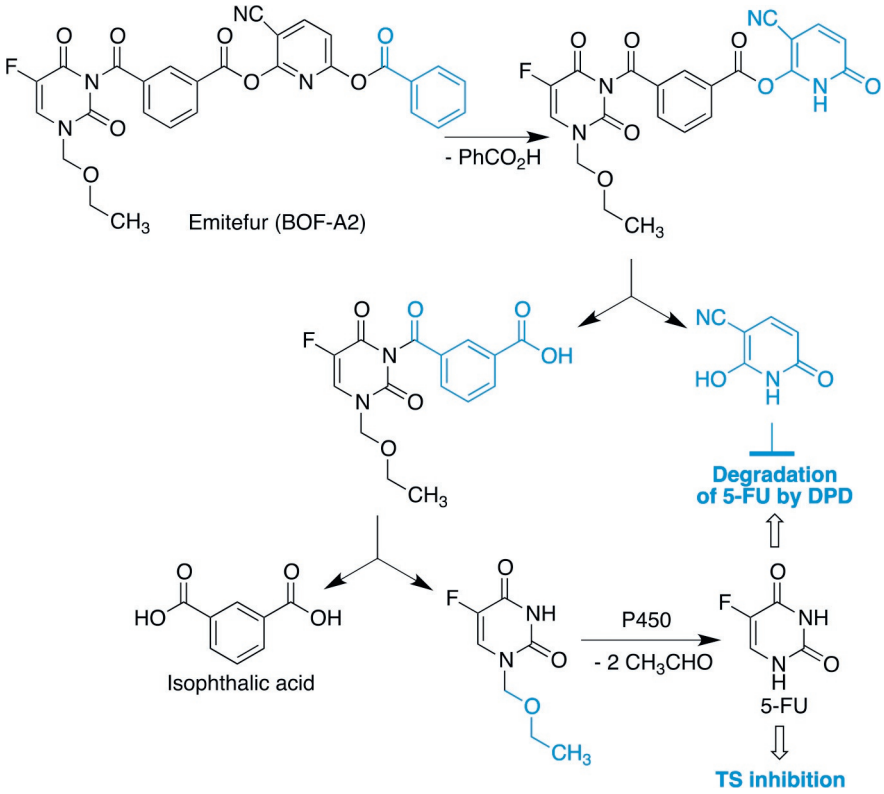


FIGURE 2.26

Bioactivation of emitefur.

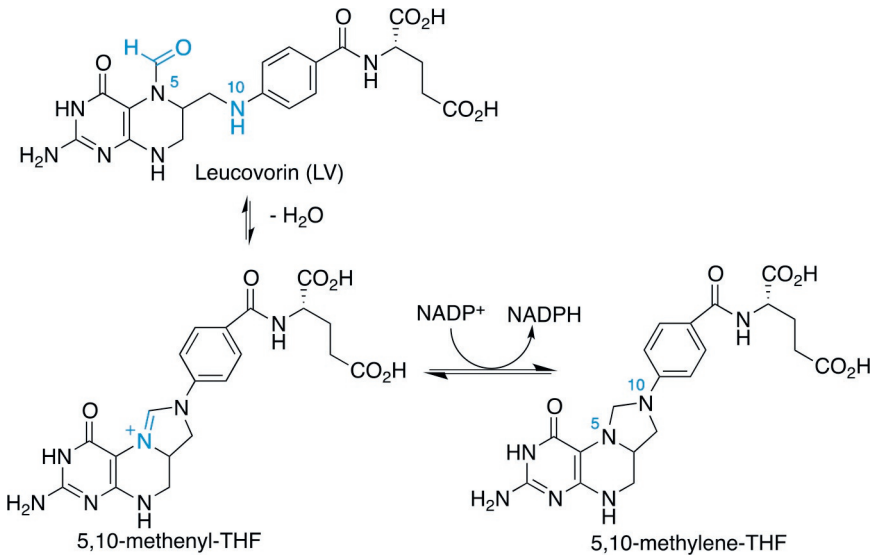


FIGURE 2.27

Biotransformation of leucovorin into 5,10-methylene-THF.

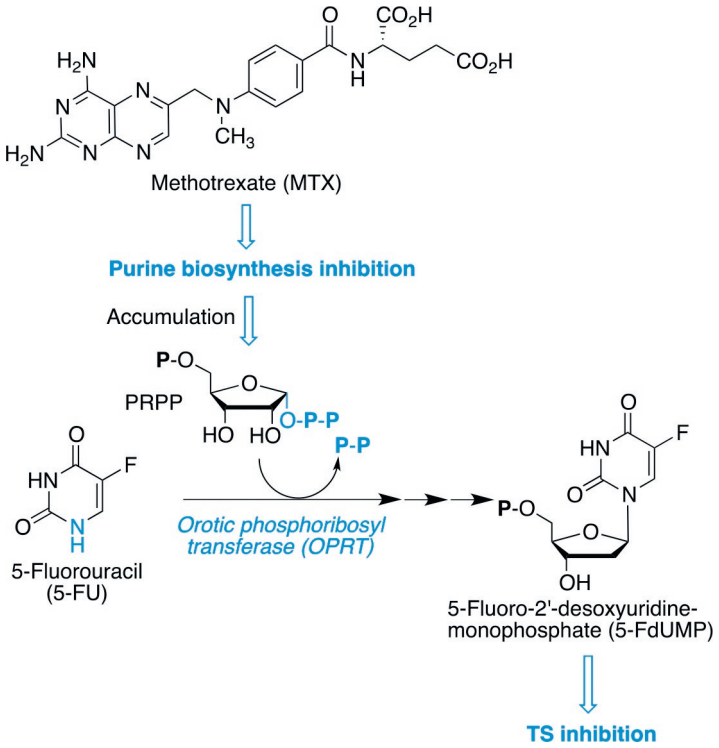


FIGURE 2.28

A strategy to enhance 5-FU activation.

Diarrhea is the most common dose-limiting toxicity associated with prolonged infusion of 5-FU. To prevent this gastrointestinal toxicity, some oral formulations have been proposed that contain the potassium salt of oxonic acid (oteracil potassium), a potent inhibitor of the phosphoriboxylation of 5-FU in the gastrointestinal mucosa. One of these formulations is S-1 (TS-1), which contains tegafur, oteracil potassium, and the previously mentioned gimestat (5-chlorodihydroxypyridine), an inhibitor of dihydropyrimidine dehydrogenase (see Section 4.4.1).[48] The combination of S-1 and cisplatin acts by the mechanisms summarized in Figure 2.29 and is approved for the treatment of gastric cancer in Japan.[49]

4.5 TRIFLURIDINE

Trifluridine (trifluorothymidine, TFT) is used as an anti-herpes drug, primarily for ocular treatments, and acting by incorporating into viral DNA. It is also an inhibitor of thymidylate synthase by the mechanism summarized in Figure 2.30. Thus, after phosphorylation to **2.28**, the initial nucleophilic attack of the Cys residue onto the substrate $C_5{=}C_6$ bond generates the enolate anion **2.29**, which evolves in this case by loss of a fluoride anion to furnish **2.30**. This intermediate bears a difluorinated α,β-unsaturated carbonyl fragment that undergoes attack by the Tyr-146 residue of the active site of thymidylate synthase, again with loss of HF, to give **2.31**. A final attack by a water, with concomitant loss of a third molecule of HF, yields the final product **2.32**.

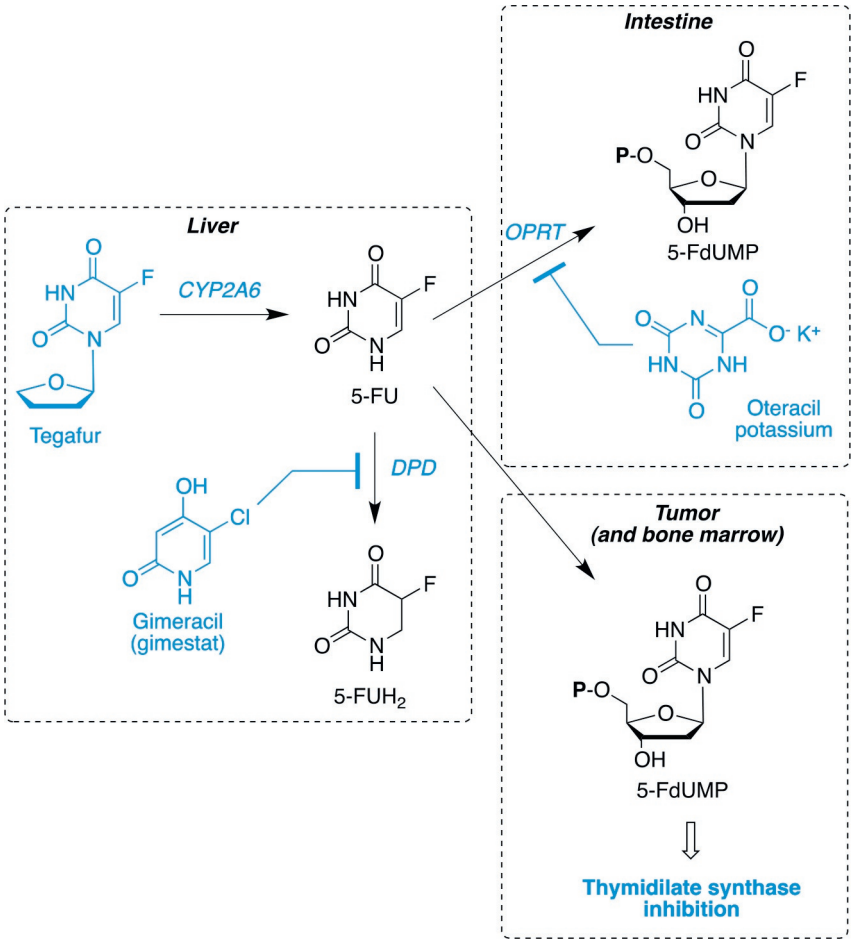


FIGURE 2.29

Mechanism of action of the S-1 combination (tegafur, oteracil potassium, and gimestat).

When taken orally, trifluridine undergoes extensive first pass hepatic metabolism by thymidine phosphorylase, which transforms it into inactive metabolite 5-trifluoromethyluracil. This observation led to the idea of associating trifluridine with tipiracil hydrochloride, a thymidine phosphorylase inhibitor (Figure 2.31). This association, known as TAS-102, is under phase II clinical trials in patients with metastatic colorectal cancer.

4.6 FOLATE-BASED THYMIDYLATE SYNTHASE INHIBITORS

As previously mentioned, TS inhibition by the fluoropyrimidines is not specific because of the effect of fluorinated nucleotides on other pathways, especially related to RNA. Also, the accumulated dUMP may compete with the antitumor drug for TS. For this reason, there has been much interest in the design

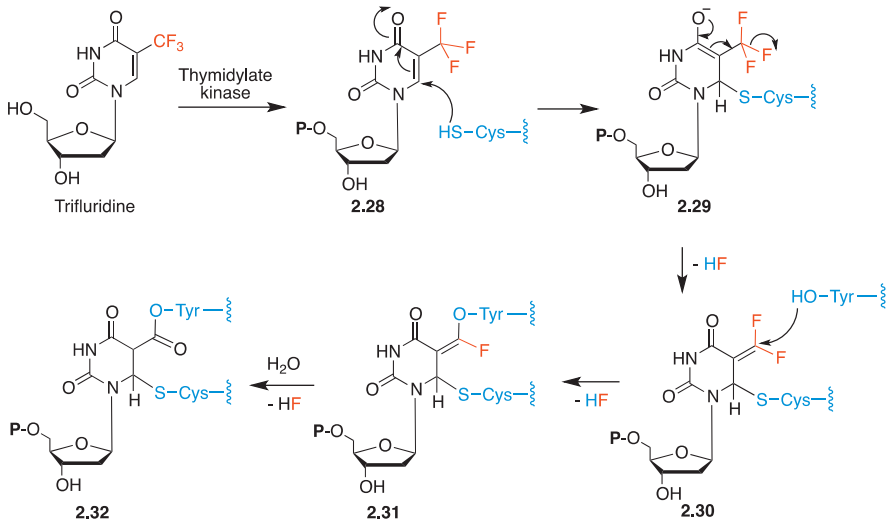


FIGURE 2.30

Mechanism of thymidylate synthase inhibition by trifluridine.

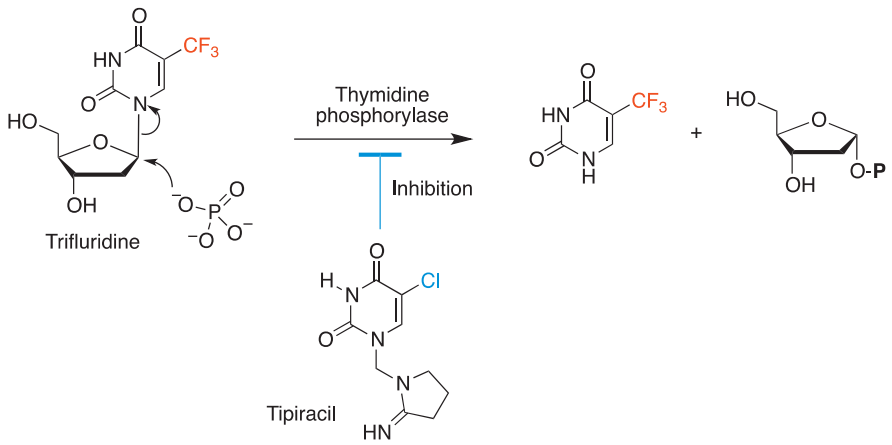
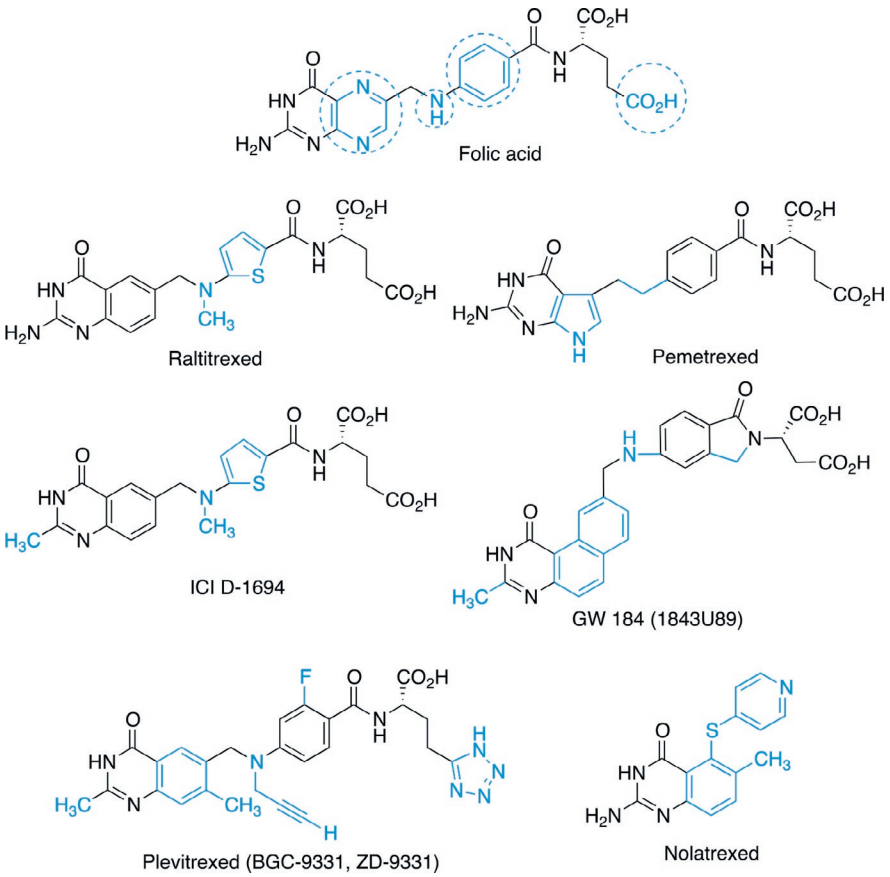


FIGURE 2.31

Basis for the TAS-102 association of trifluridine with tiparacil.

of inhibitors that recognize the folate binding site of TS, which should not have these shortcomings and thus behave as specific TS inhibitors.[50] Four of them have reached therapeutic use or are under advanced clinical evaluation—namely raltitrexed (ZD-1694, Tomudex®), pemetrexed (MTA, LY-231514, Alimta®), nolatrexed (Thymitaq®), and plevitrexed (BGC-9331, ZD-9331)—whereas the development of some related compounds, such as ICI D-1694 and 1843U89 (GW-1843), was halted at an early stage. All these compounds were rationally designed by manipulation of the folic acid structure, and they can be classified into three groups:

1. Compounds whose activity depends on both the reduced folate carrier (RFC) and folylpolyglutamate synthetase (FPGS). Examples are raltitrexed and pemetrexed.
2. Compounds whose activity depends on the RFC alone. Examples are plevitrexed and GW1843 (1843U89).
3. Compounds that do not depend on RFC nor FPGS: This category includes compounds that are water-soluble and are transported via the α-isoform of the membrane folate receptor (α-FR), such as ONX 0801 (BGC 945), and others that are lipophilic and penetrate cells by passive diffusion (nolatrexed).



Raltitrexed was the first specific TS inhibitor to be approved for clinical use, and it is employed for advanced colorectal cancer. Its structure contains two classical bioisosteric modifications, namely replacement of the pteridine ring of folate by a quinazoline unit and replacement of the benzene ring of folate by a thiophene. This drug is transported into the cells by the RFC, and its terminal glutamate residue is converted by FPGS into a polyglutamate, which is more potent as an enzyme inhibitor and is retained intracellularly, leading to a prolonged action (Figure 2.32). The closely related compound ICI D-1694 showed good activity in early clinical studies, but its use was limited by its low solubility at physiological pH. 1843U89 was another antifolate compound acting as a TS inhibitor that underwent phase I and pharmacokinetic studies in patients with advanced solid malignancies, but an unacceptably high incidence of

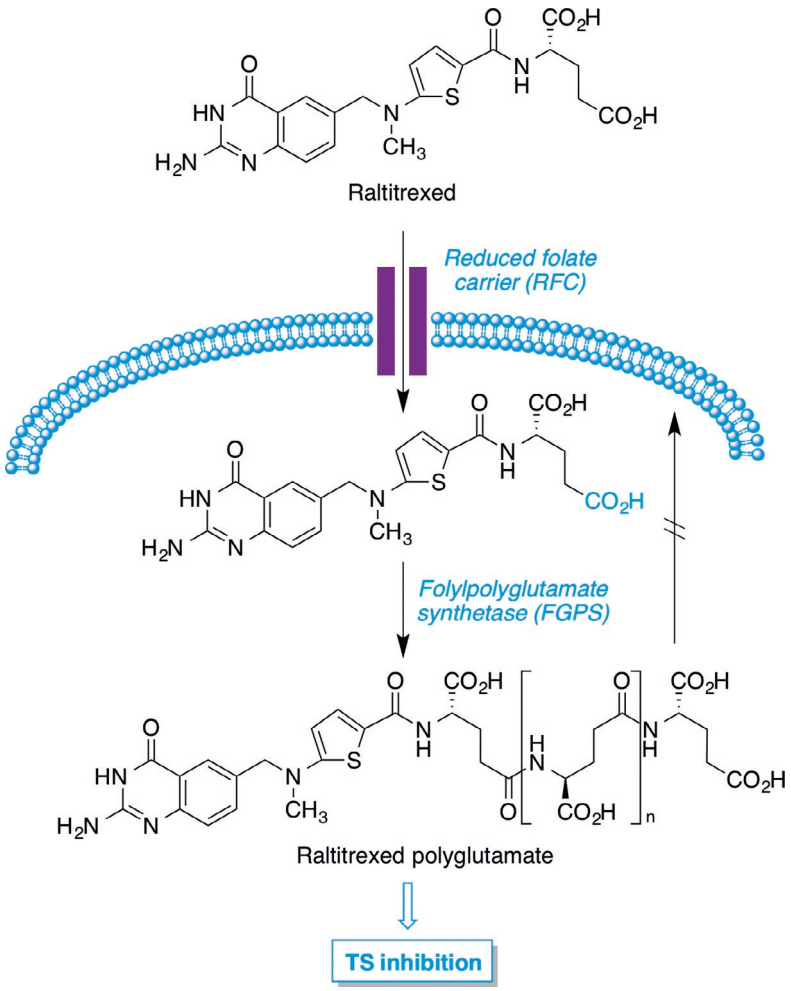


FIGURE 2.32

Active transport and polyglutamation of raltitrexed.

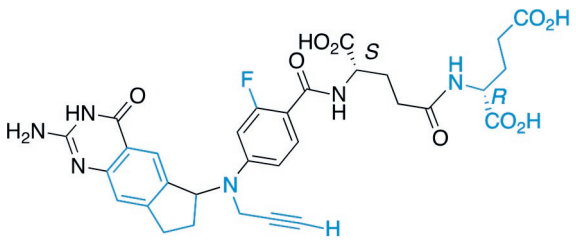
severe neutropenia and mucositis was found.[51] The multitarget drug pemetrexed (Alimta®), which inhibits TS but also several other folate-related enzymes, is discussed in Section 6.2.

Plevitrexed (BGC-9331, ZD-9331) is also a potent inhibitor of TS that is under advanced clinical evaluation, showing promising efficacy and tolerability in gastric, pancreatic, and ovarian cancers. In 2007, it was granted U.S. orphan drug designation for ovarian and gastric cancer. Plevitrexed differs from the previously mentioned compounds in several respects. One is the presence of a methyl group at C-7, which was designed from X-ray diffraction studies of TS that suggested that a 7-alkyl group would contribute to binding. Another difference is the 2′-fluorine substituent that also increased activity. A final interesting feature of ZD-9331 is the isosteric γ-carboxypropyltetrazole replacement at the glutamic portion, which prevents polyglutamation. Because this drug is active against TS in a non-polyglutamated form, it has the advantage over previously mentioned folate-based TS inhibitors of not being subject to resistance by folylpolyglutamate synthetase downregulation.

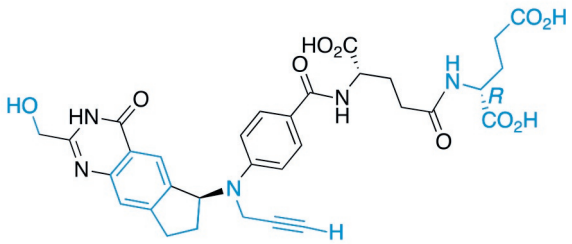
Nolatrexed (AG-337, Thymitaq®) is the inhibitor least structurally related to folate. It crosses the cell membrane by passive diffusion rather than using the RFC, and since it is not retained inside the cells because it cannot be polyglutamated, it requires a prolonged infusion (see Section 5.2). Phase II clinical trials showed activity in patients with hepatocellular carcinoma, head and neck cancer,[52] and adenocarcinoma of the pancreas. These studies prompted the EMA to grant orphan drug status to nolatrexed for the treatment of hepatocellular carcinoma, although in 2005 the FDA refused to approve the drug.

The RFC is ubiquitous and is expressed in normal tissues, and this is the main reason for the systemic toxicity of antifolate drugs. Another related membrane transporter is the α-isoform of the membrane folate receptor (MFR, α-FR), and evidence suggests that this transport mechanism contributes to their activity when the receptor is highly overexpressed or when the extracellular folate concentration is very low. Because the main factor that reduces patient tolerability to antifolate drugs is the ubiquitous expression of the RFC in normal tissues, it is expected that TS inhibitors with MFR/RFC selectivity will be better tolerated.[53] Furthermore, α-FR has the additional advantage as a target for drug discovery of being overexpressed in some epithelial tumors, especially ovarian carcinomas. The high-affinity human folate receptors (FRs), which transport folate via endocytosis, have been proposed as targets for the specific delivery of antifolates or folate conjugates to tumors.[54]

Some cyclopenta[*g*]quinazoline derivatives with activity as inhibitors of thymidylate synthase have been proven to have good α-FR/RFC selectivity and therefore are good candidates for the development of antifolates specifically targeted at α-FR-overexpressing tumors. One of these compounds is CB-300638, which was designed on the basis of the crystal structures of inhibitors bound to TS[55] and has shown promising preclinical data, including experimental proof of its selective delivery into human tumor cell lines overexpressing the α-isoform of the folate receptor.[56] This compound contains two glutamate units and cannot be further glutamated because of the unnatural *R* configuration at the α carbon of the second residue.[57] The closely related ONX 0801 (BGC 945) is also transported by the α-FR[58] and has undergone a dose-finding phase I study to evaluate its safety and pharmacokinetics in cancer patients with advanced solid tumors.[59]



CB-300638



ONX 0801 (BGC 945)

5 INHIBITORS OF DIHYDROFOLATE REDUCTASE

Folic acid and its metabolites (collectively known as folates) are coenzymes of many essential biochemical transformations. Most important, they are involved in the previously mentioned transfer of one carbon unit in the *de novo* synthesis of thymidylic acid and purine nucleotides. Folate-dependent enzymes are obvious targets for cancer chemotherapy,[60,61] but until 1980, only DHFR was exploited in this regard; in fact, it was the first enzyme to be targeted for cancer chemotherapy.

In mammals, folic acid is taken with the diet and reduced to THF by dihydrofolic reductase in two stages, using NADPH as a cofactor. Further transformations of THF lead to 5,10-methylene-THF, 5,10-methenyl-THF, 5-formyl-THF, and 10-formyl-THF (Figure 2.33), which are known as folinic acids and are involved in the transfer of one-carbon units. DHFR inhibition leads to cell death due to the essential role of folinic acids in the synthesis of thymidylate and purine bases.

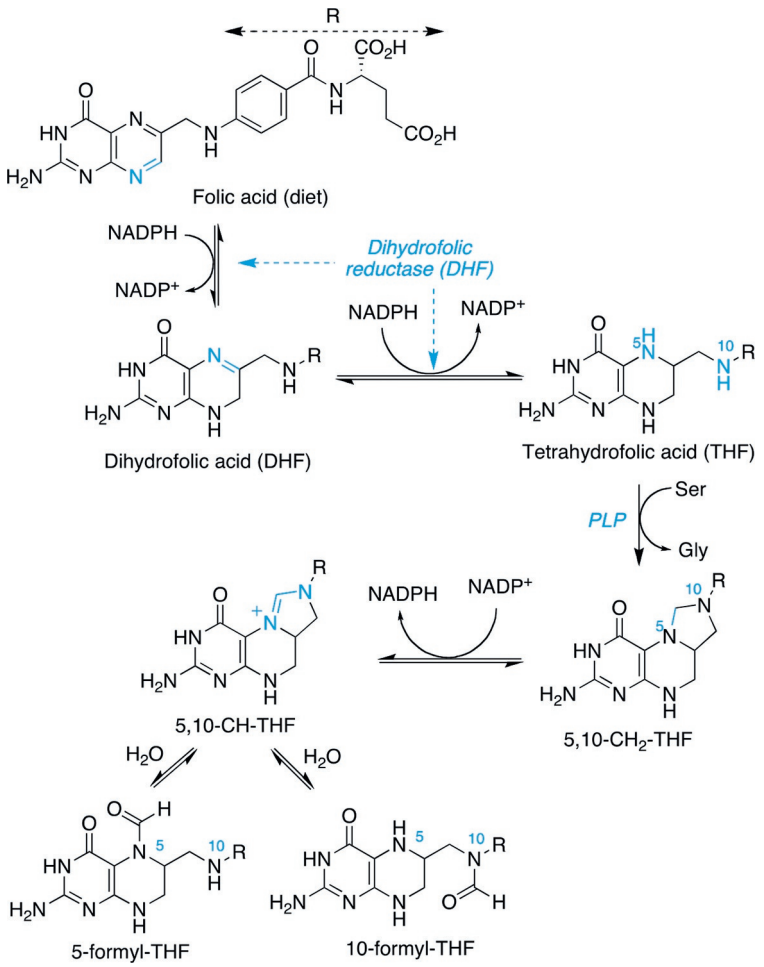


FIGURE 2.33

Biotransformation of folic acid into folinic acids.

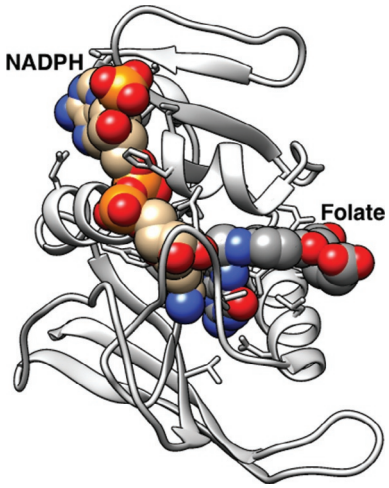


FIGURE 2.34

Dihydrofolate reductase bound to folic acid and the NADPH cofactor. Generated from Protein Data Bank reference 7DFR and displayed with Chimera 1.8.1.

DHFR is a relatively small protein with a large active site, in which DHF binds adjacent to the cofactor, NADPH, in a pocket buried deep within the enzyme. It catalyzes the transfer of the *pro-R* hydrogen of the C-4 position of the dihydropyridine ring in the cofactor onto the C=N double bonds of folic acid and dihydrofolic acid (Figures 2.34 and 2.35).

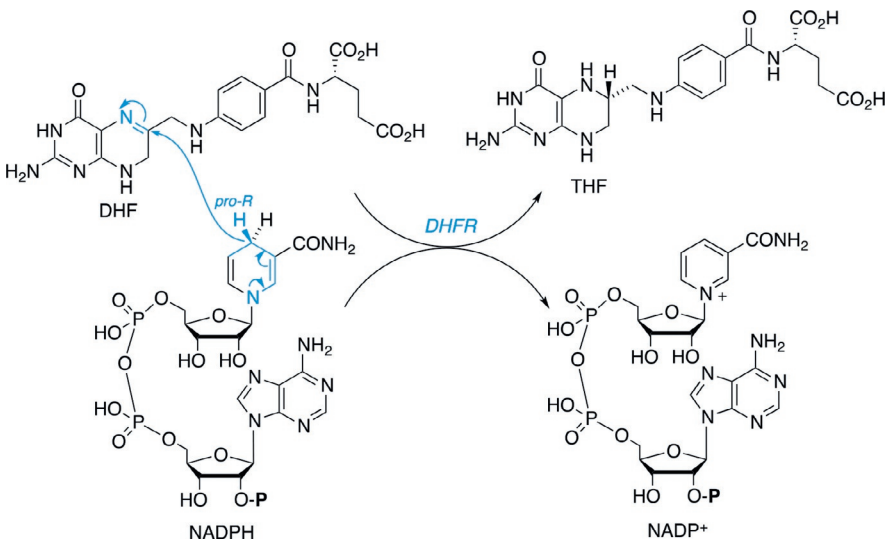


FIGURE 2.35

Reaction catalyzed by DHFR.

The most potent inhibitors of DHFR are folic acid analogs that differ from the natural ligand in that they bear a 2,4-diaminopyrimidine unit. The inhibitors in which the side chain ends in a glutamic acid residue, as in folic acid, are known as classical antifolates. Other inhibitors with lipophilic substituents are known as nonclassical antifolates.

5.1 CLASSICAL DHFR INHIBITORS

The two main classical DHFR inhibitors are aminopterin (AM) and methotrexate (MTX, amethopterin), which were designed by replacing an enol-type OH group at C-4 of the natural substrate (DHF) by an amino group. Another DHFR inhibitor bearing a glutamate side chain is pemetrexed, previously mentioned as a TS inhibitor (Figure 2.36).

In the design of methotrexate and aminopterin, the implicit assumption was made that the two ligands would bind similarly and that the 4-amino group of MTX would go to the position in the binding site normally occupied by the DHF carbonyl. However, X-ray diffraction structures of DHFR with dihydrofolate and methotrexate showed different binding modes, with the aminopteridine ring of methotrexate flipped 180 degrees about the C_2–NH_2 bond compared to that of dihydrofolate (Figure 2.37). Both ligands bind to the DHFR active site by hydrogen bonds and by additional interactions with

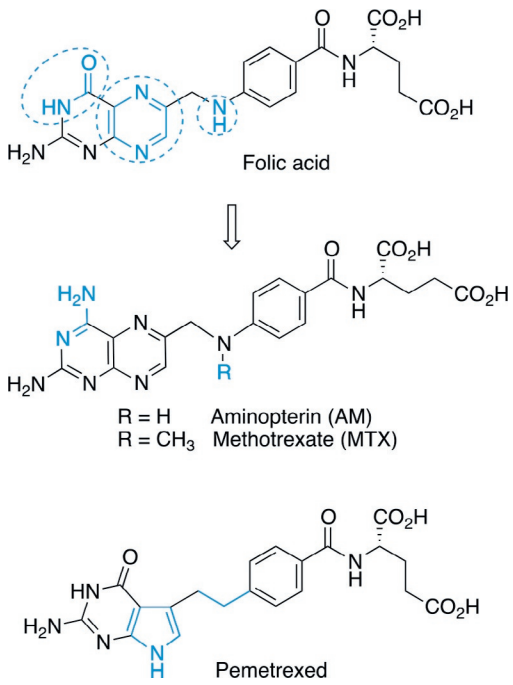


FIGURE 2.36

The design of DHFR inhibitors from folic acid.

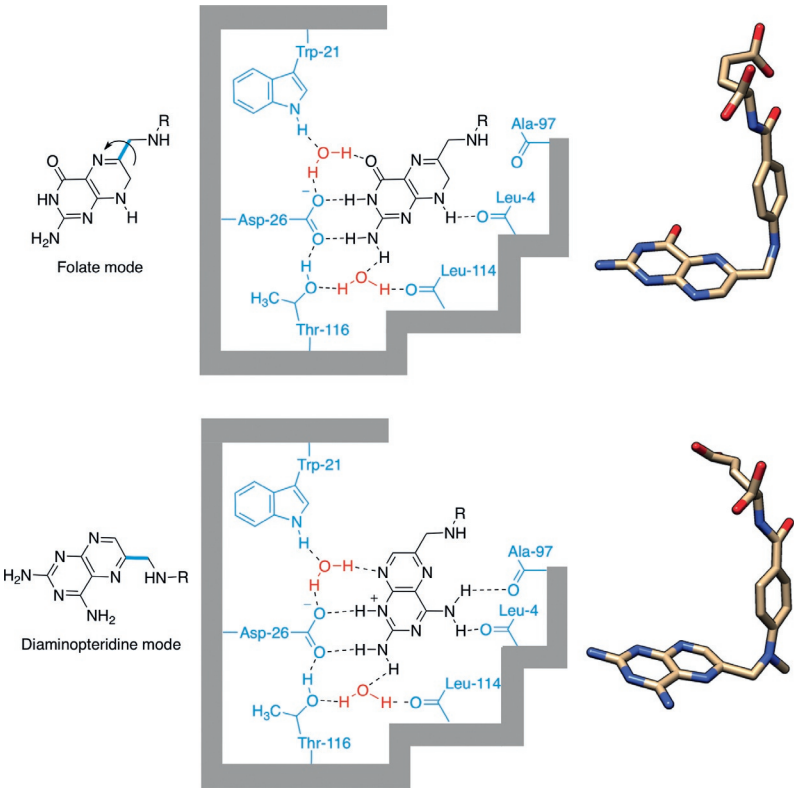


FIGURE 2.37

The two different binding modes of the heterocyclic moieties of dihydrofolate and aminopterine to DHFR. The three-dimensional structures come from the the corresponding complexes with DHFR, obtained by X-ray diffraction, and were generated from Protein Data Bank references 7DFR (folate) and 3DFR (metothrexate) and displayed with Chimera 1.8.1.

bridging water molecules. Methotrexate is approximately 3 pK_a units more basic than folic acid because it contains an electron-releasing amino group conjugated with the basic guanidine fragment instead of an electron-withdrawing carbonyl, and therefore it binds in a protonated state. The electrostatic interaction and an additional hydrogen bond involving the 4-amino group lead to a binding approximately 10^3 times stronger than that of folate.[62] The binding of methotrexate to DHFR is dependent on the presence of the NADPH cofactor and is an example of a type of enzyme inhibition known as slow, tight-binding inhibition.[63] The selective toxicity of methotrexate in malignant cells with regard to normal ones seems to be partly due to differences in the ratio of NADPH to NADP and NADH in both types of cells.[64]

The interaction of pemetrexed with DHFR has been studied based on molecular modeling and nuclear magnetic resonance studies that suggest that it can bind to the enzyme in a "2,4-diaminopteridine mode," in which the pyrrole nitrogen mimics the 4-amino group of methotrexate.

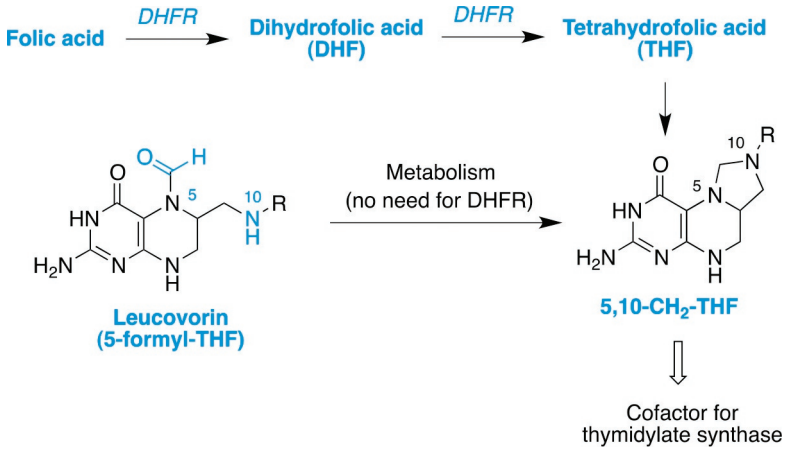


FIGURE 2.38

Bypass of DHFR inhibition by leucovorin.

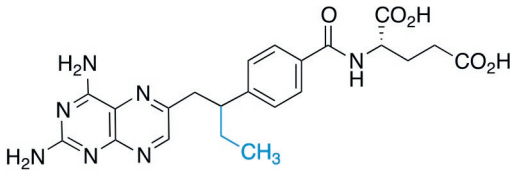
Aminopterin was the first antifolate to be introduced in cancer therapeutics in the 1950s, but it was soon verified that methotrexate is less toxic and has superior pharmacokinetic properties; this compound is now the only classical antifolate in clinical use. It is employed for the treatment of choriocarcinoma, non-Hodgkin's lymphoma, and acute lymphocytic leukemia, and also in many combination regimens. Its main side effects are myelosuppression and damage to the gastrointestinal tract, kidneys, and liver. To partly alleviate its bone marrow toxicity, methotrexate is often associated with the calcium salt of leucovorin (N^5-formyltetrahydrofolic acid), one of the folinic acids. As previously mentioned, leucovorin enters the cell via the reduced folate carrier and is metabolized to 5,10-methylene-THF without requiring the participation of DHFR, thus bypassing DHF blockade (Figure 2.38).

Other uses of methotrexate include the treatment of severe psoriasis and adult rheumatoid arthritis, being the most widely prescribed disease-modifying antirheumatic drug. Methotrexate has also been shown to induce abortion due to its ability to kill the rapidly growing cells of the placenta.

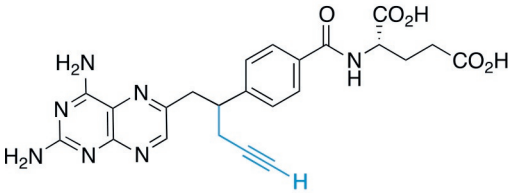
Methotrexate enters the cells via the RFC and is polyglutamated by FPGS, thereby increasing intracellular retention. Decreased polyglutamation is observed in normal versus malignant cells, which can partly explain the selectivity of methotrexate for malignant tissue. Methotrexate polyglutamates also inhibit other folate-related enzymes, including TS and also glycinamide ribonucleotide formyltransferase (GARFT) and aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT), two transformylases that participate in the purine *de novo* biosynthesis (see Section 6).

In addition to methotrexate and aminopterin, 10-alkyl-10-deaza folate analogs are also classical DHFR inhibitors. These compounds were developed on the basis of the observation that the transport mechanism for normal proliferative tissue such as intestinal epithelium distinguishes aminopterin from its *N*-alkyl derivative methotrexate, leading to higher levels of AM in the normal proliferative tissue and hence to increased toxicity. The main compounds are 10-ethyl-10-deazaaminopterin (edatrexate) and 10-propargyl-10-deazaaminopterin (pralatrexate, PDX), which have undergone several

clinical investigations.[65,66] The latter compound, under the trade name Folotyn®, was the first drug approved for the treatment of relapsed or refractory peripheral T-cell lymphoma, or peripheral T-cell lymphoma.



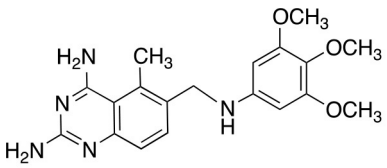
Edatrexate



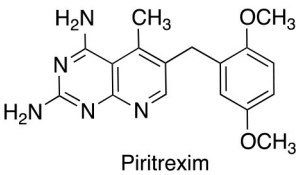
Pralatrexate (PDX)

5.2 NONCLASSICAL (LIPOPHILIC) DHFR INHIBITORS

Suppression of the glutamic chain of the folic acid skeleton leads to compounds that are not substrates for the folate active transport systems and enter the cells by passive diffusion. They have the advantage of being active in cancer cells that are resistant to methotrexate because of transport defects. On the other hand, the lack of the glutamic acid side chain prevents polyglutamation, and therefore these compounds are not retained within the cells and require more prolonged treatments. Among these compounds, trimetrexate is mainly used to treat pneumonias by *Pneumocystis carinii* and *Toxoplasma gondii*, although it is also used in the treatment of certain cancers, including colon cancer, as its glucuronate salt (Neutrexin®).[67] Piritrexim has been assayed for the treatment of psoriasis, pneumonia, and several cancers, including phase II studies for the treatment of advanced carcinoma of the urotelium.[68]



Trimetrexate



Piritrexim

6 INHIBITORS OF THE *DE NOVO* PURINE BIOSYNTHESIS PATHWAY

In contrast to pyrimidine nucleotide biosynthesis, in which a preformed heterocyclic moiety is attached to PRPP, in the case of purine nucleotides the purine ring is constructed gradually. The complete route comprises 10 steps and is summarized in Figures 2.39 and 2.40. This *de novo* pathway

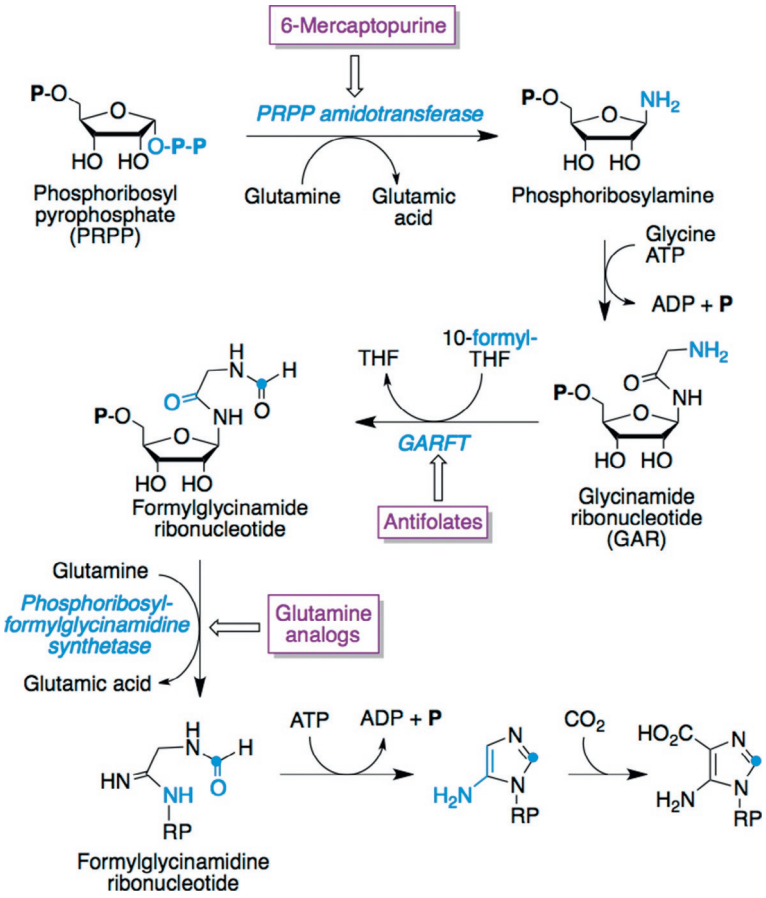


FIGURE 2.39

Initial steps of the biosynthesis of purine nucleotides.

leads to the formation of inosine monophosphate (IMP), the precursor of ATP, GTP, dATP, and dGTP necessary for RNA and DNA formation. Only the steps most relevant to antitumor drug action are discussed here.

6.1 INHIBITORS OF PRPP AMIDOTRANSFERASE

The first irreversible step in *de novo* purine biosynthesis involves the nucleophilic displacement of the pyrophosphate group of PRPP by a molecule of ammonia, generated by hydrolysis of glutamine to glutamic acid. Both reactions are catalyzed by PRPP amidotransferase, whose main inhibitors are thiopurines (e.g., 6-mercaptopurine), acting through feedback mechanisms. These antitumor drugs have a complex mechanism of action, involving the inhibition of several enzymes related to purine biosynthesis and misincorporation into nucleic acids, and are discussed in Section 6.5.

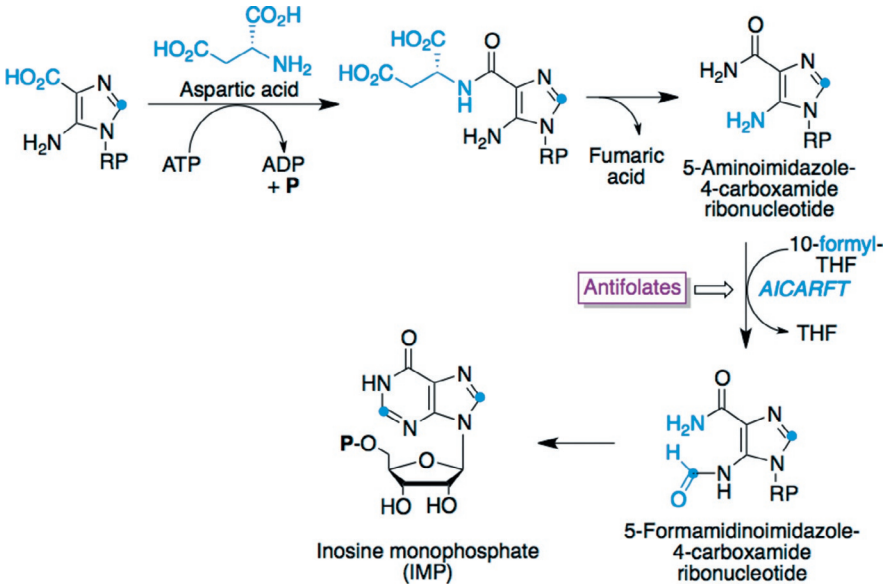


FIGURE 2.40

Final stages of the biosynthesis of purine nucleotides.

6.2 INHIBITORS OF GLYCINAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE

The third reaction in the *de novo* purine biosynthesis is the transformation of glycinamide ribonucleotide (GAR) into its formyl derivative (FGAR) using 10-formyltetrahydrofolate as the formyl donor (Figure 2.41). The enzyme that catalyzes this step is known as GARFT. In mammals, this enzyme is multifunctional, and it also catalyzes the second and fifth steps of the pathway.

The first selective and sufficiently potent GARFT inhibitor was lometrexol, designed as a folate analog lacking the 5 and 10 nitrogen atoms and therefore unable to participate in the transfer of single carbon units.[69] On the other hand, lometrexol has a 2-aminopyrimidin-4-one subunit identical to that found in the THF cofactor and, therefore, different from the 2,4-diaminopyrimidine pattern commonly present in DHFR inhibitors. Its glutamate side chain allows its ready transport into cells by means of the RFC and MFR as transport systems, and also its polyglutamation by FPGS. Lometrexol was investigated clinically, but unexpected observations of delayed cumulative toxicity[70] prompted a search for second-generation antimetabolites with a more favorable profile (Figure 2.42). Some of these compounds are LY-309887, an analog of lometrexol designed by benzene–tiophene bioisosteric replacement with a ninefold greater potency as a GARFT inhibitor; AG-2034, with an additional CH-S isosteric change and that underwent phase I studies[71]; and pelitrexol (AG-2037), an analog with the opposite configuration at C-6. In addition to being well tolerated, the latter compound shows an interesting synergism with 5-FU and has been studied in phase II trials in patients with metastatic adenocarcinoma of the colon or rectum when prior fluorouracil and leucovorin calcium therapy had failed.[72]

Pemetrexed (Alimta®, MTA), previously mentioned as an inhibitor of both TS and DHFR, was discovered during structure–activity studies of lometrexol, by removal of the C-5 carbon atom and

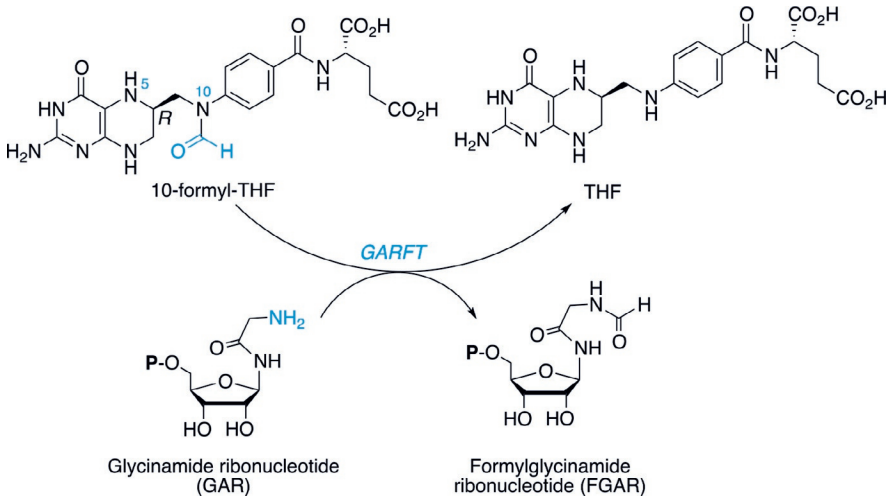


FIGURE 2.41

Reaction catalyzed by GARFT.

concomitant replacement of the ring fused to the quinazolinone unit by an indole, leading to loss of stereochemical information at C-6. It employs the RFC for entering the cells, and its polyglutamation inhibits multiple targets in the folate pathway, including the previously mentioned TS and DHFR and two enzymes from the *de novo* synthesis of purines, namely GARFT and AICARFT. This complex mechanism of action has led to its designation as MTA (multitargeted antifolate). Pemetrexed was approved in 2004 for the treatment of malignant pleural mesothelioma in association with cisplatin and as second-line treatment of non-small cell lung cancer,[73] and two indication extensions were granted in 2008 and 2009. Replacement of the 4-oxo group by a methyl (AAG 113–161) led to a further increase in activity, which was explained by a hydrophobic interaction of the 4-methyl with Phe-31 and Leu-22.[74]

A summary of the main targets for antifolate drugs and their relationships with nucleic acid biosynthesis is given in Figure 2.43.

6.3 INHIBITORS OF PHOSPHORIBOSYLFORMYLGLYCINAMIDINE SYNTHETASE

This enzyme catalyzes the reaction of formylglycinamide ribonucleotide with ammonia to give formylglycinamidine ribonucleotide, with glutamine as cofactor (Figure 2.33). The enzyme activates the amide group adjacent to the ribose ring to nucleophilic attack by its transformation into iminoether **2.28**. On the other hand, another catalytic site of the enzyme hydrolyzes glutamine to glutamic acid and ammonia, which is then channeled to the first site and reacts with **2.28** by an addition–elimination mechanism, affording the amidine **2.29** (formylglycinamidine ribonucleotide) (Figure 2.44).

Some analogs of glutamine bearing a diazomethyl moiety have antitumor activity because of their ability to inhibit several reactions in which glutamine is involved as a cofactor, specially the one catalyzed by formylglycinamidine ribonucleotide synthetase. Azaserine (*O*-diazoacetyl-L-serine)

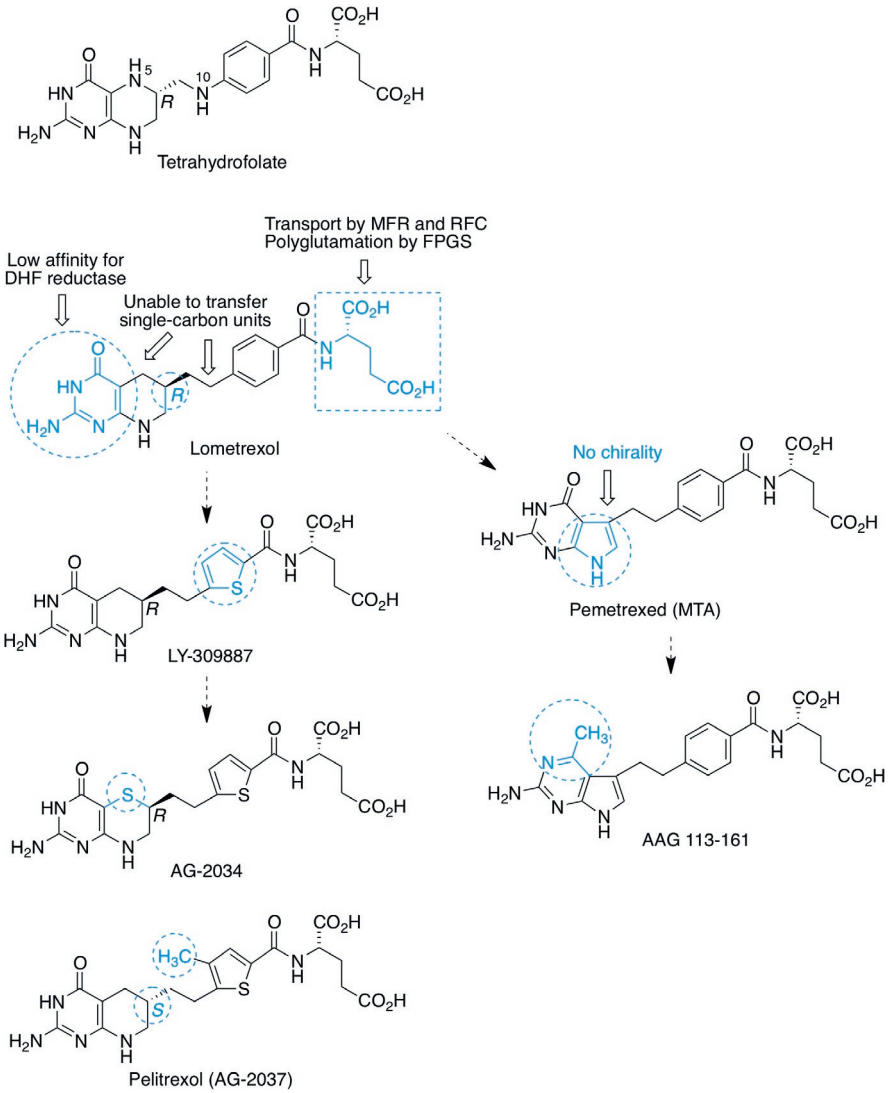


FIGURE 2.42

Representative GARFT inhibitors.

and 6-diazo-5-oxo-L-norleucine (DON) are two antitumor natural products, isolated from *Streptomyces* broths, that act as covalent inhibitors of the enzyme. Reversible attachment using the binding points normally employed by the cofactor glutamine positions the diazomethyl group close to a cysteine sulfhydryl group in the active site. After protonation, this unit is transformed into a diazonium group, which covalently links to the cysteine thiol group (Figure 2.45). Clinical trials have shown a good response of choriocarcinoma to DON, although in general, these compounds are considered too toxic for

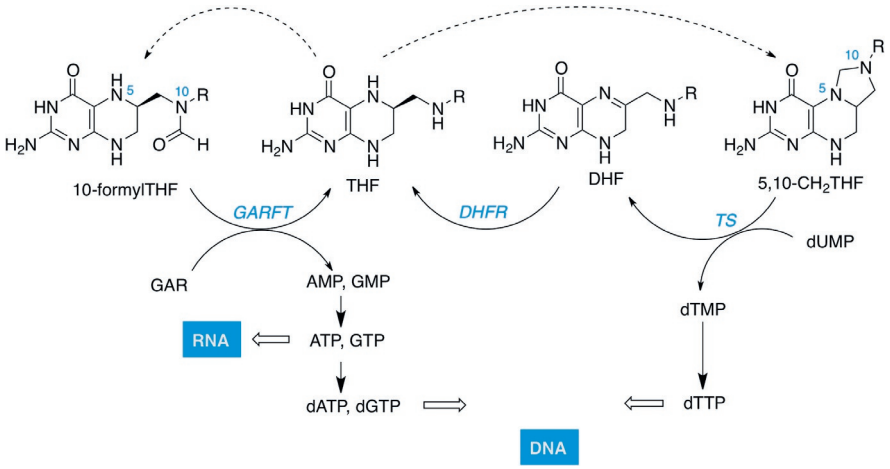


FIGURE 2.43

A summary of the main targets for antifolate drugs.

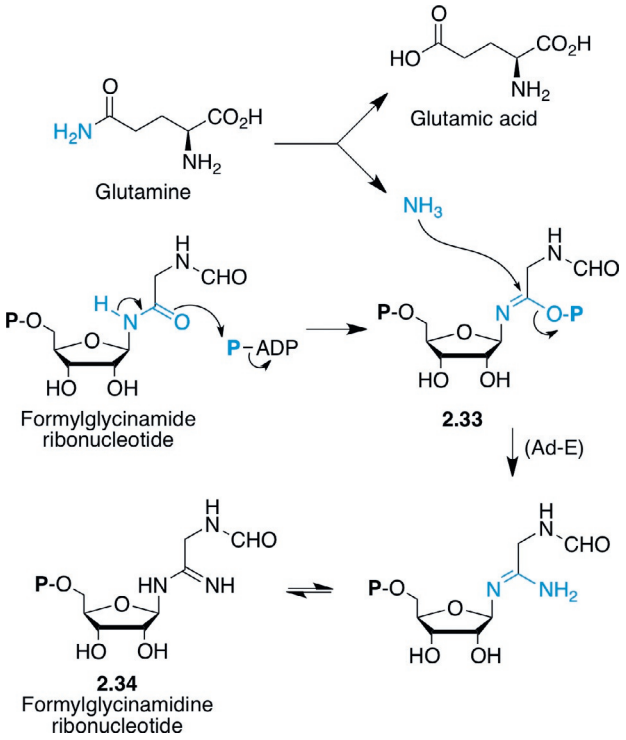


FIGURE 2.44

Biosynthesis of formylglycinamidine ribonucleotide.

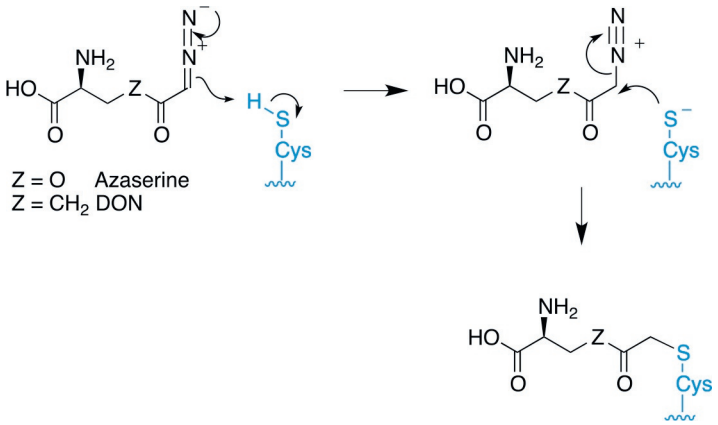


FIGURE 2.45

Covalent inactivation of phosphoribosylformylglycinamidine synthase by azaserine and DON.

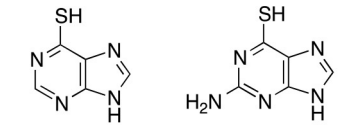
therapeutic use. Nevertheless, a phase IIa study evaluated the safety and clinical activity of the glutamine-depleting enzyme PEG-PGA (PEGylated glutaminase) in combination with DON.[75]

6.4 INHIBITORS OF 5-AMINOIMIDAZOLE-4-CARBOXAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE

Some antifolate drugs (e.g., methotrexate and pemetrexed) inhibit this enzyme, although it is not their primary target.

6.5 THIOPURINES AND RELATED COMPOUNDS

Among non-natural purine derivatives assayed as antitumor agents, 6-mercaptopurine (MP, Purinethol®) and 6-thioguanine (TG, tioguanine) are the most active. These compounds are among the oldest cancer chemotherapeutic drugs in clinical use. MP is used for lymphoblastic and myeloblastic leukemias, and the more toxic TG is employed for the treatment of acute nonlymphocytic leukemia.



MP requires intracellular metabolism by hypoxantine guanine phosphoribosyl transferase (HGPRT) to be transformed into thioinosinic acid, which shows cell cycle S-phase-specific cytotoxicity. Intracellular activation results in the inhibition of several enzymes belonging to the *de novo* purine synthesis pathway and misincorporation into DNA and RNA. Thus, thioinosinic acid, formed by incorporation of a ribose phosphate unit to MP catalyzed by HGPRT, inhibits PRPP amidotransferase, the first enzyme

in the *de novo* synthesis of purines, through a retro-inhibition mechanism. Several other enzymes that are also inhibited, resulting in lower levels of adenosine monophosphate (AMP) and guanosine monophosphate (GMP), are the following:

1. HGPRT itself because of competition between MP and its natural substrate, hypoxanthine
2. Inosinic dehydrogenase, which transforms inosinic acid (IMP) into xanthylic acid (xantosinic acid, xanthosine 5′- monophosphate), a precursor of guanylic acid (GMP)
3. Adenylosuccinate synthetase, which catalyzes the first step of the transformation of inosinic acid into adenylic acid (AMP)

In addition, thioinosinic acid is transformed into thioguanylic acid, which is misincorporated into DNA and RNA. This leads to single strand DNA breaks and DNA–protein cross-links by alteration of DNA repair mechanisms (Figure 2.46). Thioguanine acts by a very similar mechanism, after its transformation into thioguanylic acid by HGPRT.

The main degradative pathways of MP are its *S*-methylation by thiopurine methyltransferase (TPMT) and its oxidation by xanthine oxidase to an 8-oxo derivative, which is then further oxidized to give 6-thiouric acid (TUA). Allopurinol is a structural analog of hypoxanthine that is converted slowly to alloxanthine, which also inhibits the enzyme by trapping of an intermediate molybdenum(IV) species that participates in the catalytic cycle of the enzyme (Figure 2.47). Allopurinol interferes with the metabolism of MP (Figure 2.48), increasing its levels and leading to an interaction between both drugs; for this reason, patients taking allopurinol should have their MP dose reduced by up to 75%. However, the clinical benefit of this association in cancer patients taking MP is only slight, and renal damage may occur. *S*-methylation is another catabolic route of MP because the *S*-methyl derivative is not a substrate for the purine phosphoribosyl transferases.

Some heterocyclic derivatives of thiopurines have been designed to afford protection from the degradation processes described previously, two examples being the nitroimidazole derivatives azathioprine (Imuran®) and thiamiprine (Guaneran®). These compounds act as prodrugs and are presumably activated by an S_NAr mechanism involving nucleophilic attack from thiols onto the 5 position of the 4-nitroimidazole ring, followed by elimination of the thiopurine as a leaving group (Figure 2.49). None of these prodrugs are more effective as anticancer agents than the parent compounds, although azathioprine is an important immunosuppressant agent, widely used in autoimmune diseases.[76]

Another prodrug that is activated by a related mechanism is *cis*-3-(9*H*-purin-6-ylthio)acrylic acid (PTA), which is activated by glutathione through a Michael addition to the acrylic acid moiety followed by elimination (Figure 2.50).[77]

Considerable effort has been carried out to prepare other mercaptopurine and thioguanine analogs and their nucleosides in order to improve their antitumor efficacy.[78]

7 INHIBITORS OF ADENOSINE DEAMINASE

Coformycin (CF) and pentostatin (2′-deoxycoformycin, dCF, Nipent®) are two natural products isolated from *Streptomyces* species that are analogs of inosine and 2′-deoxyinosine, respectively, in which the purine ring is modified and contains a seven-membered ring. These compounds behave as very potent inhibitors of adenosine deaminase, the enzyme that degrades deoxyadenosine by its

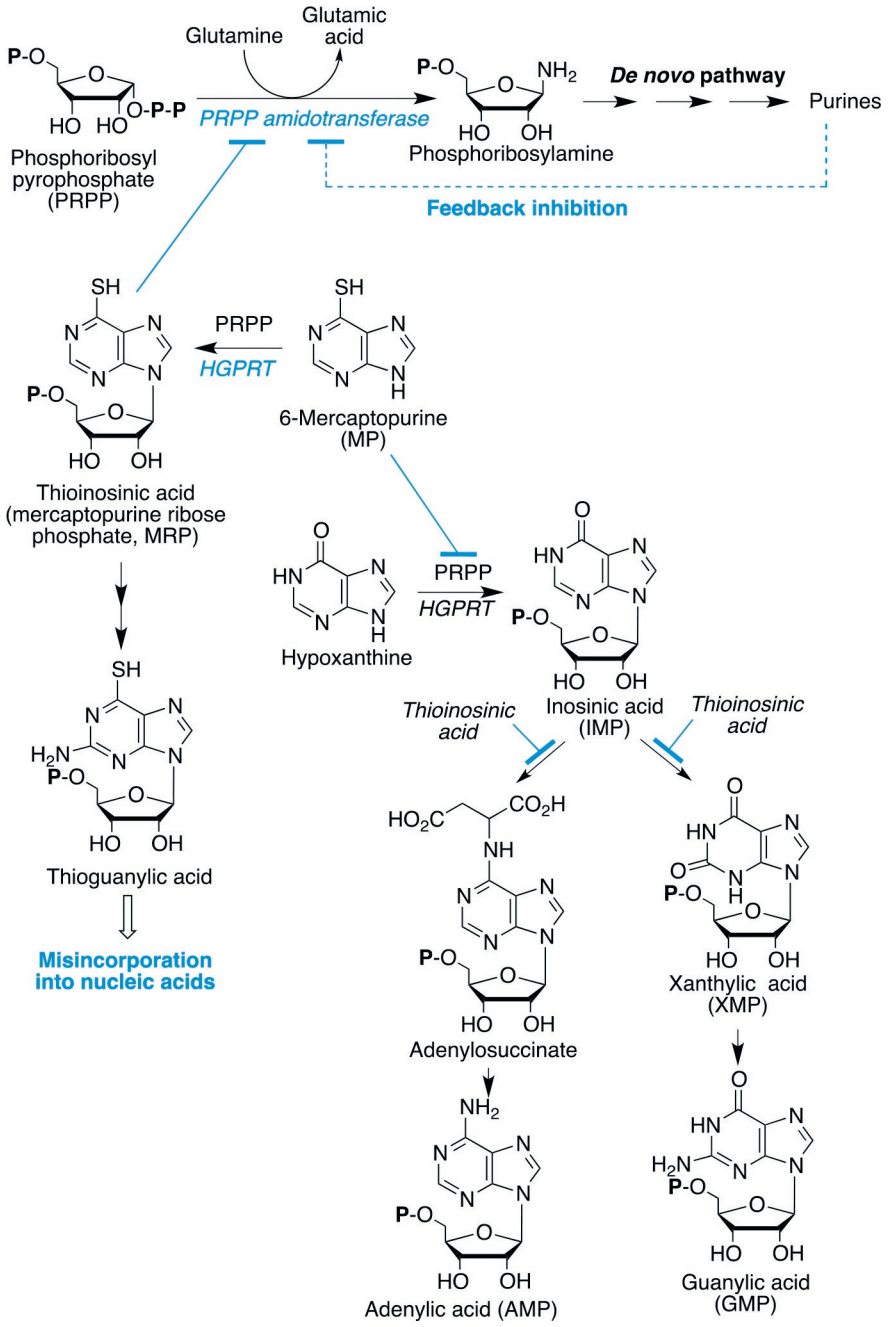


FIGURE 2.46

Reactions inhibited by 6-mercaptopurine and thioinosinic acid.

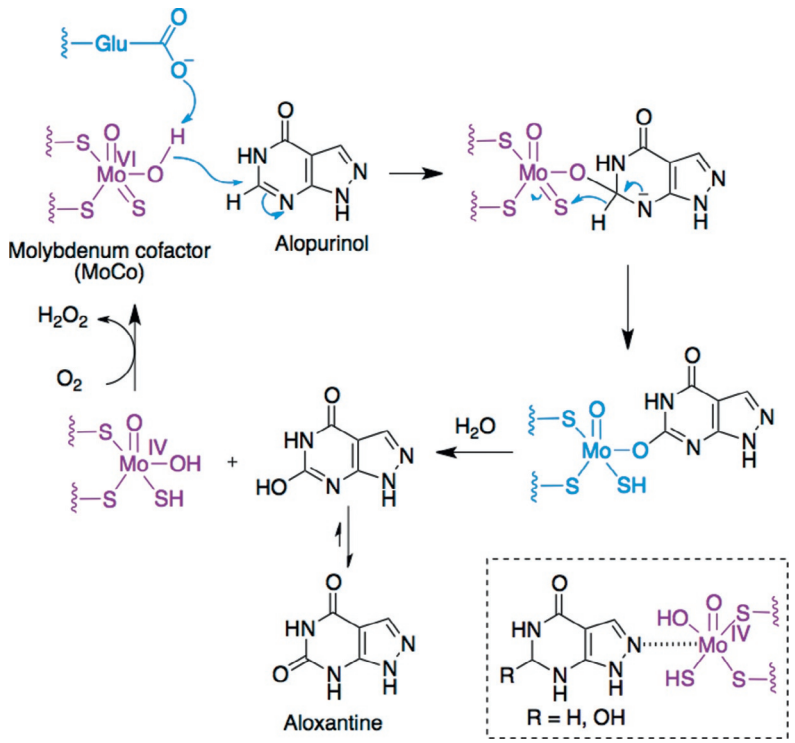


FIGURE 2.47

Inhibition of xanthine oxidase by allopurinol.

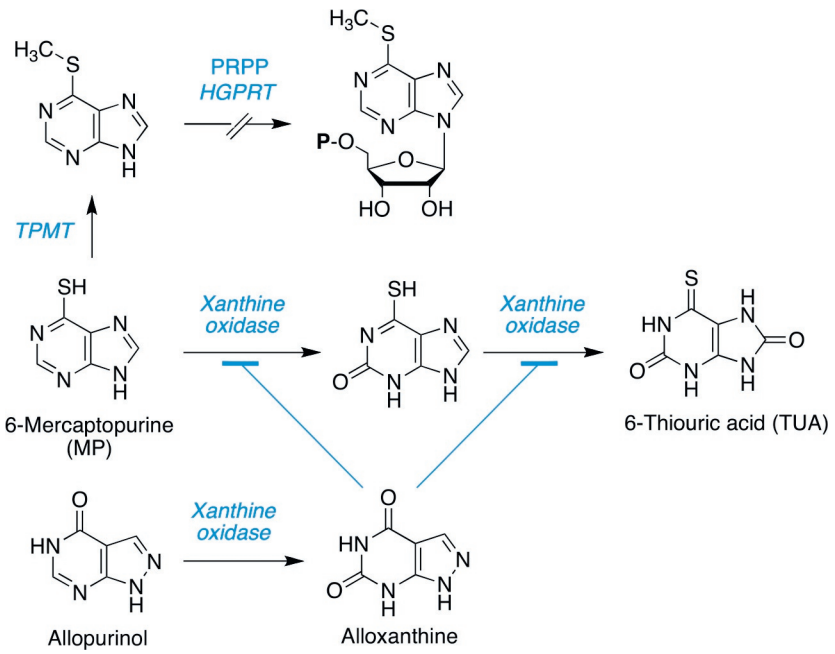


FIGURE 2.48

Main degradative pathways of 6-mercaptopurine.

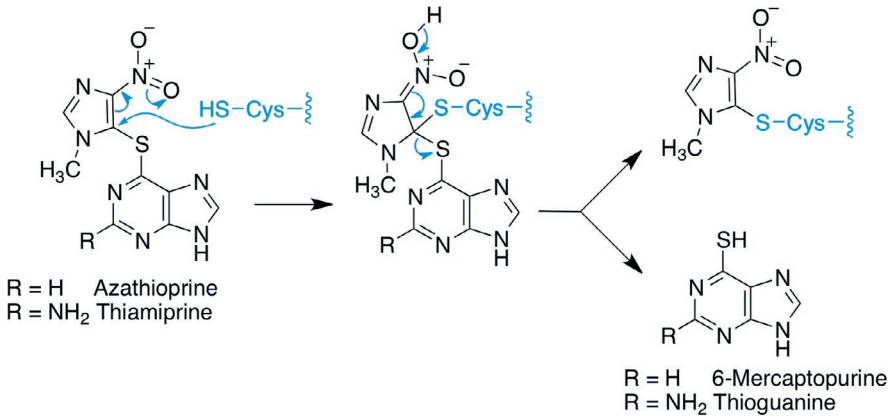


FIGURE 2.49

Bioactivation of azathioprine and thiamiprine.

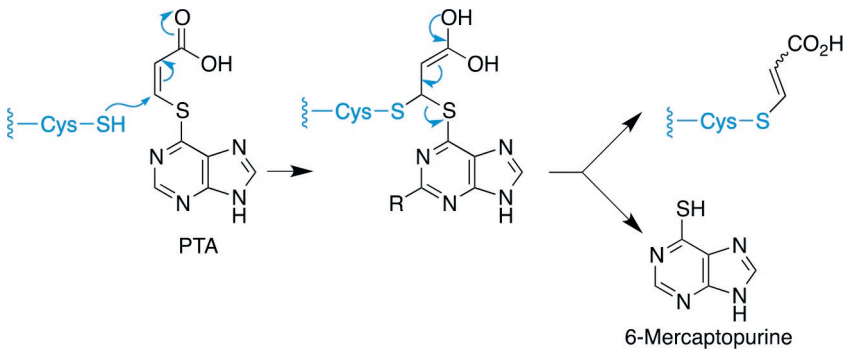


FIGURE 2.50

Bioactivation of the 6-mercaptopurine prodrug PTA.

transformation into 2′-deoxyinosine, and this inhibition is due to analogy of the drugs with the reaction transition state (Figure 2.51).

Pentostatin is used in combination with adenosine-derived antitumor drugs to increase their half-life. On its own, pentostatin is also an antitumor agent that is useful in the treatment of some types of leukemias, such as hairy cell leukemia. The mechanism of its antitumor activity is unclear and complex, and it includes the following events (Figure 2.52):

1. Inhibition of adenosine deaminase leads to accumulation of 2′-deoxyadenosine and, hence, to retroinhibition of the enzyme *S*-adenosylhomocysteine hydrolase. The subsequently accumulated *S*-adenosylhomocysteine acts as a competitive inhibitor of most of the methyltransferases that use *S*-adenosylmethionine as a cofactor, and therefore perturbs the processes related to the methylation of nucleic acids (see Chapter 8, Section 2).

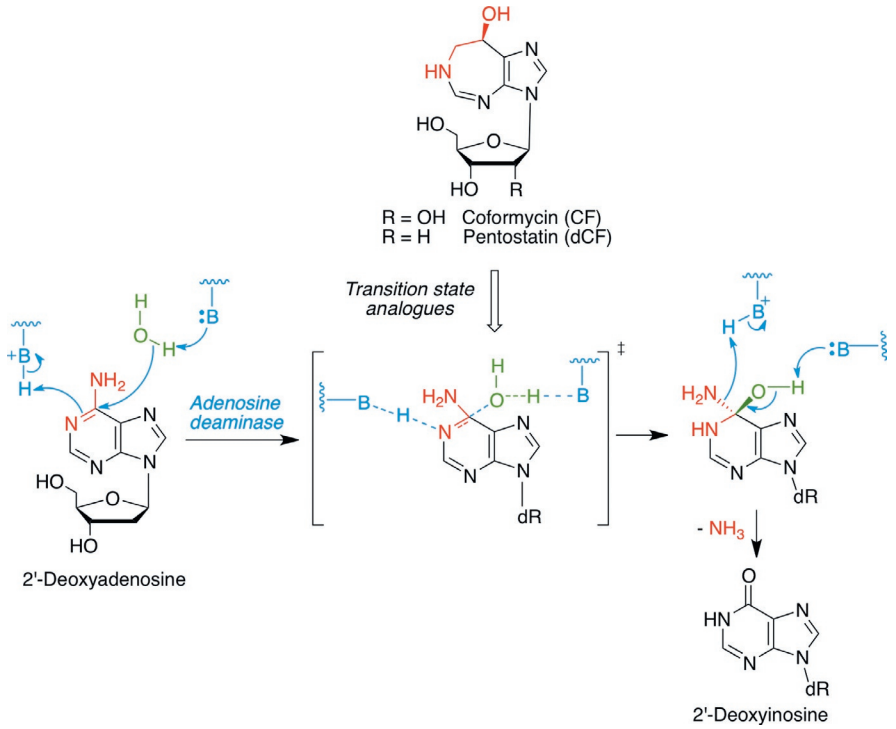


FIGURE 2.51

Inhibitors of adenosine deaminase.

2. Accumulation of deoxyadenosine also leads to high levels of deoxyadenosine triphosphate, which is an inhibitor of ribonucleotide reductase, the enzyme that removes the 2′-hydroxy group of the ribose ring during the biosynthesis of DNA.
3. Pentostatin is triphosphorylated and misincorporated into DNA.

8 INHIBITORS OF LATE STAGES IN DNA SYNTHESIS

As previously mentioned, several ribonucleoside and deoxyribonucleoside analogs are anticancer prodrugs that are activated to their triphosphates by phosphorylation catalyzed by kinases.[79] After bioactivation, the triphosphates act by misincorporation into DNA, resulting in slower chain elongation and alterations in DNA repair. The antitumor action of these compounds is due to the inhibition of DNA polymerase and other mechanisms (e.g., inhibition of ribonucleotide reductase or PNP) are known for particular compounds (Figure 2.53). A general problem associated with these drugs is that due to their cytotoxicity to lymphoid cells, significant and long-lasting immunosuppression results.

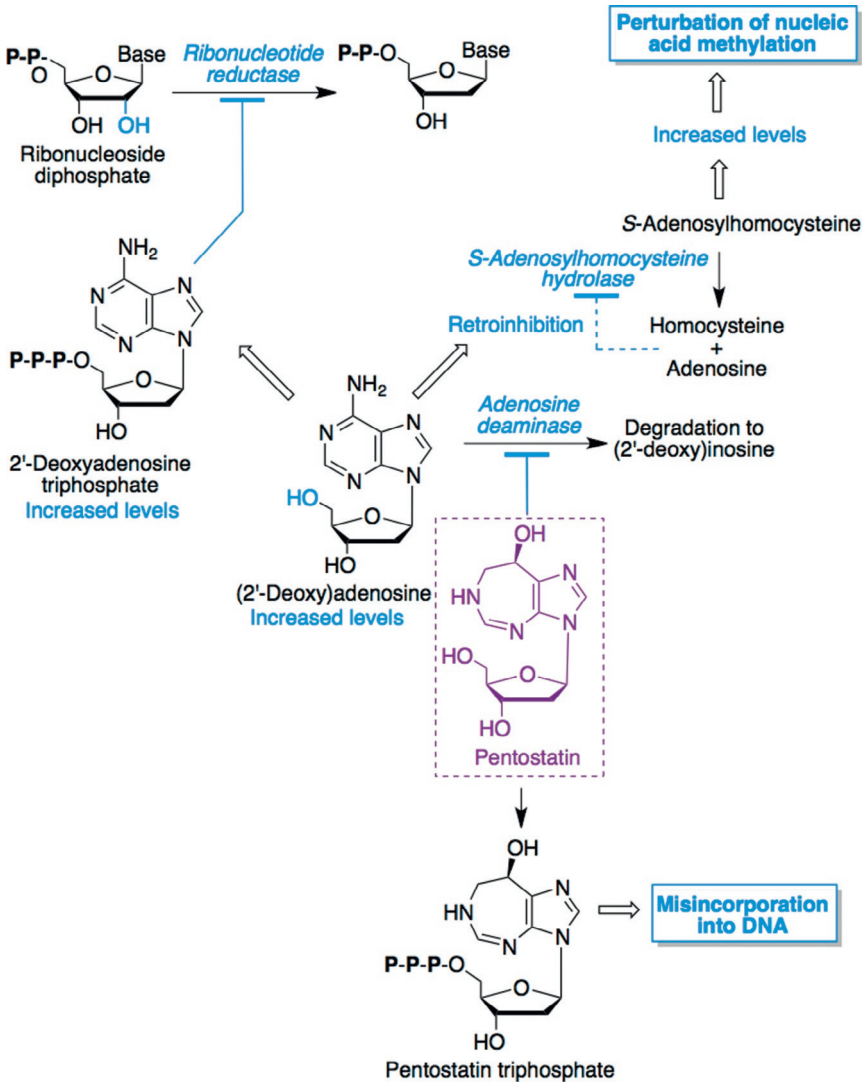


FIGURE 2.52

Events involved in the antitumor activity of pentostatin.

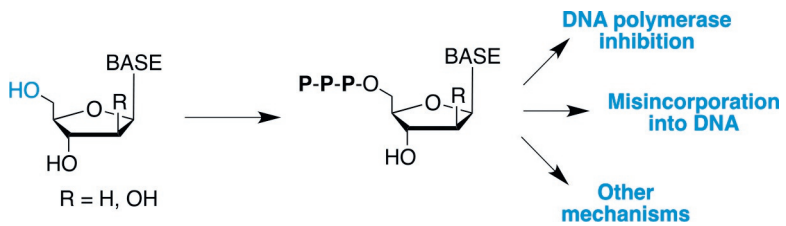
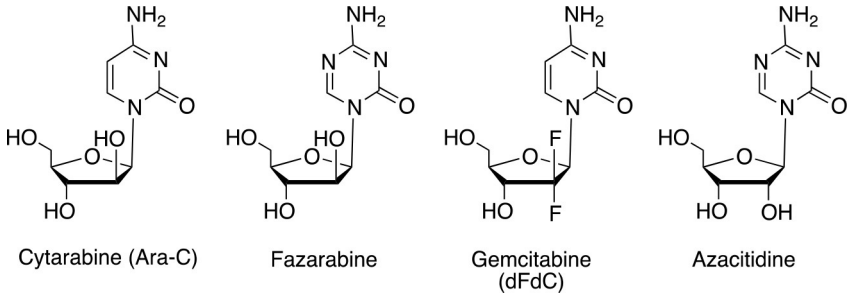


FIGURE 2.53

Antitumor mechanisms of ribonucleoside triphosphates.

8.1 PYRIMIDINE NUCLEOSIDES

The main anticancer compounds belonging to this group are cytosine or azacytosine nucleosides with a modified ribose ring, including the marine natural product cytarabine (Ara-C, Cytosar U®),[80] fazarabine (Ara-AC), gemcitabine (dFdC, Gemfar®), and azacitidine (Vidaza®), approved for myelodysplastic syndromes (MDS).



Among the arabinose-derived nucleosides, cytarabine (Ara-C), the 2′-epimer of cytidine, is useful in several leukemias, including acute myelogenous leukemia and non-Hodgkin's lymphoma. Cytarabine is employed either as a single agent or in combination with others, especially the anthracyclines, and it is the prime example of an antitumor drug specifically acting in the S phase of the cell cycle because its incorporation into DNA after being activated to the corresponding diphosphate leads to inhibition of strand elongation (Figure 2.54). Due to this S phase specificity, prolonged exposure of cells to cytotoxic concentrations is critical to achieve maximum cytotoxic activity. However, the activity of cytarabine is decreased by its rapid deamination by cytosine deaminase to the biologically inactive metabolite uracil arabinoside.[81] For this reason, the search for effective formulations and derivatives of cytarabine that cannot be deaminated and exhibit better pharmacokinetic parameters has been an active field of research.[82]

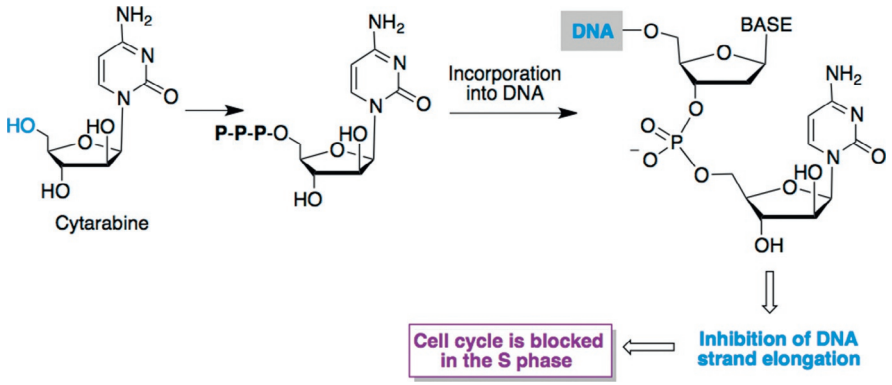


FIGURE 2.54

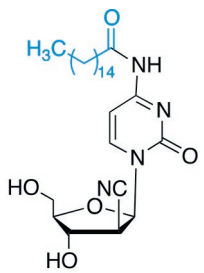
Antitumor mechanisms of cytarabine.

Fazarabine is an aza analog with a very potent activity in animal models, including solid tumors, that has been submitted to several clinical trials.[83] Gemcitabine blocks the cell cycle at the S phase similarly to cytarabine, and it is also an inhibitor of ribonucleotide reductase in its diphosphate form (see Section 3.3). Gemcitabine can be considered as the leading marketed nucleoside analog and is most commonly used to treat non-small cell lung cancer and pancreatic, bladder, and breast cancer. It has also been shown to be synergistic in combination with pemetrexed in phase III studies for pancreatic and non-small cell lung cancer because pemetrexed depletes the intracellular stores of purine and pyrimidine nucleotides, whereas gemcitabine is incorporated in nascent DNA strands.[84] The human equilibrative nucleoside transporter 1 (hENT1) plays an important role in predicting clinical outcome after gemcitabine chemotherapy for several types of cancer.[85]

Azacitidine and its 2′-deoxy analog are also triphosphorylated and misincorporated into nucleic acids, but this will be discussed in Chapter 8 in connection with the inhibition of DNA methylation.

It has been previously mentioned that one of the main drawbacks of cytarabine is its short half-life in plasma due to rapid deamination to its uracil analog by cytidine deaminase. During the search for cytarabine analogs that could overcome this problem, and also in an effort to achieve activity against solid tumors, the arabinose 2-hydroxy group was replaced with other substituents.[86] The 2-cyano derivative (2′-cyano-2′-deoxy-1-βD-arabinofuranosylcytosine, CNDAC) is particularly interesting because it acts by a novel mechanism among nucleoside analogs, involving DNA single strand breaking by a β-elimination reaction. CNDAC is less efficient than cytarabine and gemcitabine at inhibiting DNA strand elongation, and the cells can progress through the S phase, leading to incorporation of its nucleotides at internal positions of DNA (**2.30** and **2.31**). The electron withdrawing effect of the cyano group at the arabinose 2′-β position increases the acidity of the 2′-α proton and facilitates a β-elimination reaction in **2.31** involving an oxygen of the phosphate group at the 3′-β position that leads to single strand break that affords a DNA molecule lacking a 3′-hydroxyl, which prevents its repair by ligation and leads to inhibition of the cell cycle at the G_2 phase (Figure 2.55).[87]

The N^4-palmitoyl derivative of CNDAC is an oral prodrug known as sapacitabine (CYC-682, CS-682), which is activated by intestinal and plasma amidases.[88] This compound received the orphan drug designation by the FDA for the treatment of both acute myeloid leukemia (AML) and MDS. It has been suggested that clinical activity and application of sapacitabine is different from that of cytarabine or gemcitabine.[89]



Sapacitabine
(CYC-682, CS-682)

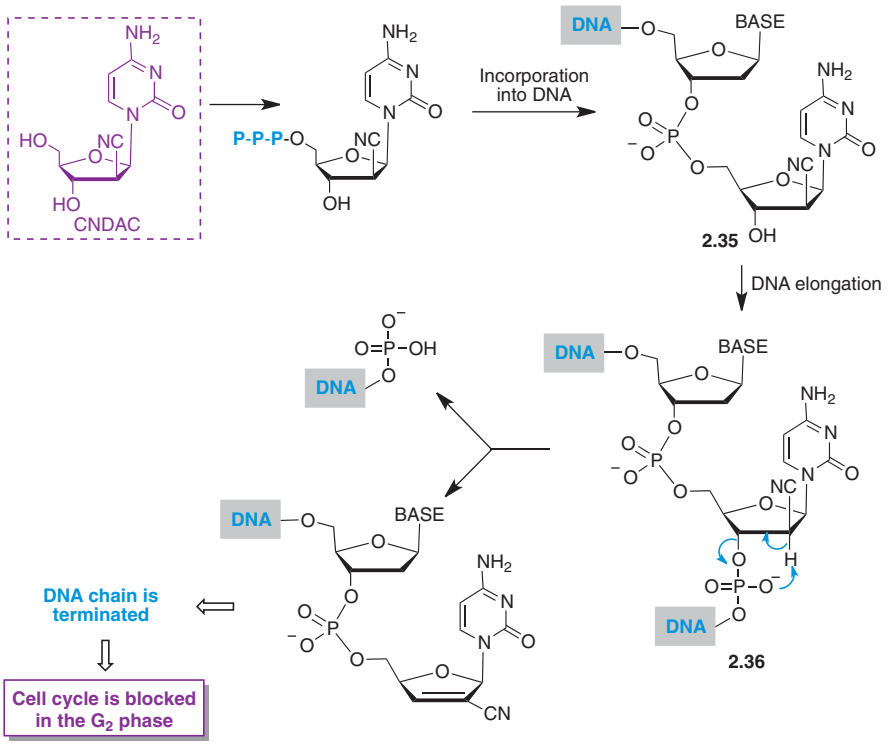
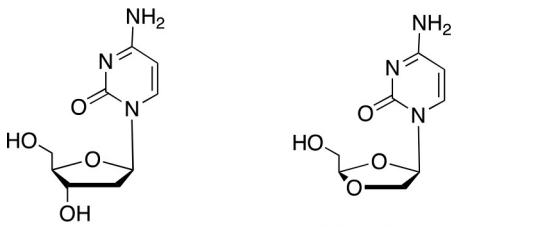


FIGURE 2.55

Mechanisms of the antitumor action of CNDAC.

Another way to avoid the action of cytidine deaminase is the use of L-nucleosides, which are not good substrates for the enzyme. For instance, troxacitabine is a dioxolane analog of β-L-deoxycytidine, which is a poor substrate for this enzyme. After activation as a triphosphate, it inhibits DNA polymerase activity and leads to a complete chain termination. This compound has shown positive responses in clinical trials in patients with metastatic renal cancer.[90]

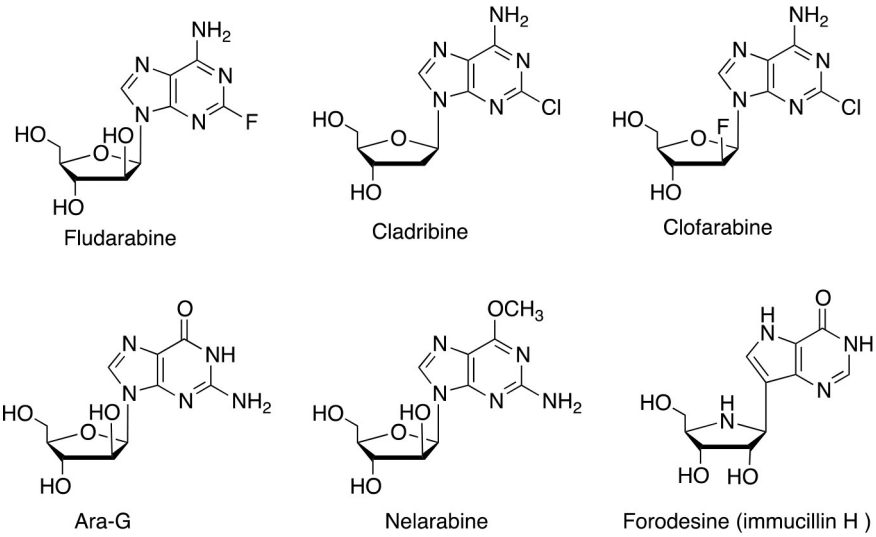


β–D-Deoxycytidine

Troxacitabine
(β-L-dioxolanecytidine, L-OddC)

8.2 PURINE NUCLEOSIDES

Fludarabine (Fludara®) and cladribine (Litak®) are used in cancer therapy, especially as second-line treatment for patients with B-cell chronic lymphocytic leukemia (CLL) who do not respond to alkylating agents. Other purine nucleosides (clofarabine, nelarabine, and forodesine) have also been introduced into clinical trials.[91] These compounds use nucleoside-specific membrane transporters to enter the cells and must then be converted into their active triphosphate forms.



Fludarabine is very insoluble and is administered as a phosphate prodrug that must be cleaved back to the nucleoside prior to entering the cell. In addition to the mechanisms common to this group of drugs, it is an inhibitor of ribonucleotide reductase and is active against several lymphoid malignancies. Cladribine is also employed in hairy cell leukemia and has the advantage compared to other adenine derivatives of being resistant to adenosine deaminase, a property that it shares with all 2-halogenated adenine derivatives. The 2′-fluoro derivative of cladribine, called clofarabine (Clolar® or Evoltra®), is more acid stable, leading to increased oral bioavailability. It was approved for treating relapsed or refractory acute lymphoblastic leukemia (ALL) in children after at least two other types of treatment have failed, and its effectiveness in AML, juvenile myelomonocytic leukemia, and other cancers is being studied. Ara-G has a very poor water solubility, and this has led to the development of its prodrug nelarabine (Arranon®), which is 10 times more soluble due to a diminished stability of its crystalline lattice, associated with loss of hydrogen bonding by the lactam moiety existing in Ara-G. Nelarabine is bioactivated by the route schematized in Figure 2.56, which also includes the subsequent steps necessary for the activity of this group of compounds. Thus, nelarabine is a substrate of adenosine deaminase, which transforms it into Ara-G. Once inside the cell, this compound is triphosphorylated at C-5′, and the resulting triphosphate (Ara-GTP) is misincorporated into DNA. Nelarabine was approved in 2005 for T-cell ALL and T-cell lymphoblastic lymphoma that has not responded to or has relapsed following treatment with at least two chemotherapy regimens. Another purine nucleoside that has entered clinical trials is forodesine (immucillin H), which was discussed in Section 3.5.

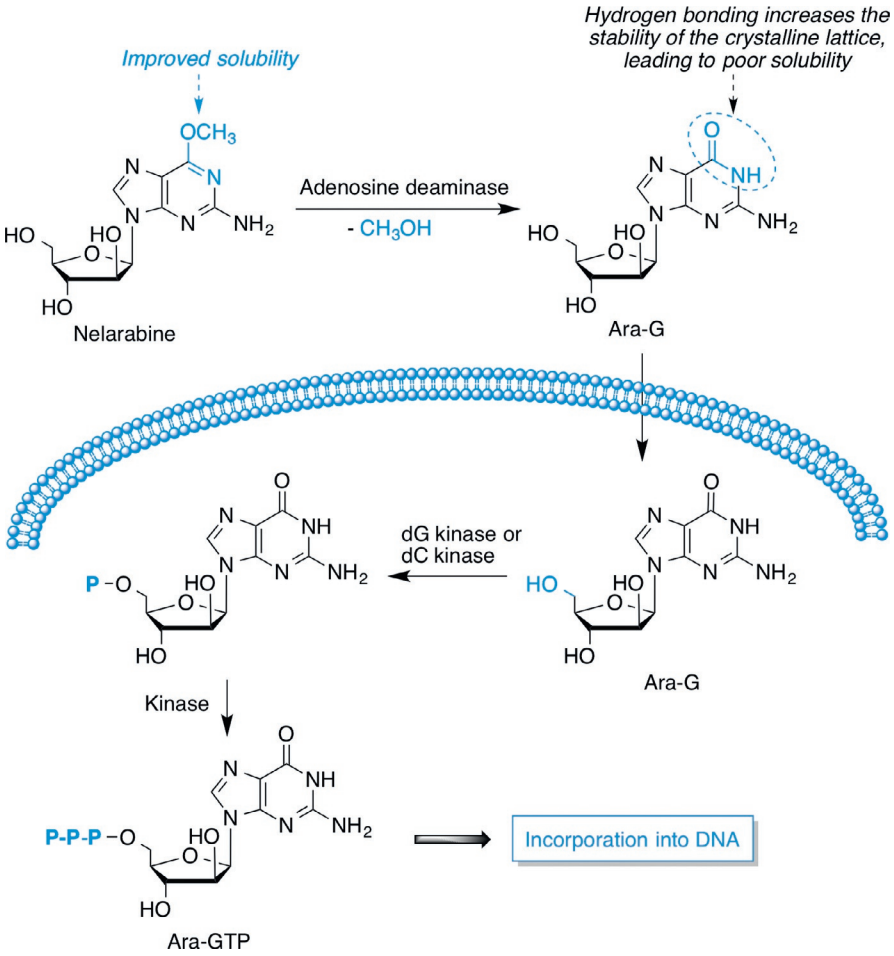


FIGURE 2.56

Intracellular transport and bioactivation of nelarabine.

9 ANTIMETABOLITE ENZYMES

L-Asparaginase is an enzyme that behaves as an antimetabolite. Its physiological role is the hydrolysis of the amino acid L-asparagine to aspartic acid and ammonia (Figure 2.57). Normal tissues can synthesize L-asparagine in amounts sufficient for protein synthesis, but some types of lymphoid malignancies take it from plasma. Treatment of these patients with L-asparaginase leads to the hydrolysis of circulating L-asparagine and prevents its uptake into the tumor cells, leading to interruption of protein synthesis and cell death. L-Asparaginase (Elspar®) is normally used for the treatment of ALL in combination with other agents, such as methotrexate, doxorubicin, cytarabine, and vincristine.

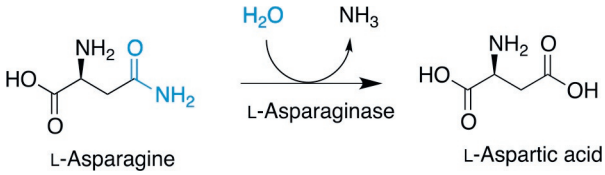


FIGURE 2.57

Reaction catalyzed by L-asparaginase.

To avoid the problems associated with the poor pharmacokinetic properties of proteins and their immnunogenicity, L-asparaginase has been modified by its covalent attachment to polyethyleneglycol (PEG)-type structures such as monomethoxy polyethyleneglycol succinimidyl units. This PEG-modified enzyme is known as pegaspargase (Oncaspar®; see also Chapter 13, Section 4.1), and it is employed for the treatment of ALL in patients who have developed hypersensitivity to the native form of L-asparaginase.

The *Erwinia chrysanthemi* asparaginase (Erwinaze®) is specifically approved as a component of a multiagent chemotherapeutic regimen for the treatment of patients with ALL who have developed hypersensitivity to *E. coli*-derived asparaginase.

REFERENCES

1 Redei I, Green F, Hoffman JP, Weiner LM, Scher R, O'Dwyer PJ. *Invest New Drugs* 1994;**12**:319.
2 For reviews, see. (a) Tsimberidou A-P, Alvarado Y, Giles FJ. *Expert Rev Anticancer Ther* 2002;**2**:437; (b) Shao J, Liu X, Zhu L, Yen Y. *Expert Opin Ther Targets* 2013;**17**:1423.
3 Hogbom M, Galander M, Andersson M, Kolberg M, Hofbauer W, Lassmann G, et al. *Proc Natl Acad Sci U S A* 2003;**100**:3209.
4 Pereira S, Cerqueira N, Fernandes PA, Ramos MJ. *Eur Biophys J* 2006;**35**:125.
5 Bernstein LR. *Pharmacol Rev* 1998;**50**:665.
6 Collery P, Keppler B, Madoulet C, Desoize B. *Cr Rev Oncol Hem* 2002;**42**:283.
7 Peri D, Meker S, Manna CM, Tshuva EY. *Inorg Chem* 2011;**50**:1030.
8 For reviews, see. (a) Saban N, Bujak M. *Cancer Chemother Pharmacol* 2009;**64**:213; (b) Madaan K, Kaushik D, Verma T. *Expert Rev Anticancer Ther* 2012;**12**:19.
9 Yarbro JW. *Semin Oncol* 1992;**19**(3 Suppl. 9):1.
10 Jiang J, Jordan SJ, Barr DP, Gunther MR, Maeda H, Mason RP. *Mol Pharmacol* 1997;**52**:1081.
11 King SB. *Free Radical Biol Med* 2004;**37**:737.
12 Reardon DA, Egorin MJ, Quinn JA, Rich JN, Gururangan I, Vredenburgh JJ, et al. *J Clin Oncol* 2005;**23**:9359.
13 For reviews, see. (a) Lanzkron S, Strouse JJ, Wilson R, Beach MC, Haywood C, Park H, et al. *Ann Intern Med* 2008;**148**:939; (b) Strouse JJ, Heeney MM. *Pedriatr Blood Cancer* 2012;**59**:365.
14 Halsey C, Roberts IA. *Br J Haematol* 2003;**120**:177.
15 Finch RA, Liu M, Grill SP, Rose WC, Loomis R, Vasquez KM, et al. *Biochem Pharmacol* 2000;**59**:983.
16 https://www.clinicaltrials.gov/ct/show/NCT00004213.
17 Atieh DM, Modiano M, Shriberg L, Brafman L, Szno M, Vahdat L. *J Clin Oncol* 2004;**22**:864.
18 Kunos CA, Waggoner S, von Gruenigen V, Eldermire E, Pink J, Dowlati A, et al. *Clin Cancer Res* 2010;**16**:1298.

19 Elford HL, Cardounel AJ, Zweier J, Henry J, Sumpter R, Oakley O, et al. *Proc Am Assoc Cancer Res* 2006;**47**:2125.
20 Szekeres T, Fritzer M, Strobl H, Gharehbaghi K, Findenig G, Elford HL, et al. *Blood* 1994;**84**:4316.
21 Taverna P, Rendahl K, Jekic-McMullen D, Shao Y, Aardalen K, Salangsang F, et al. *Biochem Pharmacol* 2007;**73**:44.
22 Fernandes PA, Ramos MJ. *J Am Chem Soc* 2003;**125**:6311.
23 Pereira S, Fernandes PA, Ramos MJ. *J Comput Chem* 2004;**25**:1288.
24 Gandhi V, Kilpatrick JM, Plunkett W, Ayres M, Harman L, Du M, et al. *Blood* 2005;**106**:4253.
25 (a) Kicska GA, Long L, Hörig H, Fairchild C, Tyler PC, Furneaux RH, et al. *Proc Natl Acad Sci U S A* 2001;**98**:4593; (b) Lee JL, Singhy V, Evans GB, Tyler PC, Furneaux RH, Cornell KA, et al. *J Biol Chem* 2005;**280**:18274.
26 For a review of the pharmacology and mechanism of action of forodesine, see Gandhi V, Balakrishnan K. *Semin Oncol* 2007;**34**:S8.
27 (a) https://www.clinicaltrials.gov/ct2/results?term=Forodesine&flds=Xabce. (b) https://clinicaltrials.gov/ct2/show/NCT00501735.
28 Duvic M, Olsen EA, Omura GA, Maize JC, Vonderheid EC, Elmets CA, et al. *J Am Acad Dermatol* 2001;**44**:940.
29 Longley DB, Harkin DP, Johnston PG. *Nat Rev Cancer* 2003;**3**:330.
30 Adjei AA. *J Clin Pharmacol* 1999;**48**:265.
31 Papamichael D. *Stem Cells* 2000;**18**:166.
32 Houghton JA, Tilman DM, Harwood FG. *Clin Cancer Res* 1995;**1**:723.
33 Longley DB, Boyer J, Allen WL, Latif T, Ferguson PR, Maxwell PJ, et al. *Cancer Res* 2002;**62**:2644.
34 For a review, see Milano G, Ferrero JM, François E. *Br J Cancer* 2004;**91**:613.
35 Ciccolini J, Evrad A, Cuq P. *Curr Med Chem Anticancer Agents* 2004;**4**:71.
36 O'Shaughnessy J, Miles D, Vukelja S, Moiseyenko V, Ayoub JP, Cervantes G, et al. *Clin Oncol* 2002;**20**:2812.
37 López-Estévez S, Ferrer G, Torres-Torronteras J, Mansilla MJ, Casacuberta-Serra S, Martorell L, et al. *Gene Ther* 2014;**21**:673.
38 LoRusso PM, Prakash S, Wozniak A, Flaherty L, Zalupski M, Shields A, et al. *Invest New Drugs* 2002;**20**:63.
39 Diasio RB, Harris BE. *Clin Pharmacokinet* 1989;**16**:215.
40 Peters GJ, van Groeningen CJ, Giaccone G. *J Clin Oncol* 2001;**19**:4267.
41 Smith IE, Johnston SR, O'Brien ME, Hickish TF, Boer RH, Norton A, et al. *J Clin Oncol* 2000;**18**:2378.
42 Hoff P, Pazdur R. *Oncologist* 1998;**3**:155.
43 Nemunaitis J, Eager R, Twaddell T, Corey A, Sekar K, Tkaczuk K, et al. *J Clin Oncol* 2000;**18**:3423.
44 Eng C, Kindler HL, Schilsky RL. *Clin Colorectal Cancer* 2001;**1**:95.
45 Leyland-Jones B, O'Dwyer PJ. *Cancer Treat Rep* 1986;**70**:219.
46 Browman GP, Levine MN, Goodyear MD, Russell R, Archibald SD, Jackson BS, et al. *J Clin Oncol* 1988;**6**:963.
47 Tomlinson SK, Melin SA, Higgs V, White DR, Savage P, Case D, et al. *BMC Cancer* 2002;**2**:9.
48 Peters GJ, Noordhuis P, Van Kuilenburg AB, Schornagel JH, Gall H, Turner SL, et al. *Cancer Chemother Pharmacol* 2003;**52**:1.
49 Kobayakawa M, Kojima Y. *OncoTargets Therapy* 2011;**4**:193.
50 For a review, see Jackman AL, Jansen G, Ng M. Jackman AL, Leamos CP, editors. *Targeted drug strategies for cancer and inflammation*. New York: Springer; 2011.
51 Schwartz G, Johnson TR, Goetz A, Burris H, Smetzer L, Lampkin T, et al. *Clin Cancer Res* 2001;**7**:1901.
52 Pivot X, Wadler S, Kelly C, Ruxer R, Tortochaux J, Stern J, et al. *Ann Oncol* 2001;**12**:1595.
53 Jackmann AL, Theti DS, Gibbs DD. *Adv Drug Delivery Rev* 2004;**56**:1111.
54 Wibowo AS, Singh M, Reeder KM, Carter JJ, Kovach AR, Meng W, et al. *Proc Natl Acad Sci U S A* 2013;**110**:15180.

55 Theti DS, Bavetsias V, Gibbs DD, Skelton LA, Jackman AL. *Proc Am Soc Cancer Res* 2001;**42**:291.
56 Theti DS, Bavetsias V, Skelton LA, Titley J, Gibas D, Jansen G, et al. *Cancer Res* 2003;**63**:3612.
57 Bavetsias V, Marriott JH, Melin C, Kimbell R, Matusiak ZS, Boyle T, et al. *J Med Chem* 2000;**43**:1910.
58 Tochowicz A, Dalziel S, Eidam O, O'Connell JD, Griner S, Finer-Moore JS, et al. *J Med Chem* 2013;**56**:5446.
59 http://www.controlled-trials.com/ISRCTN79302332.
60 For reviews of this area, see. (a) Takimoto CH. *Oncologist* 1996;**1**:68; (b) Purcell WT, Ettinger DC. *Curr Oncol Rep* 2003;**5**:114.
61 For a monograph, see Jackman AL. *Antifolate drugs in cancer therapy*. Totowa, NJ: Humana Press; 1999.
62 Goodsell DS. *Oncologyst* 1999;**4**:340.
63 Blakley RL, Cocco L. *Biochemistry* 1985;**24**:4772.
64 Kamen BA, Whyte-Bauer S, Bertino JR. *Biochem Pharmacol* 1983;**32**:1837.
65 Meyers FJ, Lew D, Lara PN, Williamson S, Marshall E, Balcerzak SP, et al. *Invest New Drugs* 1999;**16**:347.
66 (a) Kuriakose P, Gandara DR, Pérez EA. *Cancer Invest* 2002;**20**:473; (b) Hui J, Przespo E, Elefante A. *J Oncol Pharm Pract* 2012;**18**:275.
67 Blanke CD, Kasimis B, Schein P, Capizzi R, Kurman M. *J Clin Oncol* 1997;**15**:915.
68 Lassiter LK, Tummala MK, Hussain MH, Stadler WM, Petrylak DP, Carducci MA. *Clin Genitourin Cancer* 2008;**6**:31.
69 Kisliuk RL. *Curr Pharm Des* 2003;**9**:2615.
70 Sessa C, de Jong J, D'Incalci M, Hatty S, Pagani O, Cavalli F. *Clin J Cancer Res* 1996;**2**:1123.
71 McLeod HL, Cassidy J, Powrie RH, Priest DG, Zorbas MA, Synold TW, et al. *Clin Cancer Res* 2000;**6**:2677.
72 http://www.cancer.gov/clinicaltrials/MSKCC-04032.
73 Kut V, Patel JD, Argiris A. *Exp Rev Anticancer Ther* 2004;**4**:511.
74 Gangjee A, Yu J, McGuire JJ, Cody V, Galitsky N, Kisliuk RL, et al. *J Med Chem* 2000;**43**:3837.
75 Mueller C, Al-Batran S, Jaeger E, Schmidt B, Bausch M, Unger C, et al. *J Clin Oncol* 2008;**26**(May 20 Suppl.), Abstr 2533.
76 Aberra FN, Lichtenstein GR. *Aliment Pharmacol Ther* 2005;**21**:307.
77 Gunnasdottir S, Elfarra AA. *J Pharmacol Exp Ther* 1999;**290**:950.
78 Elgemeie GH. *Curr Pharm Des* 2003;**9**:2627.
79 Johnson SA. *Clin Pharmacokinet* 2000;**39**:5.
80 Arif JM, Al-Hazzani AA, Kunhi M, Jal-Khodairy F. *Biomed Biotech* 2004;**2**:93.
81 Ohta T, Hori H, Ogawa M, Miyahara M, Kawasaki H, Taniguchi N, et al. *Oncol Rep* 2004;**12**:1115.
82 Hamada A, Kawaguchi T, Nakano M. *Clin Pharmacokinet* 2002;**41**:705.
83 Ben-Baruch N, Denicoff AM, Goldspiel BR, O'Shaughnessy JA, Cowan KW. *Invest New Drugs* 1993;**11**:71.
84 Henry JR, Mader MM. *Annu Rep Med Chem* 2004;**39**:161.
85 For a review, see Santini D, Schiavon G, Vincenzi B, Cass CE, Vasile E, Manazza AD, et al. *Curr Cancer Drug Targets* 2011;**11**:123.
86 Matsuda A, Nakajima Y, Azuma A, Tanaka M, Sasaki T. *J Med Chem* 1991;**34**:2919.
87 Azuma A, Huang P, Matsuda A, Plunkett W. *Mol Pharmacol* 2001;**59**:725.
88 (a) Hanaoka K, Suzuki M, Kobayashi T, Tanzawa F, Tanaka K, Shibayama T, et al. *Int J Cancer* 1999;**82**:226; (b) Faderl S, Gandhi V, Kantarjian HM. *Curr Opin Hematol* 2008;**15**:101.
89 (a) Liu XJ, Nowak B, Wang YQ, Plunkett W. *Chin J Cancer* 2012;**31**:373; (b) *Expert Opin Investig Drugs* 2012;**21**:541.
90 Townsley CA, Chi K, Ernst DS, Belanger K, Tannock I, Bjarnason GA, et al. *J Clin Oncol* 2003;**21**:1524.
91 Robak T, Lech-Maranda E, Korycka A, Robak E. *Curr Med Chem* 2006;**13**:3165.

CHAPTER

ANTICANCER DRUGS THAT MODULATE HORMONE ACTION

3

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00003-X

1 INTRODUCTION

The recognition during the 1970s that breast and prostate tumors are subject to hormonal regulation provided the first opportunity for a targeted approach to cancer chemotherapy. Indeed, hormones, and in particular steroid hormones, are the main determinants in the induction and growth of several types of tumors, and for this reason the search for antihormones has been one of the mainstays of cancer chemotherapy. Thus, compounds acting on estrogen and androgen receptors are involved in the treatment of breast and prostatic cancers, among others, whereas corticosteroids are employed in myelomas and lymphomas because of their role in the function of lymphoid tissues.

Steroid hormone receptors are ligand-dependent transcription factors that regulate the transcription of their target genes in a highly complex process. These receptors are cytoplasmic or nuclear proteins that have a binding site for a particular steroid molecule. The steroid–receptor complexes form homodimers that bind to DNA sequences, their response elements, which are part of a gene promoter. This binding activates or represses the gene controlled by that promoter. The steroid hormone receptors consist of at least three domains:

1. One responsible for binding the hormone.
2. A zinc finger domain needed for DNA binding to the response element. A zinc finger can be defined as a protein structural motif characterized by a fold stabilized by coordination of an amino acid, usually cysteine or histidine, with a zinc ion. Zinc fingers typically act as protein–DNA binding elements.
3. Finally, a domain needed for the receptor to activate the promoters of the genes being controlled.

As shown in Figure 3.1, the sequence of events leading to the start of gene transcription by a steroid hormone is as follows: (1) binding of the hormone to the receptor; (2) formation of a homodimer from two molecules of receptor; (3) transport to the nucleus, if necessary (e.g., in the case of glucocorticoid hormones); (4) binding to the response element; (5) recruitment of coactivators; and (6) final activation of transcription factors to start transcription. The ultimate consequence is the synthesis of a molecule of mRNA and the corresponding protein, which triggers the observed biological response (see also Figure 3.14a).

2 ESTROGENS AND THEIR INVOLVEMENT IN CARCINOGENESIS

Estrogens are a family of related steroidal molecules that stimulate the development and maintenance of female characteristics and sexual reproduction, including regulation of the menstrual cycle and several other physiological functions, such as reduction of osteoporosis risk and cholesterol levels. The most prevalent forms of human estrogens are estradiol and estrone, which are produced and secreted by the ovaries, although estrone is also synthesized in the adrenal glands and other organs. The main

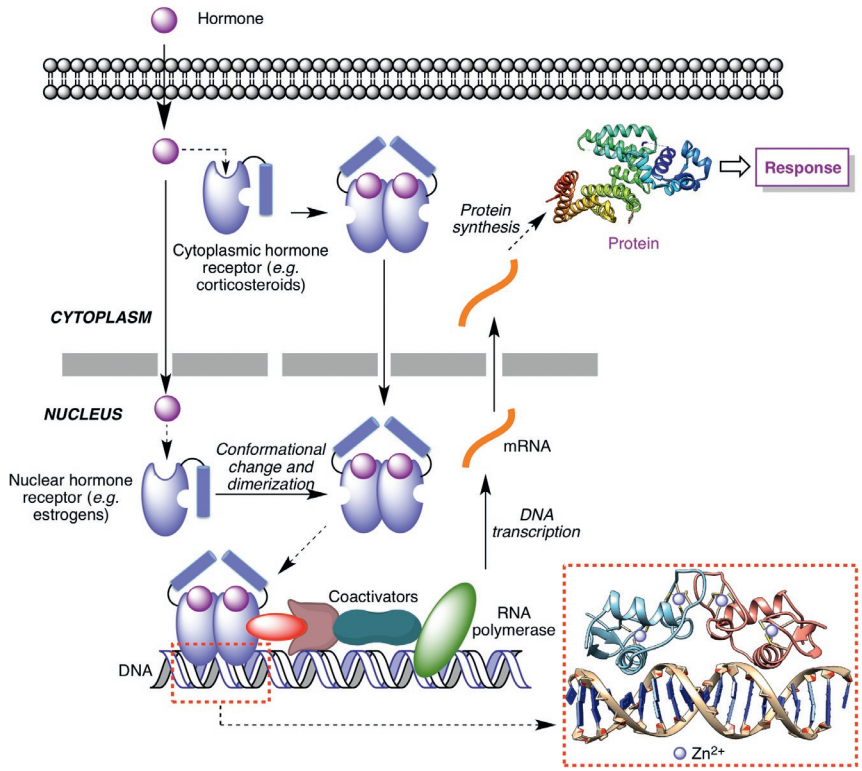
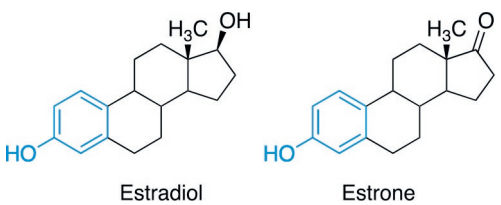


FIGURE 3.1

Sequence of events associated with steroid hormone activity. The structure of the estrogen receptor bound to DNA was generated from Proten Data Bank, reference 1HCQ and displayed with Chimera 1.8.1.

structural feature that differentiates these compounds from other steroidal hormones is an aromatic A ring bearing a phenol functional group.



Although early models proposed that the estrogen receptors (ERs) were cytoplasmic and were translocated to the nucleus after binding to the estrogen molecules, subsequent studies with monoclonal antibodies revealed that ERs were located in the nucleus.

Regulation of gene transcription by estrogens involves regions of DNA called estrogen response elements (EREs) that bind to several nuclear proteins (coactivators), forming a multi-subunit transcriptional complex. Occupation of the steroid binding domain in the receptor by the hormone is followed by receptor dimerization, which is essential for DNA binding. Upon estrogen recognition,

a conformational change takes place in the receptor protein that allows the recognition of coactivators and the start of the transcription process.

The natural estrogens induce tumors in a variety of organs in laboratory animals, and high estrogen levels increase the risk of breast and uterine cancer.[1] Estrogen receptors can be detected in approximately 60% of breast cancers [ER(+)], making them sensitive to anti-endocrine treatment. Two types of mechanisms have been proposed that explain the development of estrogen-dependent tumors, and thus it has been proven that estrogens cause both nongenotoxic and genotoxic cell proliferative effects.[2]

First, the transcription process initiated by the binding of estrogens to their receptors ultimately induces cell proliferation in some target tissues such as breast tissue, in which estrogens trigger the proliferation of cells lining the milk glands thereby preparing the breast to produce milk in case of pregnancy, and the endometrium of the uterus, in which they stimulate cell proliferation to prepare the uterus for implantation. This proliferative action, which is one of the physiological roles of estrogens, can also lead to the development of breast or uterine cancer in cells from tissues that possess a DNA mutation that increases the risk of developing cancer because they will proliferate (along with normal cells) in response to estrogen stimulation.

An additional mechanism that explains the carcinogenesis by estrogens is the generation of mutagenic species in their metabolism.[3] Strong evidence supports that tumors may be initiated by metabolic conversion of estradiol (E_2) to the catechol metabolite 4-hydroxyestradiol (4-OHE_2), which is further oxidized to estradiol-3,4-quinone (E_2-3,4-Q) (Figure 3.2).[4] Furthermore, the oxidation process generates radical oxygen species via a nonenzymatic pathway, leading also to cell damage by oxidative stress (see Chapter 4, Section 2).

Estradiol-3,4-quinone reacts with DNA as a Michael substrate forming a bond between its C-1 atom and the N-7 atom of guanine, affording hydroquinone **3.1**. The positive charge generated at the guanine N-7 position facilitates the hydrolysis of the glycosidic bond of **3.1**, leading to purine derivative **3.2** and depurinized DNA (**3.3**) (Figure 3.3).

Alternatively, the nucleophilic attack to the E_2-3,4-Q may involve the N-3 atom of adenine residues, leading to hydroquinone **3.4**. Similarly to **3.1**, these covalent adducts are unstable under hydrolytic conditions and evolve to give purine derivatives and depurinized DNA **3.3** (Figure 3.4).

As shown in Figure 3.5, the alternative catechol metabolite 2-hydroxyestradiol (2-OHE_2) produces the estradiol-2,3-quinone (E_2-2,3-Q), which also gives DNA adducts by forming a covalent bond with a nitrogen atom of a purine base, but these adducts are much less reactive than those derived from E_2-3,4-Q and have less relevance in the carcinogenesis due to estradiol.[5]

The link between ovarian function and breast cancer has been known for more than a century, and endocrine therapy can be considered as the oldest, safest, and best established systemic treatment for breast cancer. Many breast and endometrial tumors are estrogen dependent, and their treatment is based on the modulation of these hormones, which can be achieved directly by administration of antiestrogens or indirectly by inhibition of aromatase, the enzyme responsible for the biosynthesis of estrogens. Finally, estrogen production can also be controlled by inhibition of the release of luteinizing hormone (see Section 7).

Recognition of estradiol by the ligand-binding domain of the ER involves a combination of polar and nonpolar interactions. Thus, the A ring and the A/B interface interact with the side chains of Ala-350, Leu-387, and Phe-404, whereas the D ring contacts with Ile-424, Gly-521, and Leu-525. The hydroxyl at the phenolic ring of ring A establishes hydrogen bonds with the carboxylate of Glu-353, the guanidinium group of Arg-394 and a water molecule. The hydroxyl group at the C-17 position of the D ring establishes a hydrogen bond with the His-524 residue (Figure 3.6).

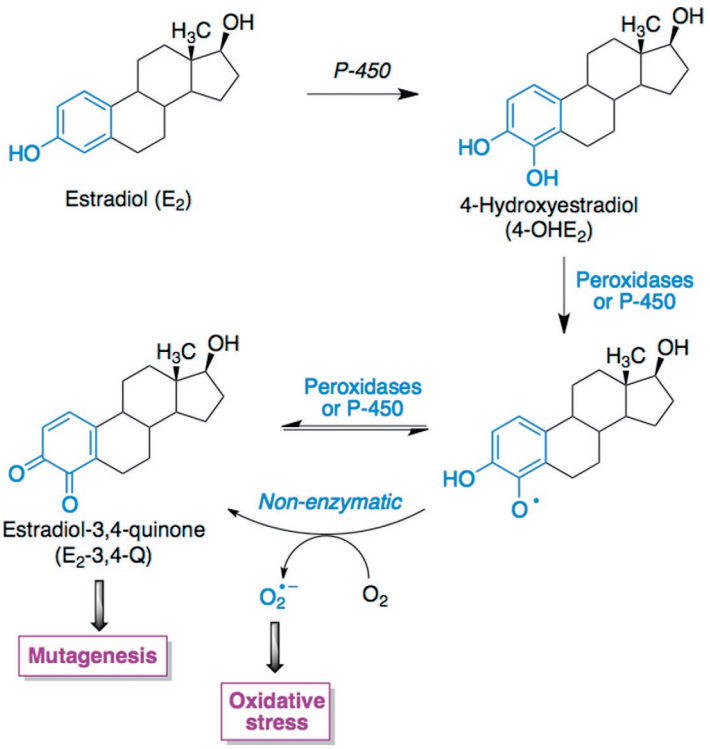


FIGURE 3.2

Mutagenic species produced in the metabolism of estradiol.

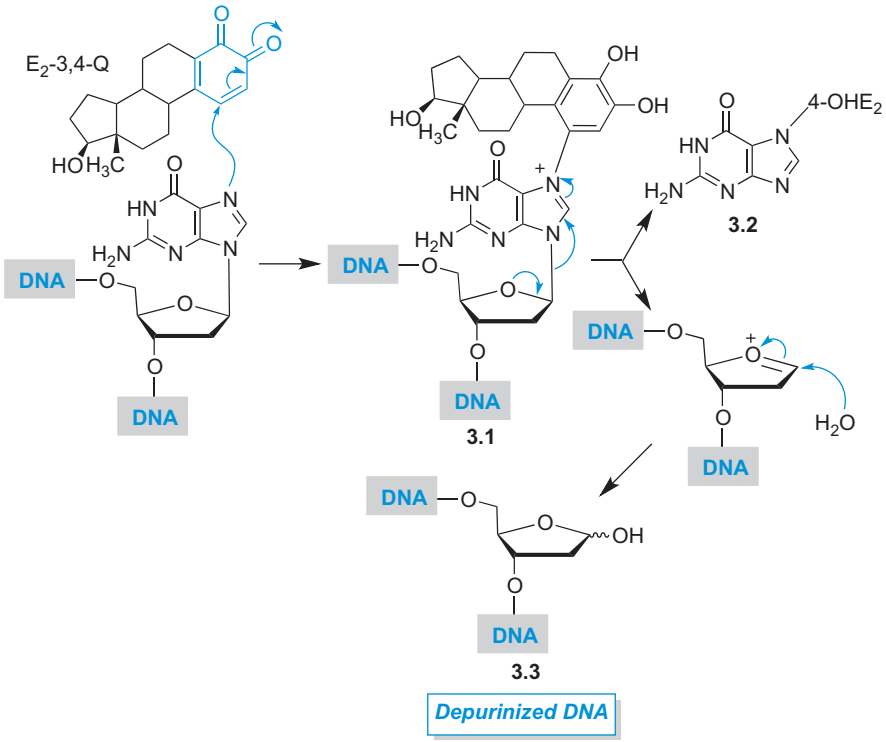


FIGURE 3.3

DNA depurinization by estradiol-3,4-quinone.

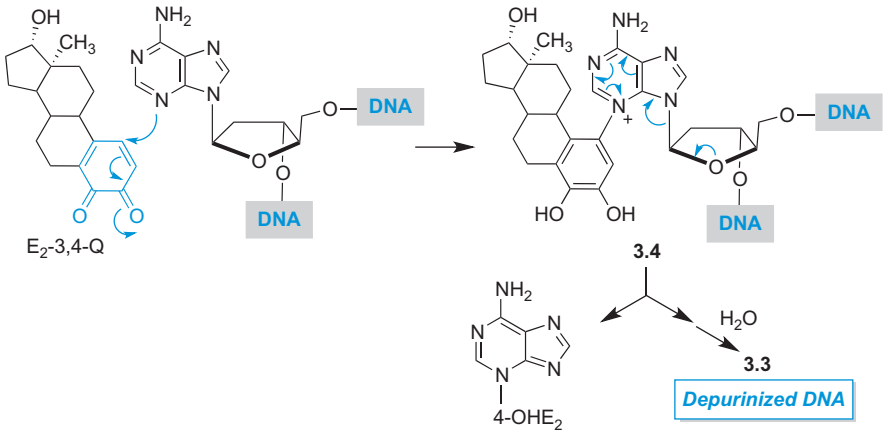


FIGURE 3.4

Alternative mechanism for DNA depurinization by estradiol-3,4-quinone.

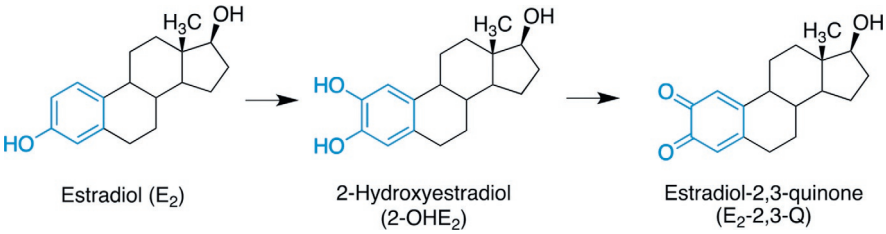


FIGURE 3.5

Metabolic conversion of estradiol into estradiol-2,3-quinone.

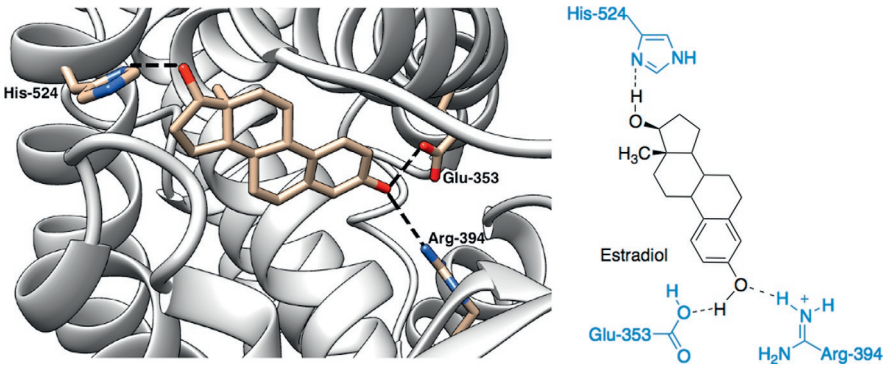


FIGURE 3.6

Binding of estradiol, a typical estrogen, to the estrogen receptor. The three-dimensional structure was generated from Protein Data Bank reference pdb 1A52 and displayed with Chimera 1.8.1.

3 ANTIESTROGENS AS ANTITUMOR DRUGS

Antiestrogens can be defined as compounds that prevent the stimulation of transcription by the estrogen receptor complexes. Two main types of antiestrogens are known:

1. Nonsteroidal antiestrogens, which interfere with the transcription process by binding to the hormone recognition site in the estrogen receptor, preventing the induction of the conformational change necessary for recognition of the coactivators. Because estrogen receptors of different target tissues vary in chemical structure, these compounds may show mixed biological responses and can behave as antagonists in one estrogen target tissue and as agonists in another. Despite not being completely selective, compounds of this group are often designed as "selective estrogen receptor modulators" (SERMs).
2. Pure antiestrogens, which are analogs of the natural hormones that bear long, flexible side chains at C-7. These compounds bind to the estrogen receptor and prevent receptor dimerization and binding to DNA, probably because the side chains bind to the receptor outside the steroid-binding region.

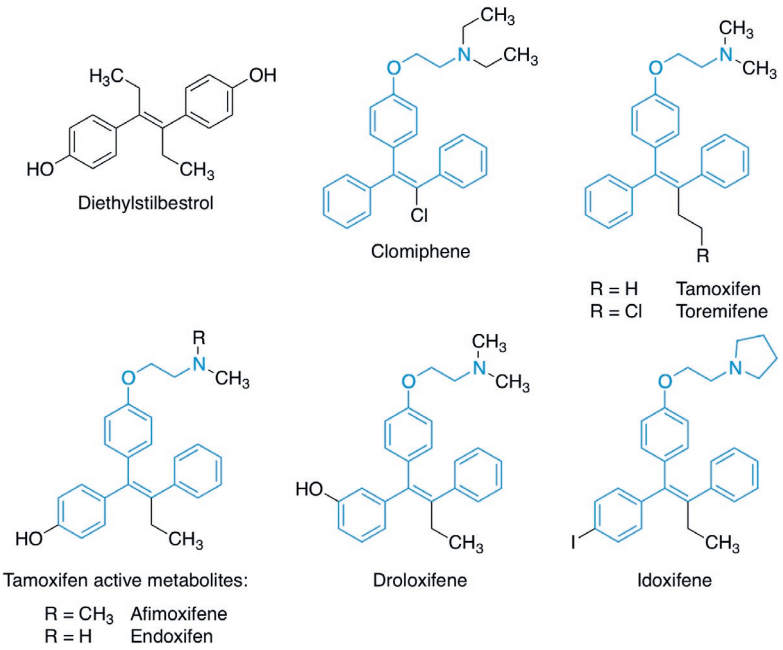
3.1 NONSTEROIDAL ANTIESTROGENS (SELECTIVE ESTROGEN RECEPTOR MODULATORS)

The discovery of this group of compounds is a good example of serendipity. They are derivatives of the triphenylethylene system and were developed by molecular manipulation of diethylstilbestrol, the prototype nonsteroidal estrogen agonist. The key structural features of this group of compounds, which are essential for activity, are the presence of a triphenylethylene core and a basic aminoether side chain at the 4 position of one of the phenyl rings.[6]

The first-discovered antiestrogen was clomiphene, but its development for the treatment of advanced breast cancer was discontinued because of concerns about potential side effects. Tamoxifen was initially originated in the course of research on fertility in the 1960s and later became the first antiestrogen to be approved in Great Britain for the treatment of advanced breast cancer in 1974; a similar approval was given by the U.S. Food and Drug Administration (FDA) 3 years later. Since then, this drug, which is formulated as a citrate salt (Valodex®, Nolvadex®), has become the standard therapy for all types of estrogen receptor-positive breast cancer. In the 1990s, it was also the first cancer chemopreventive agent approved by the FDA for the reduction of breast cancer in pre- and postmenopausal women with high risk[7] (for a more detailed description of the use of tamoxifen in cancer chemoprevention, see Chapter 15, Section 4.1). Tamoxifen has a relatively low affinity for the estrogen receptor, and it is metabolized in the liver by the cytochrome P450 isoforms CYP2D6 and CYP3A4 into active metabolites such as 4-hydroxytamoxifen (afimoxifene) and *N*-demethyl-4-hydroxytamoxifen (endoxifen), which have a much higher affinity and compete with estrogens for binding to the estrogen receptor.[8] Tamoxifen also binds to other targets, such as the microsomal antiestrogen binding site (AEBS), protein kinase C, calmodulin (CaM)-dependent enzymes and acyl-coenzyme A: cholesterol acyltransferases, more simply referred to as ACAT.[9]

Due to the nonspecific activation of estrogen receptors in different tissues, tamoxifen has estrogenic agonist effects in bone and endometrium. For this reason, several other triphenylethylene-derived SERMS, which have a reduced agonist profile on breast and gynecological tissues[10] and were first

developed to treat menopause symptoms, have been studied. Other SERMS belonging to the family include toremifene (Fareston®),[11] droloxifene,[12] and idoxifene.[13]



Most of these compounds may lead to long-term toxic effects. For instance, tamoxifen induces liver cancer in rats after prolonged administration, which has been attributed to the generation of DNA-alkylating species from the metabolism of the stilbene framework. It has been proposed that in addition to other reactions, cytochrome P450 hydroxylates tamoxifen at the allylic position of the ethyl side chain leading to alcohol **3.5**, which can generate the highly delocalized allylic cation **3.6** and therefore alkylate DNA to give product **3.7** through an S_N1 mechanism (Figure 3.7).

This mechanistic proposal also explains the lack of carcinogenicity of toremifene, which can be attributed to destabilization of the positive charge in **3.8** by the inductive effect of the chlorine substituent at the position adjacent to the allylic carbon (Figure 3.8). Despite this advantage, toremifene is not often used due to other adverse side effects. Clinical trials with idoxifene and droloxifene have shown that they are no more efficacious or safer than tamoxifen.

Because of the toxic effects associated with the central double bond in triphenylethylene derivatives, a new family of antiestrogens was developed in which the incorporation of this double bond into a cyclic system increases its chemical and metabolic stability. Another structural difference of these compounds compared to the traditional triphenylethylene derivatives is the presence of a ketone group bridging the phenyl ring that contains the basic side chain. The main representative of this family is raloxifene (Evista®), which was identified as an antiestrogen and was first approved by the FDA only for the prevention of osteoporosis, whereas studies on its use as a treatment for breast cancer were discontinued. Interest in raloxifene as a means for breast cancer prevention was renewed and resulted in

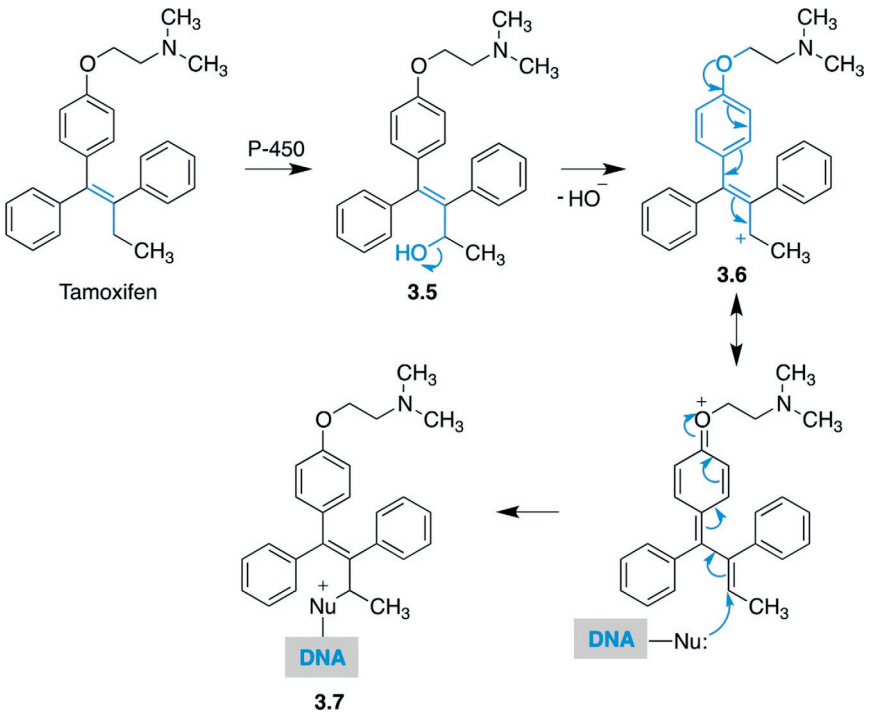


FIGURE 3.7

Mechanism proposed to explain tamoxifen long-term toxicity.

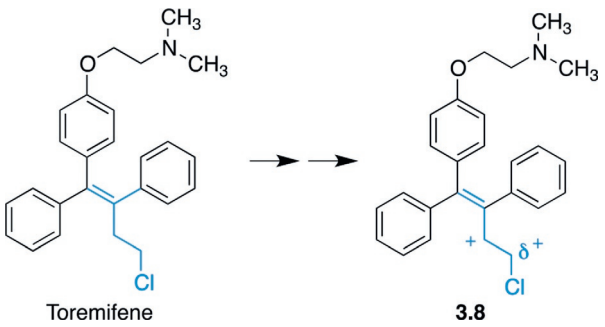
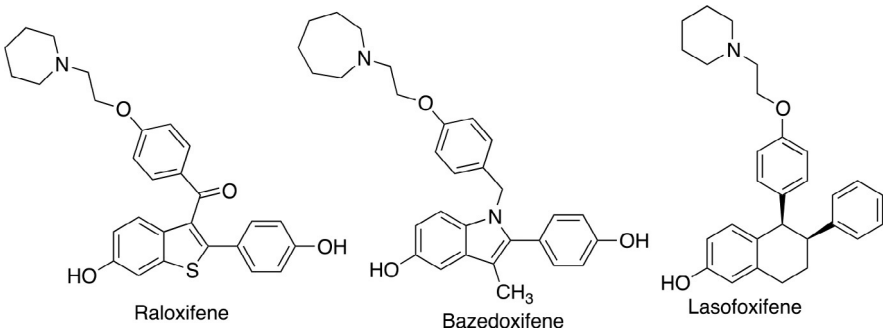


FIGURE 3.8

Destabilization of the electrophilic species derived from toremifene.

FDA approval of raloxifene in 2007 for the treatment/prevention of osteoporosis and for risk reduction of invasive breast cancer in postmenopausal women at high risk;[14] similar approval was given by the European Medicines Agency (EMA) in 2009. Related drugs that were initially introduced for osteoporosis and that have shown promise in the prevention or treatment of breast cancer are bazedoxifene[15] and lasofoxifene.[16]



Inhibition of the estrogen receptor by triphenylethylene derivatives and by their cyclic analogs has been rationalized using the structures of their complexes with the receptor, as determined by X-ray diffraction data.[17] The agonists and antagonists bind at the same site but with different binding modes, as shown by a comparison of Figures 3.6 and 3.9. Recognition of raloxifene by the ligand-binding domain of the receptor involves the same interactions previously described for estradiol—namely the same hydrophobic interactions with the side chains of Ala-350, Leu-387, Phe-404, Ile-424, Gly-521, and Leu-525, plus some additional ones due to the side chain and three polar interactions. The first of these polar interactions occurs between the hydroxyl at the phenolic ring and the carboxylate of Glu-353, the guanidinium group of Arg-394 and a water molecule. A second hydrogen bond involves the imidazole ring in His-524, which rotates to accommodate the difference in position of the hydroxyl group in raloxifen with regard to C-17 in estradiol. A third polar interaction, absent in estradiol, is established between the basic group present in the side chain and the carboxylate group of Asp-351.

After binding of an agonist to the ligand-binding domain of the estrogen receptor, a conformational change occurs in which the helix H12 is placed against the ligand-binding cavity, projecting its inner,

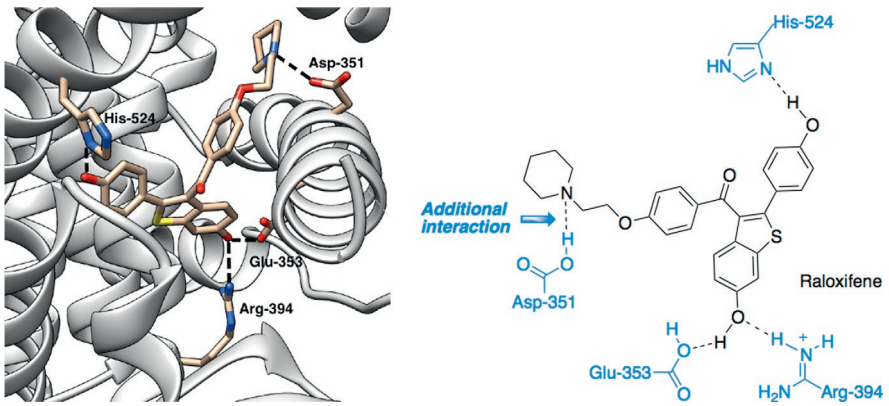


FIGURE 3.9

Binding of raloxifene, a typical antagonist, to the estrogen α receptor. The three-dimensional structure was generated from Protein Data Bank, reference 1ERR, and displayed with Chimera 1.8.1.

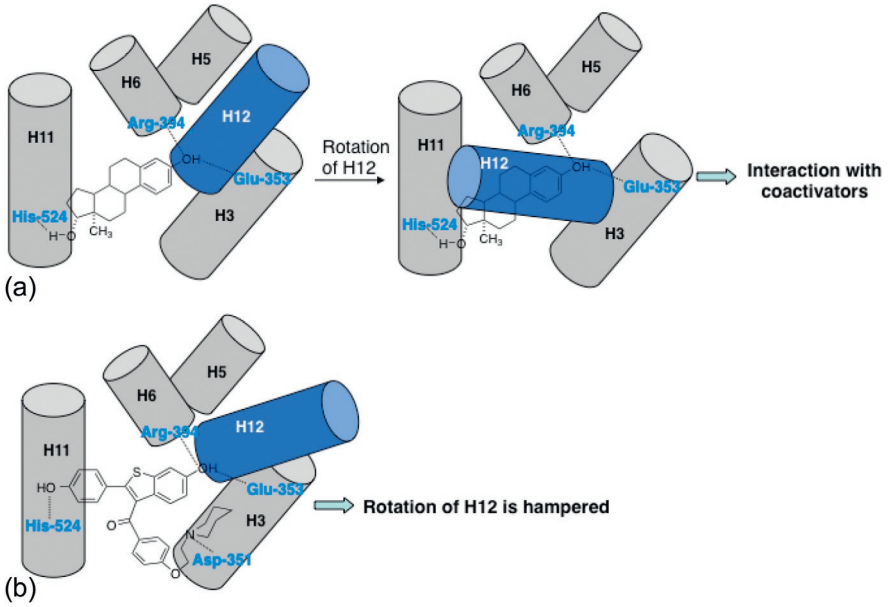
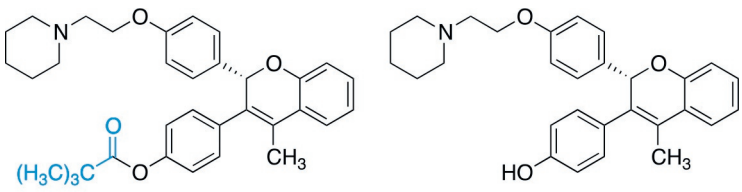


FIGURE 3.10

Conformational changes following the activation of the estrogen receptor and their inhibition by raloxifene.

hydrophobic surface toward the ligand. The outer, charged surface, which is essential for the interaction of the receptor with coactivators, is left outside (Figure 3.10a).[18] The alignment of H12 over the cavity is prevented by the binding of antagonists, exemplified by raloxifene, because their side chain is too long to fit the binding cavity and protrudes from the pocket between H3 and H11, preventing the folding of the helix H12 and hence the transcriptional activation function of the estrogen receptor (Figure 3.10b). This helix displacement seems to be a common feature of steroidal and nonsteroidal antiestrogens with a bulky side chain. A more realistic view of these three protein structures is shown in Figure 3.11.

Acolbifene (EM-652), the active species derived from the prodrug EM-800, is a fourth-generation SERM, behaving as a pure antagonist that does not stimulate endometrial tissue. This compound has been studied clinically for the prevention of cancer in premenopausal women at high risk of breast cancer.[19]



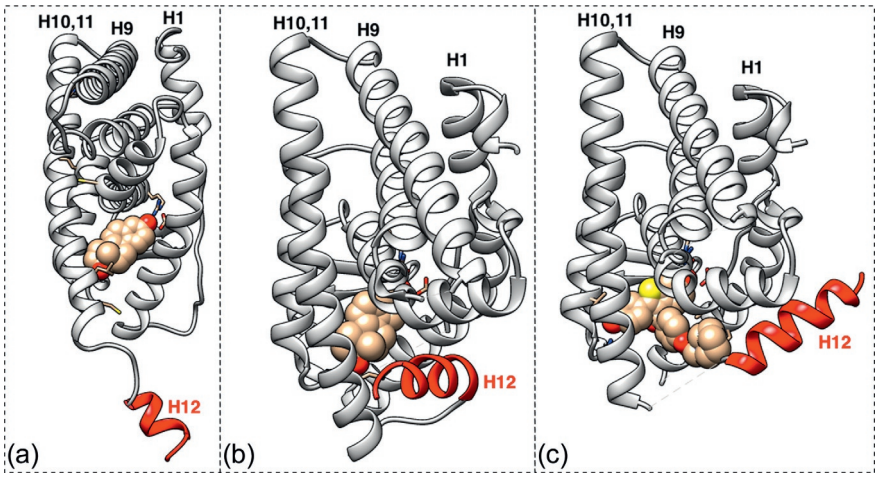


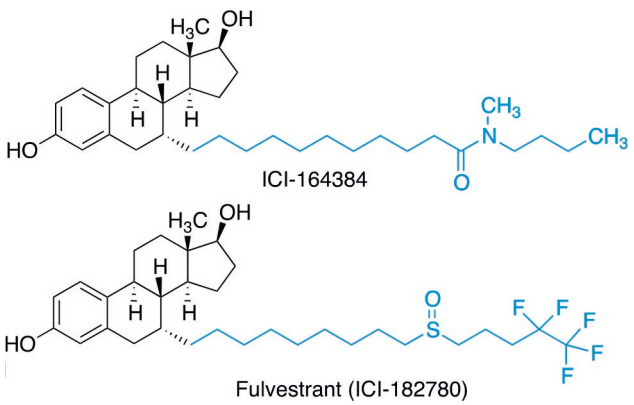
FIGURE 3.11

(a) The human estrogen α receptor bound to estradiol, prior to H12 rotation (pdb 1A52). (b) The same receptor bound to estradiol, after H12 rotation (pdb 1ERE). (c) The same receptor bound to raloxifene (pdb 1ERR).

3.2 STEROIDAL ANTIESTROGENS

The previously mentioned estrogen receptor modulators, especially tamoxifen and toremifene, have been the preferred first-line hormonal therapy for estrogen-responsive postmenopausal breast cancer, but they have several disadvantages related to their partial estrogenic agonistic activity. These include tumor stimulation in some patients at the initial stages of treatment (tumor flare) and increased hot flashes, endometrial cancer, and thromboembolism. These limitations stimulated the search for pure estrogen receptor antagonists.

The main family of selective estrogen antagonists are steroids bearing a long lipophilic chain at C-6, represented by ICI-164384 and fulvestrant (ICI-182780, Faslodex®), the latter of which was approved in 2002 for the treatment of hormone-positive metastatic breast cancer.[20,21]



As in the case of other antagonists, the binding of ICI-164384 or fulvestrant to the estrogen receptor obstructs the folding of the H12 helix of the receptor and therefore prevents its interaction with coactivators. In this case, the H12 rotation is physically prevented by the presence of the bulky C-7 side chain of the antagonist (Figure 3.12).

Fulvestrant is a competitive inhibitor of estradiol, binding at the estrogen receptors with an affinity of 89% that of estradiol. A consequence of fulvestrant binding is the impairment of the dimerization of estrogen receptors, leading to accelerated receptor degradation due to the lower stability of the monomer (Figure 3.13).[22]

Figure 3.14 summarizes the events associated with fully activated transcription by estrogen receptor agonists, partially inactivated transcription by SERMs, and full inactivation by antiestrogens. In the

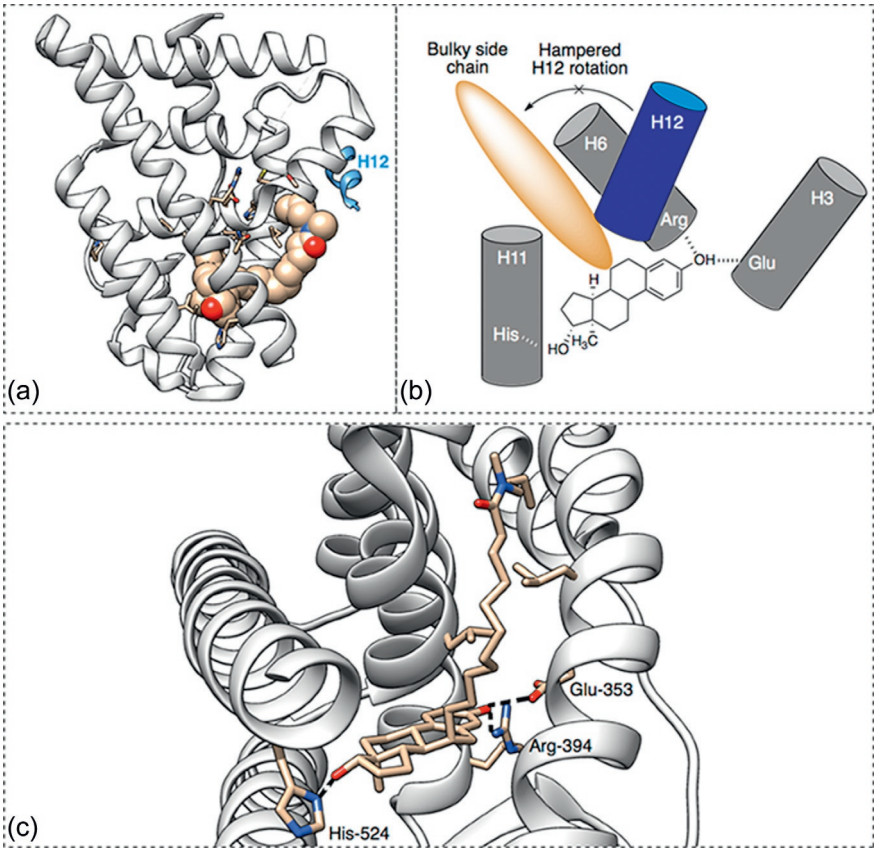


FIGURE 3.12

(a) Binding of ICI-164384 to the estrogen β receptor. (b) Schematic depiction of the blockade of the H12 chain of the receptor by the antagonist side chain. (c) ICI-164384 binds to the receptor active site by the same hydrogen interactions as estradiol, but its C-7 side chain protrudes from the cavity. The three-dimensional structures were generated from Protein Data Bank reference 1HJ1 and displayed with Chimera 1.8.1.

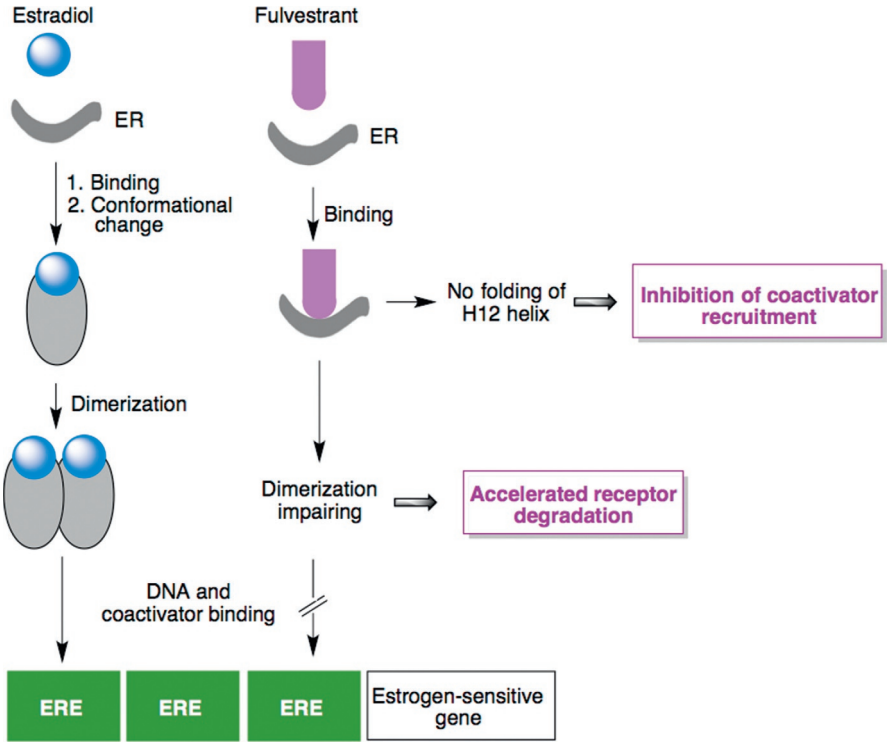


FIGURE 3.13

Mechanism of action of fulvestrant.

resting state, the estrogen receptor has a single domain called activating function (AF1) available for binding with coactivators and/or corepressors. After binding of estradiol (Figure 3.14a), a second activating function (AF2) is exposed, the complex dimerizes and migrates to the cell nucleus, where it binds to the estrogen response element (ERE) of DNA, leading to recruitment of coactivators by both activation functions with the subsequent stimulation of RNA polymerase activity and fully activated transcription. After the binding of SERMs, exemplified by tamoxifene (T) (Figure 3.14b), activation of AF2 does not take place and therefore coactivator recruitment and transcription activation are only partial. The pure steroidal antagonists, such as fulvestrant (Figure 3.14c), bind to the estrogen receptor with high affinity, leading to a conformational change in the receptor that results in the formation of a complex in which neither of the AF1 and AF2 activation functions is active. This complex does not dimerize, which facilitates its degradation. Also, migration to the cell nucleus is markedly reduced, preventing coactivator recruitment and transcription activation.

In the absence of estrogen, some growth factors may also produce estrogen receptor activity, which provides a possible pathway for endocrine-resistant breast tumors. This stimulation is due to phosphorylation of the AF1 region by a cascade of events that involve some kinases. One of these kinases is the human epidermal growth factor receptor 2 (HER2) that, when overexpressed in tumors, can led to

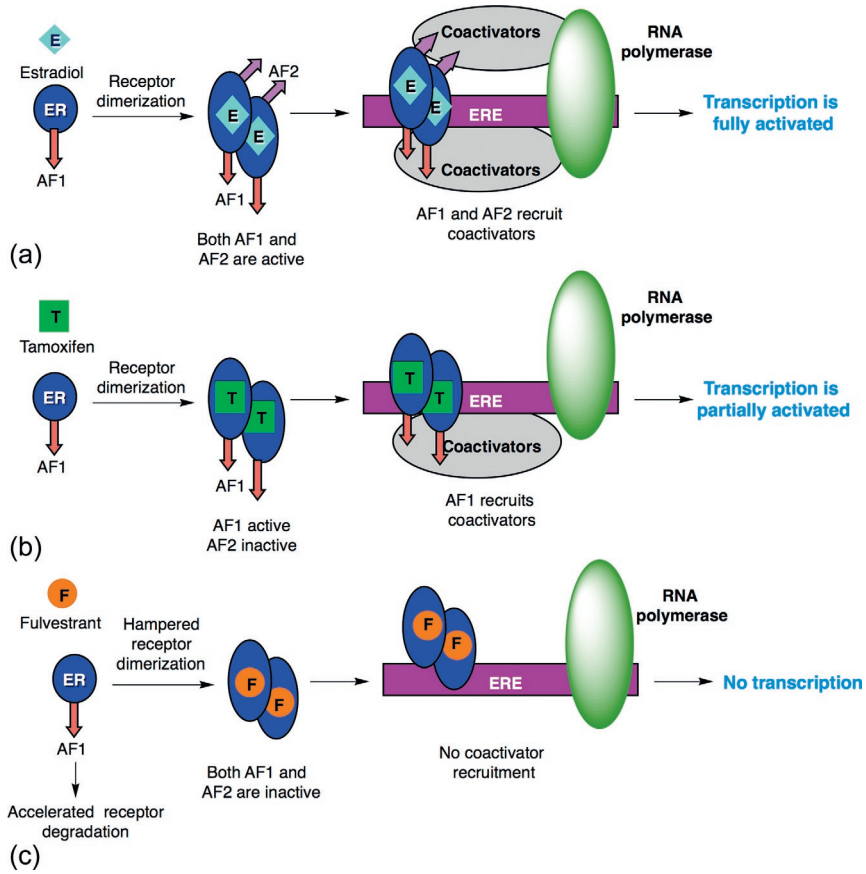


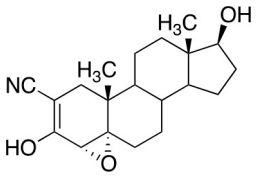
FIGURE 3.14

Events following the interaction of the estrogen receptors with agonists (a), modulators (b), and antagonists (c).

resistance to hormonal therapy,[23] especially when tamoxifen is employed.[24] In these circumstances, trastuzumab (Herceptin®), a monoclonal antibody against HER2 (see Chapter 10, Section 4.2), is used in combination with paclitaxel.

Antiestrogens, principally tamoxifen, and aromatase inhibitors (which are discussed in Section 4), have been used as first- and second-line therapy in patients with advanced postmenopausal breast cancer for many years. If patients acquire resistance to these treatments, further endocrine treatment is achieved by merely substituting the current medication with a different antiestrogen or aromatase inhibitor. Trilostane (Modrenal®), another steroidal compound, offers an alternative endocrine treatment due to its unique mode of action. It is an inhibitor of 3β-hydroxysteroid dehydrogenase, the enzyme that transforms pregnenolone into progesterone, and also an allosteric modulator of the estrogen receptor, probably binding directly to the DNA-binding domain.[25] Due to the fact that progesterone is a gestagen and also a biosynthetic precursor of all other types of steroidal hormones, trilostane is used to treat

Cushing's disease (which is characterized by high levels of cortisol) in dogs and cats, but it has also been approved in some countries for the treatment of postmenopausal advanced breast cancer following relapse from initial hormone therapy. Clinical trials examined the potential for its use in premenopausal breast cancer, as well as in other malignancies such as prostate cancer.[26]



Trilostane

4 AROMATASE INHIBITORS

An alternative strategy for achieving antiestrogenic effects is the inhibition of aromatase, the enzyme responsible for the biosynthesis of estradiol and estrone from androgens.[27] In principle, this strategy has the advantage over the use of antiestrogens of blocking indirectly the previously discussed two pathways involved in the generation of tumors by the estrogenic hormones: estrogen receptor activation and the generation of carcinogenic metabolites (Figure 3.15). On the other hand, some aromatase inhibitors (AIs) have found application as suppressors of estrogen production in the course of treatments with anabolic steroids, a strategy that is based on the fact that diminished levels of estradiol result in an increase of luteinizing hormone and, consequently, of testosterone.

Aromatase catalyzes the loss of the C-19 methyl group as a formic acid molecule, allowing the creation of the aromatic A ring that is characteristic of estrogens (Figure 3.16). Aromatase inhibitors are employed for the therapy of breast cancer in postmenopausal women, for whom the primary estrogen source is aromatase activity in the breast, bone, vascular endothelium, and central nervous system. Whereas the use of AIs in premenopausal women leads to incomplete estrogen suppression and increased gonadal stimulation, in postmenopausal women aromatase levels are not under gonadotropin regulation and this avoids complications arising from the feedback regulatory mechanism that increases luteinizing hormone and follicle-stimulating hormone after aromatase inhibition.[28]

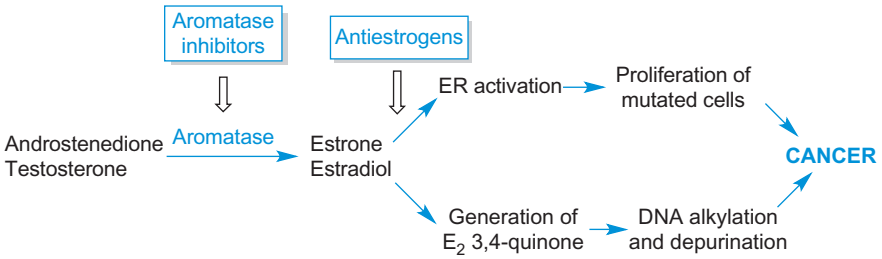


FIGURE 3.15

Pathways involved in tumorigenesis by estrogens.

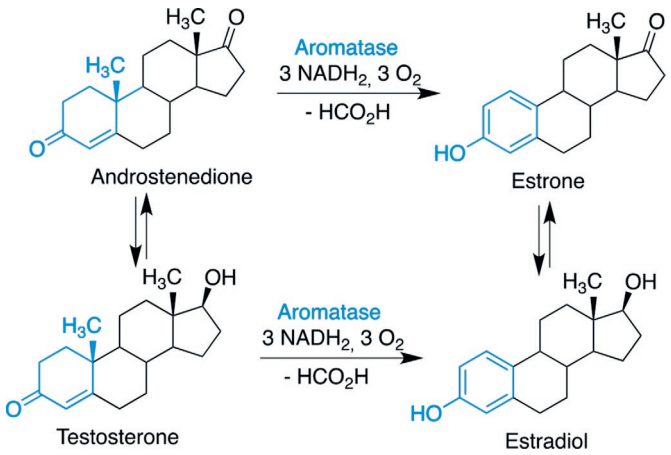


FIGURE 3.16

Transformations catalyzed by aromatase.

Aromatase inhibition, especially by third-generation drugs, results in near complete estrogen deprivation. For this reason, some of the drugs discussed here have improved clinical outcomes compared to tamoxifen in breast cancer treatment due to the previously mentioned estrogenic agonistic effects of tamoxifen and to the genotoxicity of the metabolites of estradiol, especially its quinone derivatives.[29] AIs are now considered to be the standard treatment for postmenopausal women with hormone receptor-positive breast cancer. The combination of AIs and bisphosphonates to compensate their pro-osteoporotic effects has not been fully evaluated, and other possible long-term effects remain unknown.[30,31] A important drawback is that resistance to AIs inevitably occurs in metastatic settings after prolonged suppression of estrogen production.

4.1 AROMATASE MECHANISM OF ACTION

Aromatase, also known as estrogen synthase, belongs to the group of microsomal cytochrome P450 enzymes responsible for hydroxylation metabolic processes. The overall process comprises a series of three oxidative steps. The first two are the insertion of two hydroxyl groups at the C-19 methyl group of its substrates leading to **3.9** and then to gem-diol **3.10**, which is dehydrated to aldehyde **3.11**. The third reaction is only partly understood, and it involves loss of the C-19 carbon atom as a molecule of formic acid, with concomitant aromatization of ring A.

One mechanistic possibility is that a third hydroxylation takes place at C-2β, yielding intermediate **3.12**, which would rearrange to **3.14** through the intermediacy of the cyclic hemiacetal **3.13**. Loss of a molecule of formic acid, driven by the generation of the aromatic A ring, would finally culminate the process, yielding the estrogens estradiol and estrone (Figure 3.17). However, experiments with isotopically marked **3.12** [2β-^{18}O-,19-^{3}H]-2β-hydroxy-10β-formylandrost-4-ene-3,17-dione] failed to show incorporation of β-hydroxyl to formic acid under enzymatic and nonenzymatic conditions.[32]

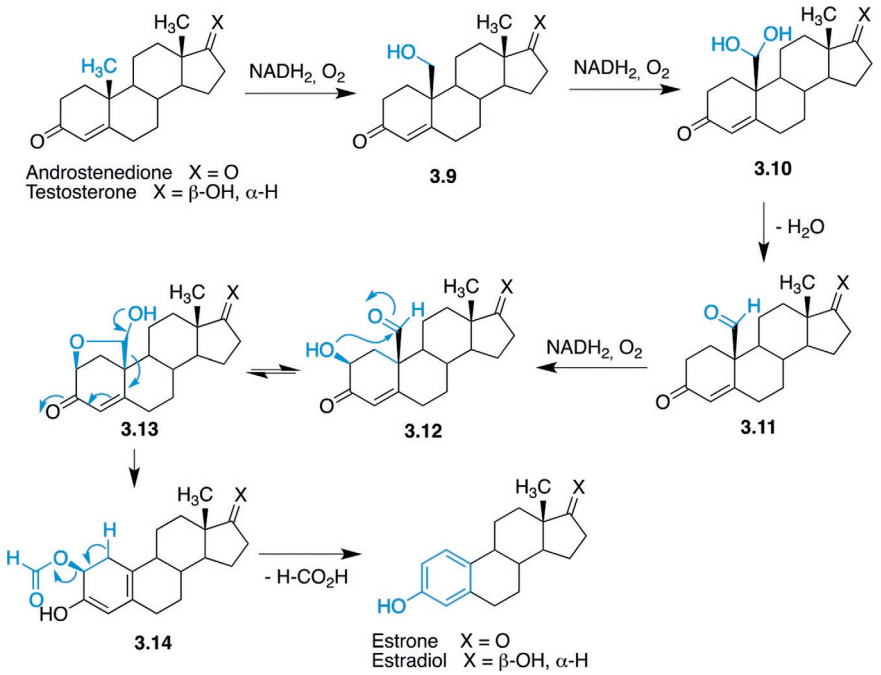


FIGURE 3.17

Initial mechanistic proposal to explain aromatase activity, which was falsified by isotopic labelling experiments.

An alternative mechanism involves chemical changes in the heme group at the catalytic site. Similarly to other cytochrome P450 enzymes, the catalytic site of aromatase contains an Fe(III) heme group that, after reduction to Fe(II) affording species **3.15**, binds to an oxygen molecule that becomes activated, giving **3.16**. A further one-electron reduction leads to peroxide anion **3.17**, which can undergo a nucleophilic attack onto the formyl group of aldehyde **3.11**. The adduct **3.18** thus generated, probably as its enol tautomer **3.19**, evolves to **3.20** and **3.21** by loss of a molecule of formic acid via the ionic mechanism shown in Figure 3.18, or perhaps through a radical pathway, yielding the estrogen hormones. This mechanism is consistent with all experimental data and is considered more likely than the one via 2-hydroxylation previously discussed and other alternatives that have been proposed.[33]

4.2 STEROIDAL AROMATASE INHIBITORS (TYPE I INHIBITORS)

Aromatase inhibitors are normally classified as steroidal (type I) or nonsteroidal (type II). Numerous steroidal agents that exhibit competitive, irreversible, or mechanism-based inhibition of aromatase have been developed.[34] Mechanism-based inhibitors, known as *aromatase inactivators*, are bound to the catalytic site, where they are transformed into electrophilic intermediates that become irreversibly attached to the enzyme, blocking its activity. These inhibitors have distinct advantages in drug design because they are highly enzyme specific, produce prolonged inhibition, and exhibit minimal

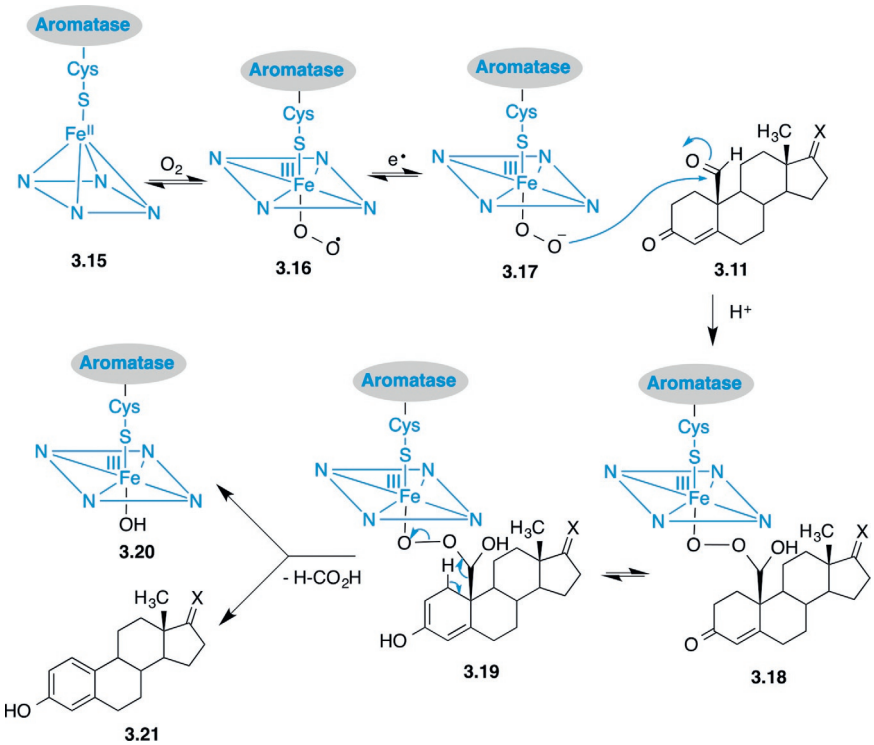


FIGURE 3.18

Alternative mechanism accounting for aromatase activity.

toxicities. For this reason, the steroidal aromatase inhibitors in clinical use behave as mechanism-based irreversible inhibitors.[35] Although the precise chemical details are sometimes unknown, many types of compounds are available that contain latent electrophilic groups intended to be activated by aromatase. The most relevant are summarized here.

4.3 C-19 MODIFIED SUBSTRATE ANALOGS

One example of C-19 modified substrate analogs is the propargyl derivative plomestane, for which two main types of mechanisms have been proposed. The first one postulates its oxidation by aromatase to give the C-19 carbonyl derivative, leading to the Michael acceptor **3.22**, a substrate for nucleophilic attack at the enzyme active site to give **3.23**. The second mechanism is based on the one proposed for the inactivation of cytochrome P450 enzymes by terminal acetylenic compounds and involves epoxidation of the acetylene chain by aromatase to give the unstable oxirene **3.24**, which reacts with aromatase after rearrangement to ketene **3.26** to give **3.27** (Figure 3.19). Although it was found to be effective and produce few adverse effects in preliminary studies, clinical data related to plomestane are very scarce because of the "technical problems" that were encountered in the course of its development.[36]

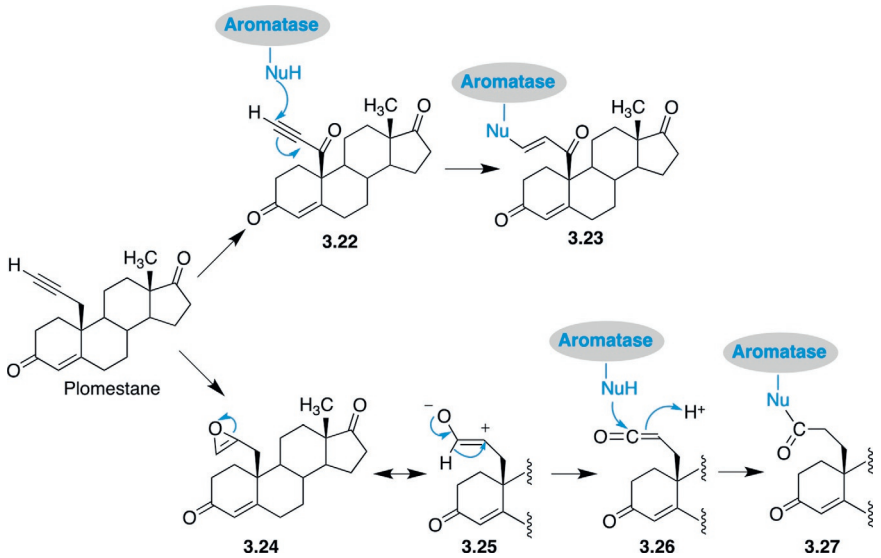


FIGURE 3.19

Aromatase inhibition by C-19 modified substrate analogs.

4.4 4-HYDROXYANDROSTENEDIONE DERIVATIVES

The main representative of this group is formestane (Lentaron®). This compound was first described as a competitive inhibitor, but subsequent evidence proved that its binding to aromatase was irreversible. The presence of the C-19 methyl group is essential because the 19-nor derivative is not an aromatase inactivator, which suggests that the 19-oxygenated metabolites are the inactivating species. The 4-hydroxy group is also essential, with the ethers and esters of formestane at O-4 being inactive. One possible mechanism that is consistent with these observations is summarized in Figure 3.20, although the low activity found for the intermediate formyl derivative **3.28** would seem to cast some doubt on this proposal.

Formestane is a second-generation steroidal aromatase inhibitor and the first one to reach clinical use during the early 1990s.[37] Its main drawback is that it must be administered intramuscularly in order to avoid its first-pass glucuronidation at the C-4 hydroxyl—a problem that renders it unsuitable for widespread clinical use.

4.5 STEROIDS WITH ADDITIONAL UNSATURATIONS AT THE A AND B RINGS

The first member of this class of compounds to be recognized as an aromatase inhibitor was testolactone. Subsequently were identified 1,4-androstadiene-3,17-dione and related compounds, including 1,4,6-androstatrien-3,17-dione (ATD) and 4-androstene-3,6,17-trione ("6-oxo"). Among other more

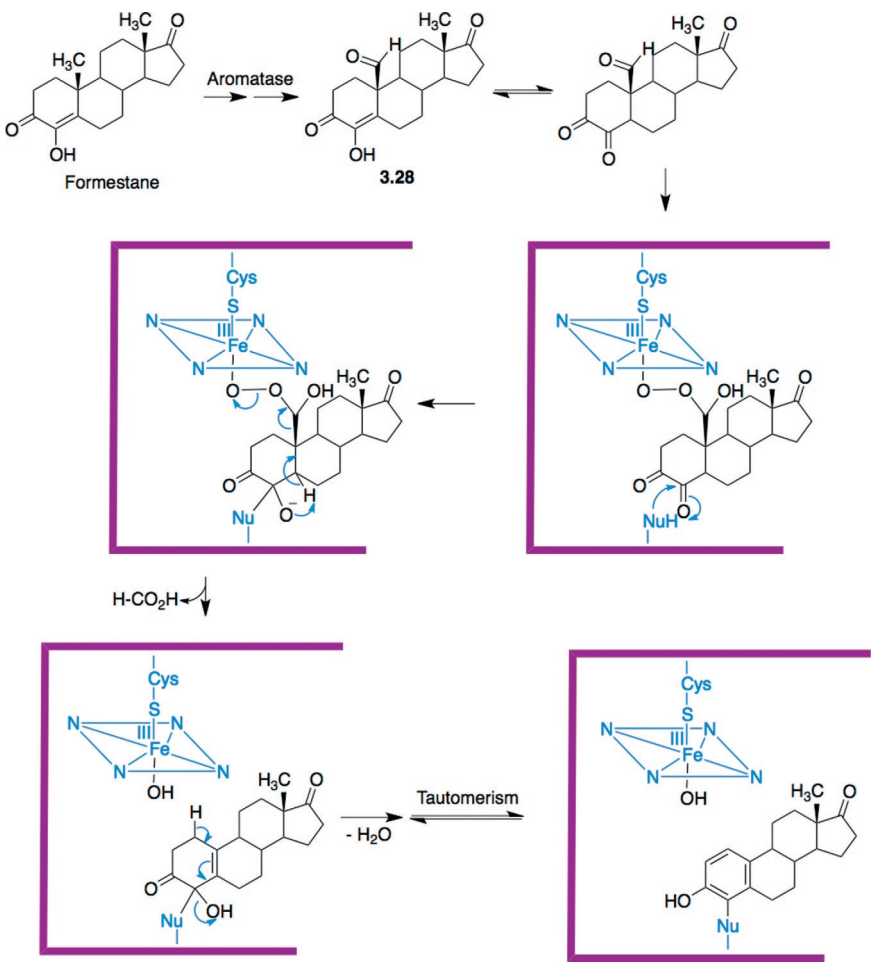


FIGURE 3.20

Aromatase inhibition by formestane, a 4-hydroxyandrostenedione derivative.

highly unsaturated compounds, the most relevant is the 6-methylene derivative, known as exemestane.[38] The use of testolactone (Teslac®) in the treatment of breast cancer started in 1960, although its ability to inhibit aromatase was not discovered until 1979. It is a weak inhibitor with a moderate clinical response that has precluded its widespread use. Exemestane (Aromasin®), the first example of an irreversible aromatase inhibitor, was reported in 1987 and approved in some countries for the treatment of advanced breast cancer in postmenopausal women in whom antiestrogenic therapy has failed. It has the advantages over formestane of being more potent and, especially, allowing oral administration,[39] although it has important adverse effects.

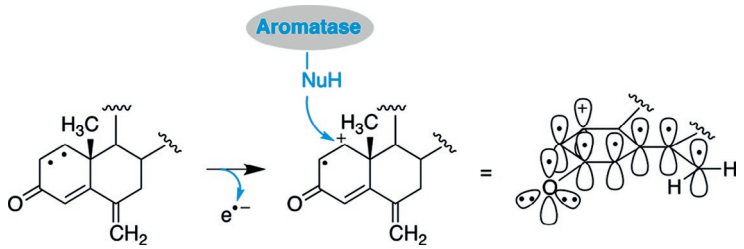
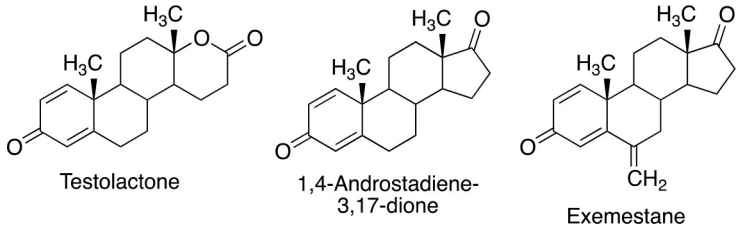


FIGURE 3.21

Inactivation of aromatase by exemestane.



The presence of the double bond at C-1 is essential for activity, and it has been suggested that the mechanism of irreversible inactivation involves its oxidation to a cation radical that would then be intercepted by a nucleophilic group of the active site. The generation of this radical would be facilitated by stabilization of the unpaired electron by delocalization across the adjacent unsaturated carbonyl system (Figure 3.21).

4.6 STRUCTURE–ACTIVITY RELATIONSHIPS IN STEROIDAL AROMATASE INHIBITORS

The spatial requirements for interaction of steroidal compounds with the active site of aromatase are very restrictive, allowing only small structural changes on the A ring and at C-19. Some exceptions to this rule are the incorporation of small polar substituents at the C-4 position, such as a hydroxyl group, or the addition of aryl functionalities at the 7 position of the steroid. Inhibitors with such modifications exhibit enhanced affinity for the enzyme.[34] Several enzyme structure–function studies have revealed two regions that are important parts of the active site and contribute to the binding of the substrate and inhibitors: the I helix, which comprises the portion from Cys-299 to Ser-312, and a hydrophobic pocket that comprises the portion from Ile-474 to His-480 (Figure 3.22).

4.7 NONSTEROIDAL AROMATASE INHIBITORS (TYPE II)

This group of inhibitors comprises compounds structurally different from the substrates that are able to bind to the active site of aromatase through the coordination of a heterocyclic nitrogen atom, usually an imidazole or triazole ring, to the iron atom of the heme group of the enzyme. Here, we use fadrozole as a

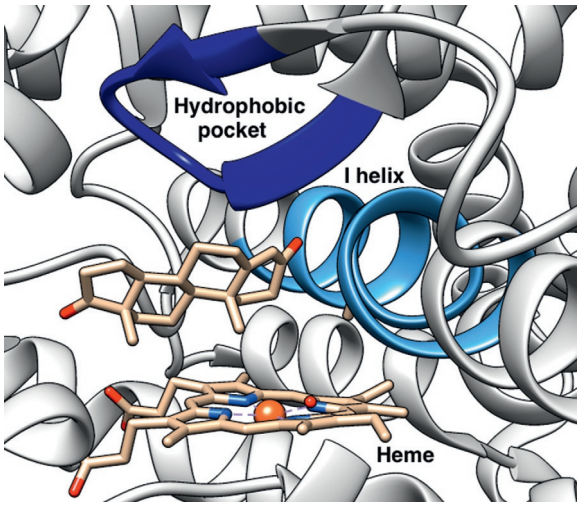


FIGURE 3.22

Androstenedione at the active site of human placental aromatase.[40] The structure was generated from Protein Data Bank reference 3EQM and displayed with Chimera 1.8.1.

model compound for describing the interaction of azole derivatives with aromatase. The N-2 atom of fadrozole is involved in a coordinate bond with the heme iron of aromatase, having also favorable interactions with the side chains of Ileu-305, Ala-306, and Thr-310, whereas the cyano group appears to be hydrogen-bound to the Ser-478 hydroxyl. Three-dimensional quantitative structure–activity relationship (QSAR) data for fadrozole derivatives and other studies support the presence of hydrogen bonding and hydrophobic interactions in the active site of the enzyme (Figure 3.23)[41a], although more recent molecular docking studies do not fully agree with this conclusion.[41b]

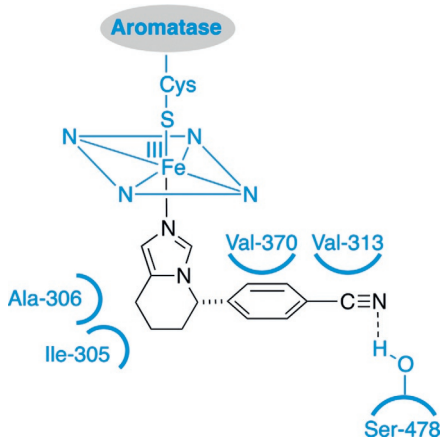
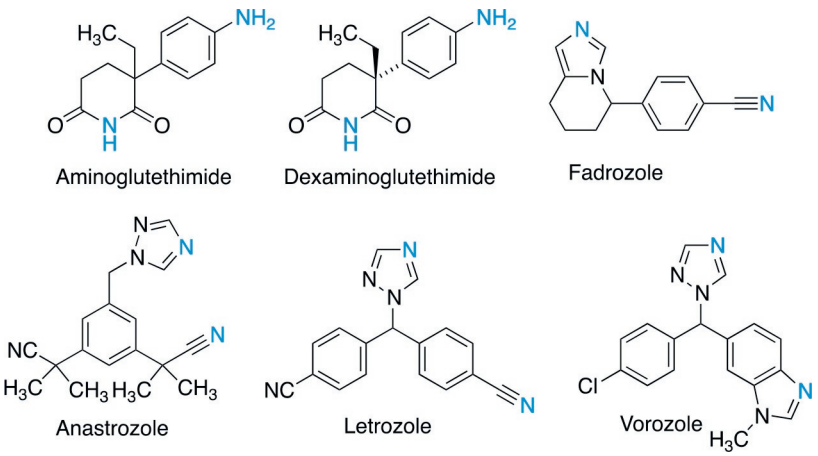


FIGURE 3.23

Interaction of fadrozole with the aromatase active site.

Due to the similarity of aromatase with other essential enzymes of the cytochrome P450 group, selectivity is the main problem to be solved. The structures of the main drugs belonging to this group are given here. The atoms involved in binding to the cytochrome iron and the serine hydroxyl are shown in color.



The first nonsteroidal aromatase inhibitor was aminoglutethimide (Cytadren®, Orimeten®), a derivative of the sedative agent glutethimide that was initially introduced as an anticonvulsant agent. Aromatase inhibition by this compound was discovered serendipitously in the late 1950s when it was observed that it blocked adrenal steroidogenesis. This drug was the first aromatase inhibitor approved by the FDA for use in breast cancer treatment, but it was withdrawn after reports of adrenal insufficiency. This toxic effect is due to the nonspecific effects of iminoglutethimide, which, by inhibiting several cytochrome P450 enzymes, affects a number of hydroxylation steps in the metabolic conversion of cholesterol to active steroid products in adrenal steroid biosynthesis.

The second nonsteroidal aromatase inhibitor to reach the clinic was the previously mentioned fused imidazole derivative fadrozole (Afema®), which causes the suppression of aldosterone—a disadvantage that limits its administration to lower doses than that required to achieve the total inhibition of aromatase activity. The third generation of nonsteroidal aromatase inhibitors includes the triazole derivatives anastrozole (Arimidex®), vorozole (Rivizor®), and letrozole (Femara®), which are very potent and specific and allow almost complete estrogen suppression.[42] They have the advantage over aminoglutethimide and fadrozole of not showing affinity for other cytochrome P450-related steroidogenic enzymes because the presence of two electron-withdrawing nitrogen atoms in the heterocyclic ring renders it too electron-deficient for aromatic oxidation. They are usually stable to metabolism but also have adverse secondary effects. Anastrazole and letrozole are mainly used in postmenopausal women who are unable to take tamoxifen because of a high risk of thromboembolism or endometrial abnormalities. Development of vorozole as a second-line tumor therapy was discontinued.

Some ER-positive tumors respond well to AI therapy, but others do not; the reason for these differences is not well understood. A clinical trial of AI therapy response paired with genomic studies provided new insights into the genetic mechanisms of AI resistance and may lead to personalized breast cancer treatment with aromatase inhibitors.[43]

5 STEROID SULFATASE INHIBITORS

It was previously mentioned that in postmenopausal women, who show the highest incidence of breast cancer, estrogens are produced in adipose tissues and in the breast by the action of aromatase on androstenedione. However, the clinical response to aromatase inhibitors is not as high as may be expected, and often it is not superior to the one obtained with antiestrogens or with other antihormones. In addition to the somatic alterations of ER-positive breast cancers, there appears to be no relationship between the clinical response and the degree of suppression of circulating estradiol levels, which suggests that other factors must be involved in tumor growth.[44]

The enzyme called steroid sulfatase (STS) also plays a pivotal role in steroid biosynthesis. This enzyme regulates the formation of estrone by hydrolysis of estrone sulfate (E_1S) and also controls the hydrolysis of dehydroepiandrosterone sulfate (DHEA-S) to dehydroepiandrosterone (DHEA). The latter compound can be reduced to 5-androstenediol (adiol), a steroid with potent estrogenic properties. On the other hand, there is evidence that in postmenopausal women, DHEA is an important source of androstenedione via the peripheral action of 3β-hydroxyesteroid dehydrogenase/isomerase (3β-HSD/isom), as shown in Figure 3.24. Consequently, steroid sulfatase inhibitors can be useful for breast cancer therapy, although, in contrast to aromatase inhibitors, they are still in an early stage of development.[45,46]

Among many structurally diverse steroid sulfatase inhibitors that have been developed, the most successful candidates are those bearing an aryl sulfamate group (Ar-OSO_2NH_2), which is considered as the pharmacophore for irreversible inhibition to the enzyme. Estrone-3-*O*-sulfamate (EMATE) was the first potent steroid sulfatase inhibitor, but this agent was not developed because of its estrogenic properties. The development of nonsteroidal mimics led to the discovery of irosustat (667-coumate, STX-64, BN83495), the first STS inhibitor that entered clinical trials for treating postmenopausal women with breast cancer.[47] Irosustat rapidly disappears from plasma because of its low stability, presumably due to a facile E1cB elimination of sulfamate anion to give the corresponding coumarin, but it shows a long half-life in blood. This increased stability has been ascribed to binding of the drug to carbonic anhydrase II in erythrocytes, where the hydrophobic environment in which the coumarin ring system is placed according to modeling studies hampers the generation of charged intermediates through the E1cB mechanism.[48] Docking studies suggest that during the course of the inactivation mechanism, a sulfamoyl group is transferred to the *gem*-diol form of formylglycine 75 of steroid sulfatase (Figure 3.25).[49]

Despite much interest in irosustat and its second-generation analogs,[50] the results of phase I/II clinical studies led to the discontinuation of the development of irosustat in monotherapy in 2011, although the study of its combination with other hormonal therapies will continue.[51]

Another current goal for the treatment of hormone-dependent breast cancer is the development of dual aromatase–sulfatase inhibitors (DASIs). The potential advantages of a single chemical agent with the ability to interact with multiple biological targets were highlighted some years ago.[52] In the case of DASIs, this goal is being pursued by the introduction of the critical sulfamate unit in structures with known aromatase-inhibiting properties.[53,54]

6 ANDROGEN-RELATED ANTITUMOR AGENTS

Androgens are steroidal hormones that stimulate and control the masculine primary and secondary characteristics. They exert their action by binding to a nuclear receptor called the androgen receptor (AR),[55] and the complex acts as a transcription factor, in a similar way to estrogens. In the absence of

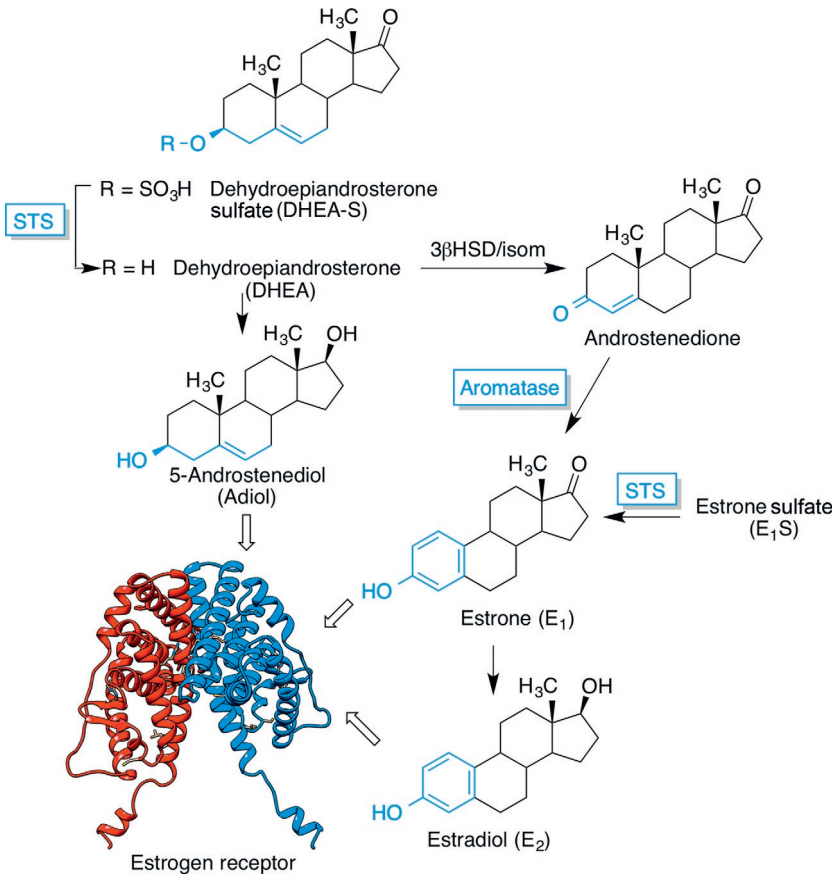


FIGURE 3.24

Role of steroid sulfatase in the biosynthesis of estrogens.

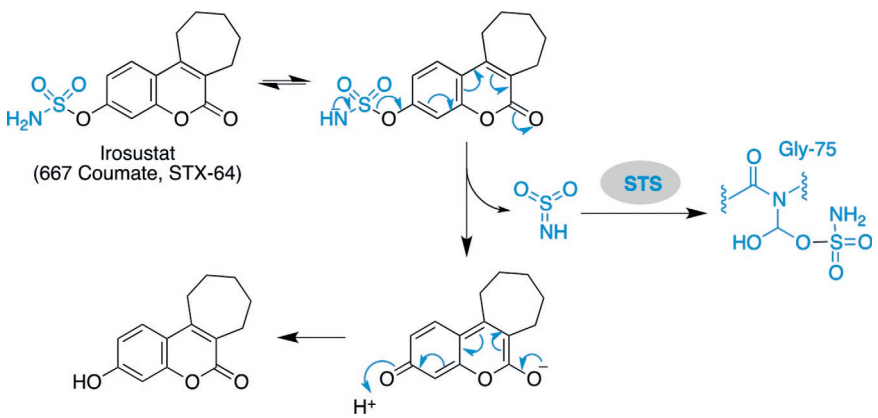
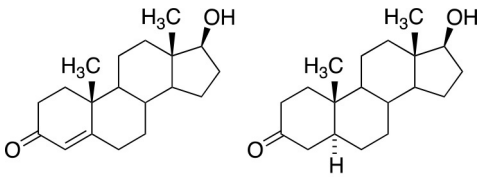


FIGURE 3.25

Metabolism of irosustat, a steroid sulfatase inhibitor.

ligand binding, AR is bound to heat shock proteins in the cytoplasm, but androgen binding leads to dissociation of these complexes followed by the usual process of dimerization, translocation to the nucleus, DNA binding, coactivator recruitment, and activation of transcription of androgen-regulated genes. The main androgens are testosterone and its reduced metabolite 5α-dihydrotestosterone, which has a higher affinity for the androgen receptor and 3- to 10-fold greater molar potency than testosterone.



Prostate cancer, the most common cancer and the second most common cause of death from cancer in males in Western countries, shows a high sensitivity to androgen deprivation therapy. Indeed, it can be considered as the most endocrine-sensitive solid neoplasm, although advanced disease eventually progresses to castration-resistant prostate cancer (CRPC). Reduction of AR signaling may be achieved by direct androgen depletion (castration via surgical orchiectomy or treatment with luteinizing hormone-releasing hormone agonists), by blockage of the AR with antiandrogens, or by combination treatments.[56] Resistance to these treatments may be associated with the presence of a variant of the androgen receptor having a single F876L amino acid substitution, as has been recently found for enzalutamide and abiraterone (discussed later). Detection of the androgen receptor splice variant 7 messenger RNA (AR-V7) in circulating tumor cells from men with advanced prostate cancer has been proposed as a biological marker to avoid the use of these drugs.[57]

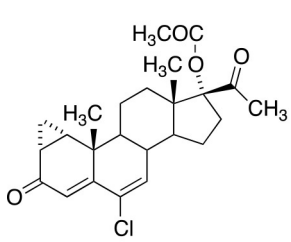
6.1 ANTIANDROGENS

6.1.1 Steroidal Antiandrogens

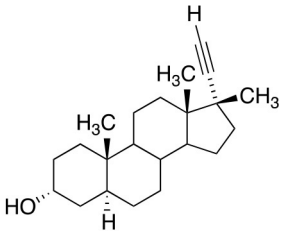
Modulation of testosterone levels can be achieved by inhibition of enzymes involved in androgen biosynthesis or by administration of antiandrogens.[58] Androgen antagonists, employed in the treatment of prostate cancer, benign prostate hyperplasia, and as topical anti-alopecia agents, bind to the androgen receptor and prevent binding of the natural steroids; however, they do not produce the correct conformational change in the receptor that is essential to elicit normal changes in gene expression.[59]

Cyproterone is a pregnane-derived steroidal antiandrogen that was initially developed as a synthetic gestagen to be used as a contraceptive, but the observation of feminization of the offspring in gestating rats led to its identification as a competitive inhibitor of the androgen receptor. Cyproterone acetate (Cyprostat® or Androcur®) is the main steroidal antiandrogen in clinical use for prostatic carcinoma. However, its side effects of gynecomastia and edema, attributed to its analogy with natural gestagens and glucocorticoids, respectively, stimulated the search for compounds with pure antiandrogenic action (selective androgen receptor modulators).

Apoptone (HE3235), a dihydrotestosterone analog, exhibits a wide range of effects, including alteration of androgen receptor signaling and reductions in levels of intratumoral androgens,[60] and it has also entered clinical phase I/II trials in patients with CRPC. Galeterone is a dual androgen receptor antagonist and a CYP17A1 inhibitor that is discussed in Section 6.2.



Cyproterone acetate

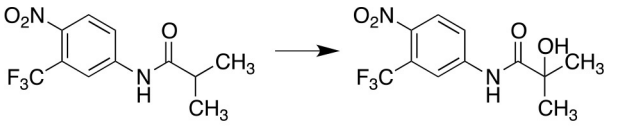


Apoptone (HE 3235)

The crystal structure of the ciproterone acetate–androgen receptor complex shows that the steric bulk from the drug 17α-acetate group displaces the Leu-701 side chain, resulting in the expansion of the receptor binding cavity by generation of an additional hydrophobic pocket surrounded by the Leu-701, Leu-704, Ser-778, Met-780, Phe-876, and Leu-880 residues (Figure 3.26a). As a consequence, the H11 and H12 helices are displaced. Hydrogen bonds with Arg-752, Gln-711, and Asn-705 also contribute to the complex stabilization (Figure 3.26b).

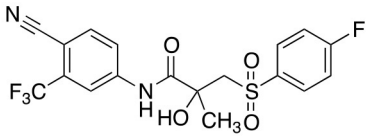
6.1.2 Nonsteroidal Antiandrogens

Flutamide (Eulexin® and Drogenil®) was the first nonsteroidal antiandrogen to be developed. It is a prodrug whose active metabolite (hydroxyflutamide) acts by inhibiting the binding of testosterone and 5α-dihydrotestosterone (DHT) to the androgen receptor. Molecular modeling studies have attributed the greater affinity of this metabolite to its dominant conformation, induced by intramolecular hydrogen bonding (see later). Another related antiandrogen that is in clinical use for the treatment of prostate cancer is bicalutamide (Casodex®), whose structure allows similar hydrogen bonding.



Flutamide

Hydroxyflutamide
(flutamide active metabolite)



Bicalutamide

In all these compounds, binding to the receptor is similar to that of testosterone. Thus, the aromatic ring on nitrogen occupies the same region of the receptor as the testosterone A ring, via hydrogen bonding of the nitro or cyano groups with Gln-711 and Arg-752 and stacking interactions with Phe-778. The hydroxy group interacts by hydrogen bonding with the same region of the receptor as the testosterone C17-OH, albeit less efficiently due to the loss of one hydrogen bond because of its involvement in intramolecular hydrogen bonding. The R substituent protrudes from a pseudocyclic structure generated by intramolecular hydrogen bonding and blocks the rotation of the H12 chain of the receptor, being thus responsible for the antagonistic effect (Figure 3.27).[61]

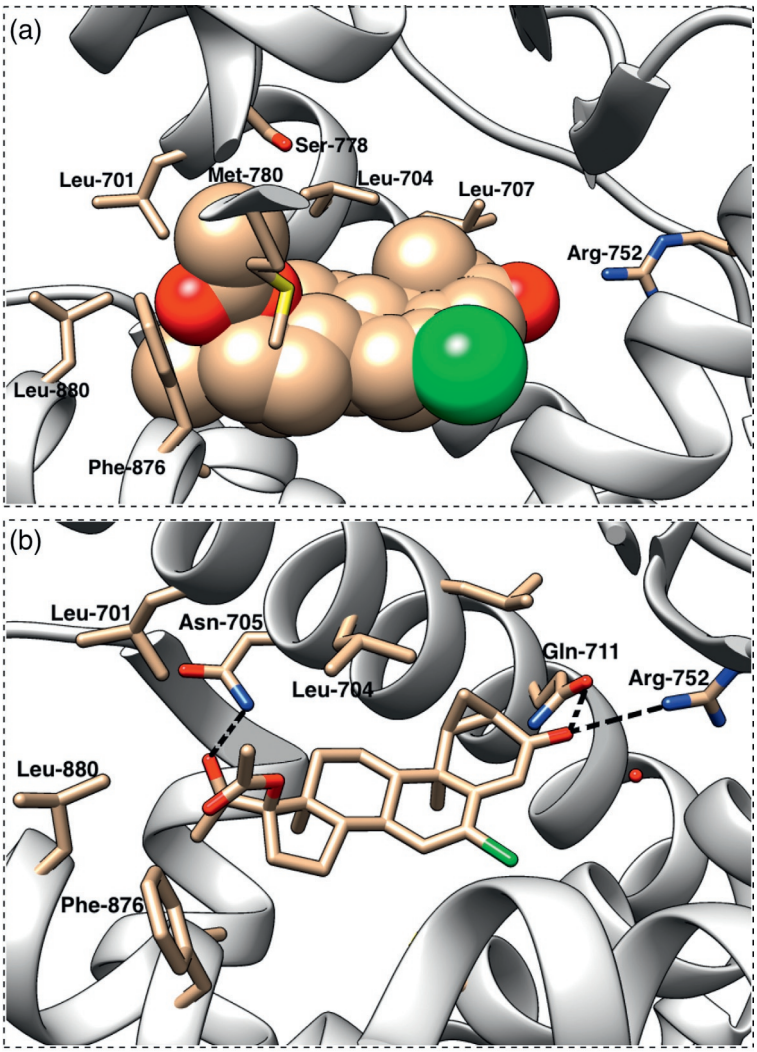


FIGURE 3.26

Hydrophobic cavity (a) and hydrogen bonding interactions (b) in the ciproterone acetate–androgen receptor complex. Generated from Protein Data Bank reference 2OZ7 and displayed with Chimera 1.8.1.

In another family of antiandrogens, the previously mentioned pseudo five-membered ring has been replaced by hydantoin or thiohydantoin moieties. Members of this class of compounds include nilutamide, enzalutamide, and ARN-509. Nilutamide (Nilandron® and Anandron®) has the advantage over flutamide of having a higher half-life that allows its administration only once daily. Enzalutamide (MDV3100, Xtandi®) is another androgen receptor antagonist[62] that has a higher affinity for the androgen receptor than the first-generation nonsteroidal antiandrogens. It was approved by the FDA in 2012 for the treatment of CRPC and has been shown to reduce the progression of the disease by more

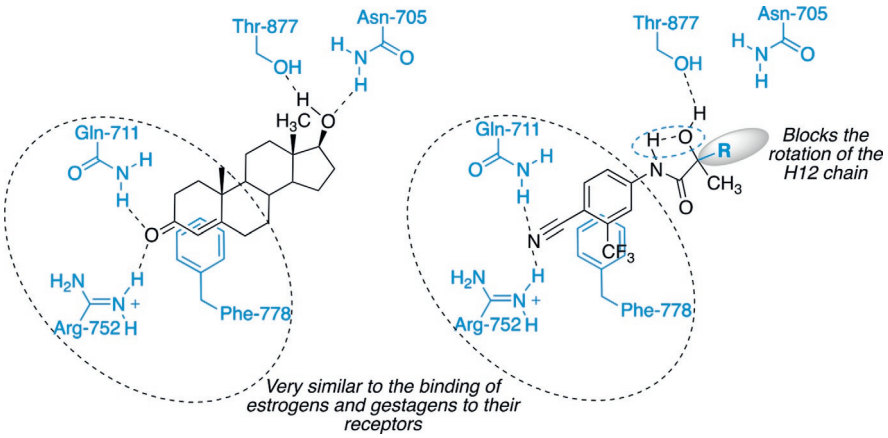
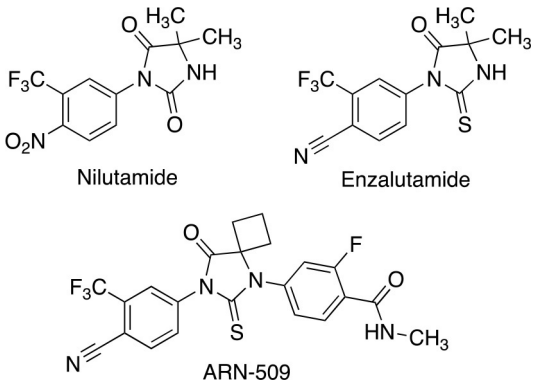


FIGURE 3.27

Comparison between the binding modes of testosterone and bicalutamide to the androgen receptor.

than 80%, making it more efficient than other antiandrogens.[63] ARN-509 is another thiohydantoin derivative that, in contrast to the first-generation antiandrogen bicalutamide, exhibits no agonist activity in prostate cancer cells that overexpress androgen receptor.[64] This compound has entered a phase I/II clinical trial to determine its antitumor activity, safety, and tolerability in patients with advanced CRPC. ODM-201 is another AR antagonist that showed good tolerability and high anticancer activity in phase I/II trials and, contrary to other antiandrogens, does not enter the brain.[65]



6.2 INHIBITORS OF ANDROGEN BIOSYNTHESIS

Another approach to achieve androgen deprivation is based on the inhibition of one of the stages of androgen biosynthesis. As for other steroidal hormones, androgen biosynthesis is a very complex process, requiring the concerted participation of a large number of enzymes. The main steps that serve as targets of anticancer drugs discussed in this section are summarized in Figure 3.28.

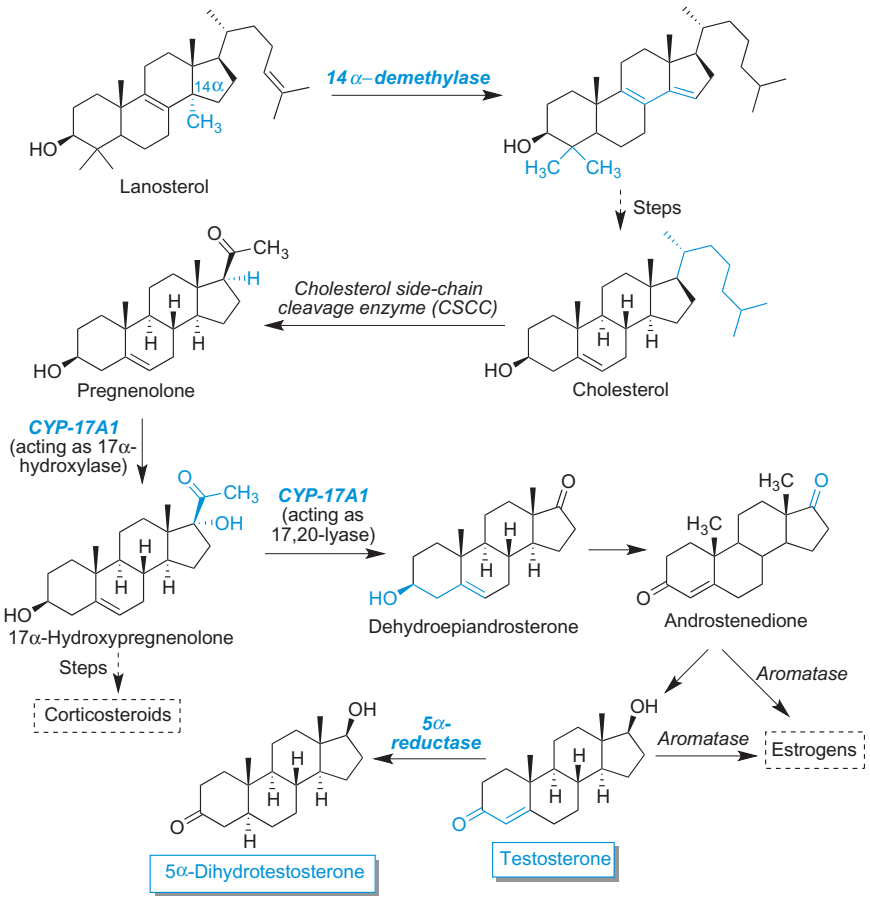
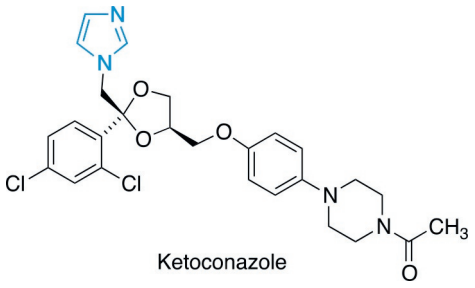


FIGURE 3.28

Antiandrogens that interfere with the conversion of lanosterol into steroidal hormones.

6.2.1 Inhibitors of 14α-Demethylase

Ketoconazole is an imidazole derivative primarily employed as an antifungal agent because it inhibits the biosynthesis of ergosterol, a key component of fungal membranes.



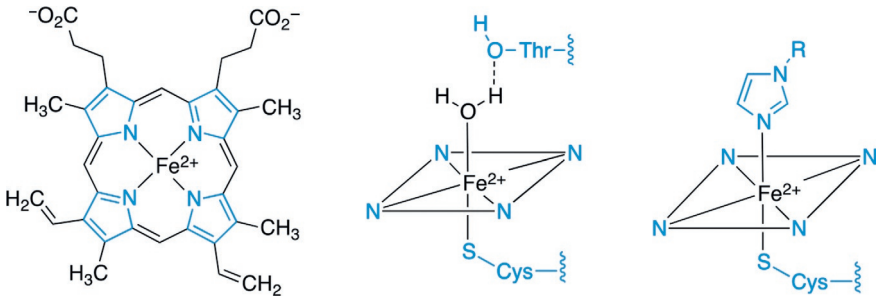


FIGURE 3.29

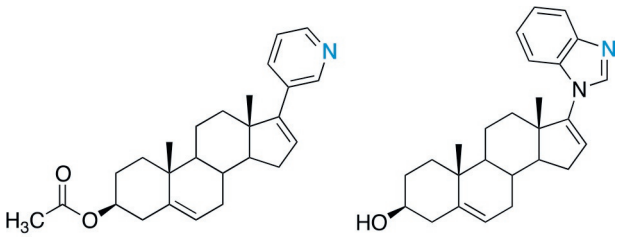
Binding of the imidazole ring of ketoconazole to the hemo group in 14α-demethylase.

The basis for the activity of ketoconazole and related antifungal imidazoles and triazoles is the inhibition of 14α-demethylase, a cytochrome P450 enzyme necessary for the conversion of lanosterol to ergosterol in fungal cells. Because this enzyme is also present in mammalian cells, where it is essential for the transformation of lanosterol into cholesterol, the precursor to all steroidal hormones, high doses of ketoconazole lead to androgen deprivation.[66] Its use for the treatment of metastatic prostate cancers that do not respond to antiandrogens normally involves short treatments due to its toxicity, in association with corticoids to prevent adrenal insufficiency associated with the inhibition of corticosteroid synthesis. Ketoconazole acts by coordination of the unsubstituted imidazole nitrogen atom to the iron atom in the active site of the cytochrome, displacing a coordinated water molecule (Figure 3.29).

6.2.2 Inhibitors of CYP17A1 (17α-hydroxylase and C(17,20)-lyase)

CYP17A1 is a cysteinato-heme enzyme that belongs to the cytochrome P450 superfamily. It contains a heme group, which is covalently linked to the protein through the sulfur atom of a proximal cysteine. The heme is the reactive center to activate molecular oxygen and to oxidize the substrate. This enzyme shows 17α-hydroxylase and C(17,20)-lyase properties and catalyzes the two steps of the transformation of pregnenolone into dehydroepiandrosterone, via 17α-hydroxypregnenolone as an intermediate (Figure 3.30).

The first CYP17A1 inhibitors to be studied clinically were the steroids abiraterone acetate and galeterone. Abiraterone was the first compound acting by this mechanism to be commercialized, under the trade name Zytiga®.



Abiraterone acetate Galeterone

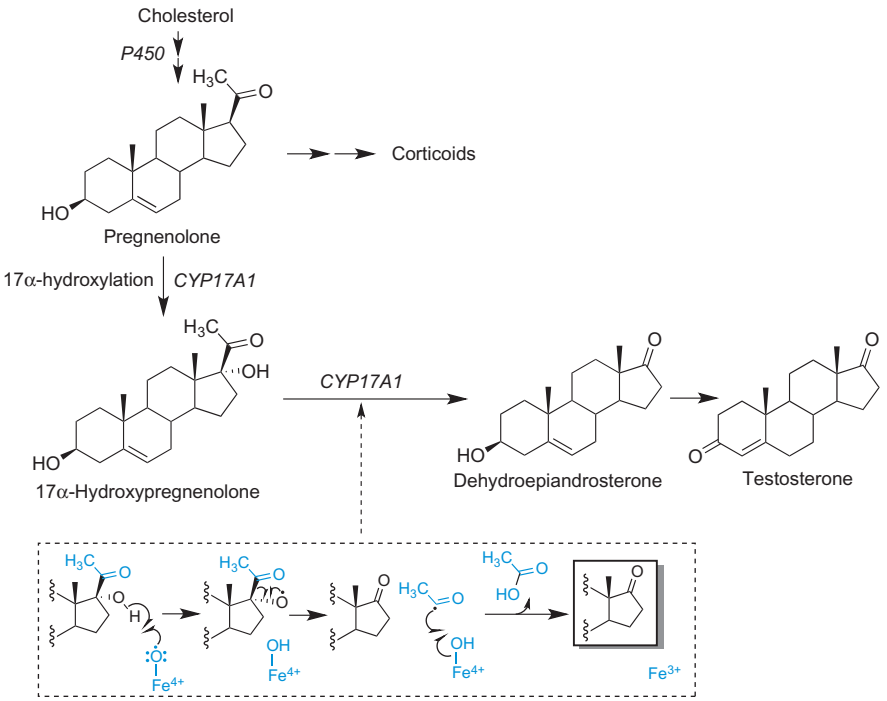


FIGURE 3.30

Reactions catalyzed by CYP17A1 and mechanism of the oxidative deacetylation at C-17.

Abiraterone is an irreversible inhibitor of CYP17A1.[67] Because this is a key enzyme in the production of androgens and estrogens in the adrenal glands and tumor tissues, abiraterone inhibits both adrenal and intratumoral androgen synthesis. A study in humans showed that repeated treatment with abiraterone in men with intact gonadal function can successfully suppress testosterone levels to the castrate range, although this level of suppression may not be sustained in all patients due to compensatory hypersecretion of luteinizing hormone (LH).[68] After several clinical trials to determine its usefulness,[69] abiraterone acetate was tested in patients with CRPC in a phase III trial that demonstrated an overall survival benefit, confirming that CRPC is hormone-driven.[70] These results were the basis of its FDA approval for treatment of prostate cancer in 2011.

Galeterone (TOK-001, VN/124-1) can be regarded as an analog of abiroterone and has a similar binding mode to CYP17, although its benzene moiety occupies an additional hydrophobic pocket. Furthermore, it has a unique, dual mechanism of action involving both androgen receptor antagonism and CYP17A1 inhibition. This compound is being tested in advanced clinical trials for CRPC.[71]

The way in which these inhibitors bind to CYP17A1 is illustrated here for the case of abiraterone. The pyridine nitrogen at the C-17 substituent of this compound binds iron, forming an approximately 60° angle above the heme plane, and simultaneously the 3β-hydroxy substituent interacts with the aspargine-202 residue in the F helix (Figure 3.31).[72]

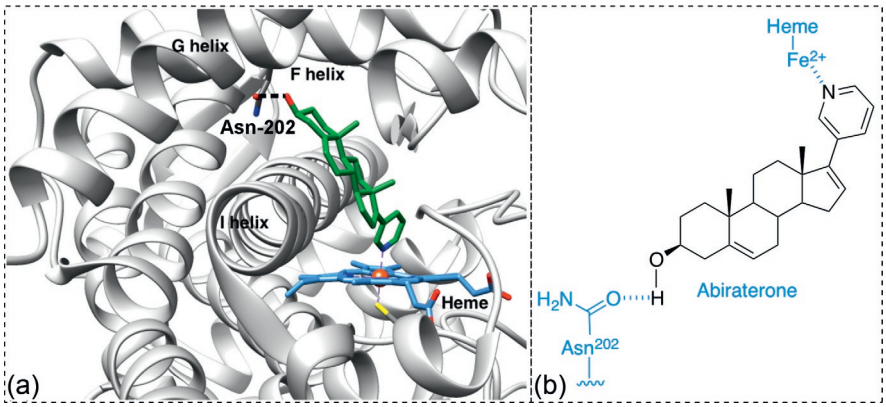
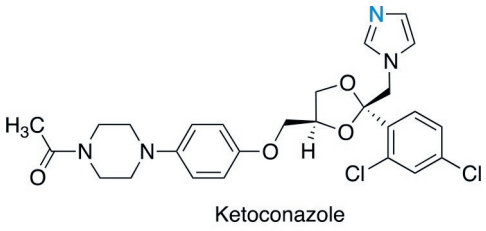


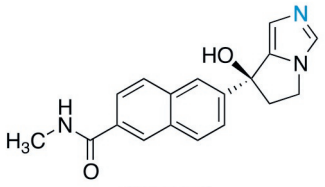
FIGURE 3.31

Binding of abiraterone to the active site of CYP17A1. The three-dimensional structure was generated from Protein Data Bank reference 3RUK and displayed with Chimera 1.8.1.

Despite the good anticancer activity of the steroidal CYP17 inhibitors, they have some drawbacks associated with their stereoidal structures, including first-pass metabolism, leading to poor oral bioavailability, and affinity for several steroid receptors. Nonsteroidal CYP17 inhibitors would overcome these problems and are therefore viewed as promising alternatives. The antifungal drug ketoconazole is an antimycotic agent that shows nonselective inhibition toward several CYP enzymes, including CYP17A1. Despite its toxicity associated with indiscriminate CYP inhibition, this drug is still employed in several countries for the treatment of prostate cancer, in combination with hydrocortisone to compensate for the inhibition of corticoid biosynthesis. Orteronel (TAK-700) is another nonsteroidal selective nonsteroidal inhibitor of CYP17A1[73] that is currently in phase III clinical trials for metastatic, hormone-refractory prostate cancer.



Ketoconazole



Orteronel

6.2.3 Inhibitors of 5α-Reductase

Approximately 95% of testosterone entering the prostate is converted to the more potent androgen DHT by the enzyme 5α-reductase; therefore, the androgenic activity in the prostate is due to this enzyme, whose expression is largely restricted to the prostate. Blockade of the activity of 5α-reductase leads to the inhibition of testosterone action on urogenital sinus tissue derivatives, notably the prostate, without blocking peripheral androgenic action due to testosterone. To date, the main use of 5α-reductase inhibitors (5ARIs) is the treatment of alopecia and benign prostate hyperplasia, but there is much interest in their potential use as cancer chemopreventive agents. Despite their efficacy in the treatment of benign prostatic hyperplasia, the popularity of inhibitors of 5α-reductase is limited by their association with adverse sexual side effects.

5α-Reductase is associated with the nuclear membrane and requires hydride donation from NADH, which acts as a cofactor and is transformed into NAD^+ (Figure 3.32).

Finasteride (Proscar®, Propecia®), the first inhibitor of this enzyme to reach the market, is used to treat benign prostatic hyperplasia and androgenic alopecia in men. It is believed to be a mechanism-based inhibitor acting through the mechanism shown in Figure 3.33, which involves the addition of hydride to the unsaturated lactam system of finasteride followed by trapping of the highly electrophilic NAD^+ molecule by enol **3.29** generated in the first step.[74]

Dutasteride (GI-198745, Avodart®) is an analog of finasteride that behaves as a dual inhibitor of 5α-reductase type 1 and 2 isozymes. This compound is approved for benign prostate hyperplasia and has been proposed for the chemoprevention of prostate cancer in men at high risk.[75]

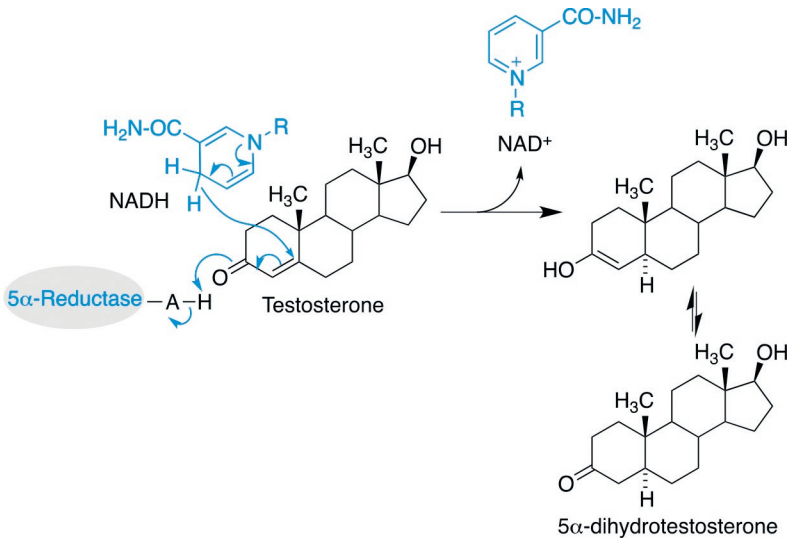


FIGURE 3.32

Reduction of testosterone to 5α-dihydrotestosterone by 5α-reductase.

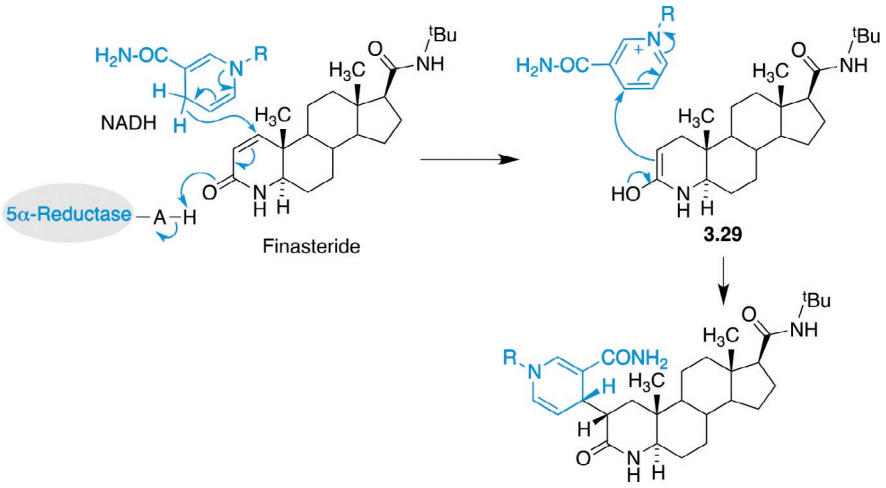
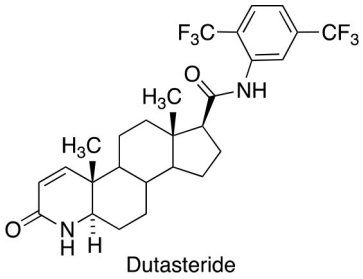


FIGURE 3.33

Irreversible inhibition of 5α-reductase by finasteride.



7 REGULATION OF GONADOTROPIN-RELEASING HORMONE: CONTROL OF THE HYPOTHALAMIC–PITUITARY–GONADAL AXIS

7.1 INTRODUCTION

Testosterone production in men is controlled by the hypothalamic–pituitary–gonadal axis. Secretion of gonadotropin-releasing hormone (GnRH, LHRH) from the hypothalamus stimulates the pituitary gland to release luteinizing hormone (LH), which acts on testicular Leydig cells to produce testosterone. The strategies currently employed for achieving a reduction of testosterone levels for the treatment of prostatic cancer,[76] including the strategies already discussed, are summarized in Figure 3.34.

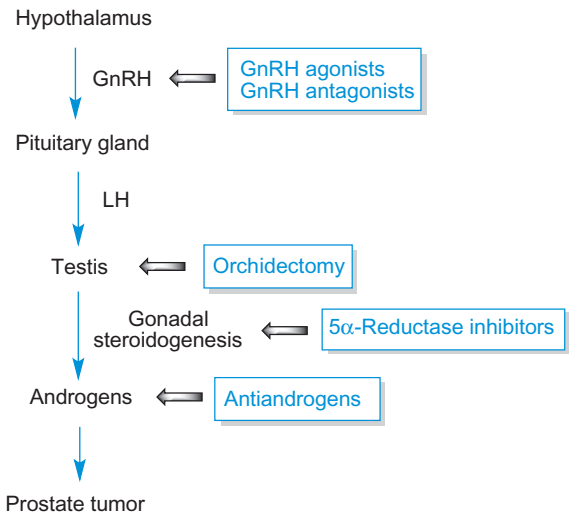


FIGURE 3.34

A summary of the strategies used to reduce testosterone levels.

In women, LH liberation stimulates the onset of ovulation in the first phase of the menstrual cycle and stimulates the production of progesterone in the second phase. Another pituitary hormone known as follicle-stimulating hormone (FSH) stimulates the secretion of estrogens in the ovary, although small amounts of LH are also required. A summary of these steps and the drugs used in breast and gynecological cancers, some of them already discussed, is presented in Figure 3.35.

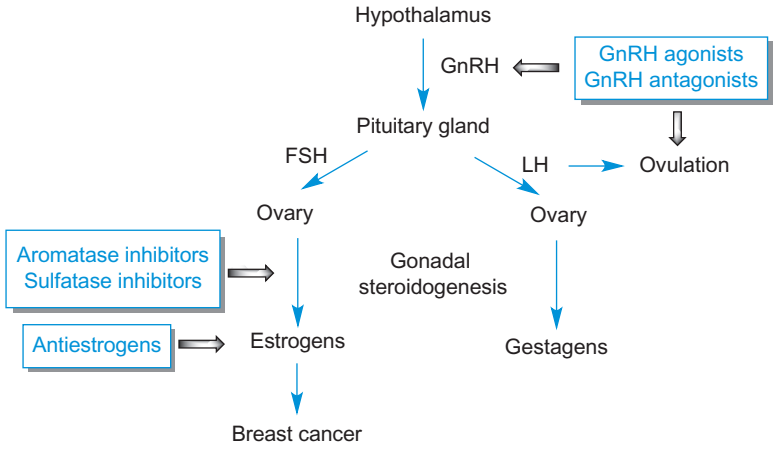


FIGURE 3.35

A summary of the strategies used to reduce estrogen and gestagen levels.

7.2 GnRH (LHRH) AGONISTS

The main clinical use of these drugs is the treatment of prostatic carcinoma. When a GnRH agonist is given in pulses by injection, it mimics the natural action of luteinizing hormone-releasing factor (LH-RF, also known as LHRH, gonadorelin, gonadotropin-releasing factor, gonadoliberin, or luliberin), and it induces the release of LH from the pituitary gland. However, if a GnRH agonist is given continuously, the pituitary is first stimulated, but after some days the response ceases. During the period of initial stimulation, more LH is released and consequently there is a surge in testosterone production, called a flare, during which time approximately 1 in 10 men with metastatic cancer may experience a temporary worsening of their symptoms. For this reason, GnRH agonists are associated with an antiandrogen or an inhibitor of androgen synthesis prior and during the first weeks of the treatment. After a few days, the pituitary becomes desensitized by the continuous presence of the hormone, loses its membrane receptors for GnRH, and stops releasing LH, leading to a decline of testosterone production and ending in levels similar to those achieved by orchidectomy.[77]

GnRH is a decapeptide with two isoforms differing in three amino acids, the most important of which is isoform I. Because the *in vivo* half-life of GnRH is very low (4 minutes) due to fast hydrolysis of the bond between amino acids 6 and 7, a search began for related but more stable molecules. The main strategy employed involved replacement of the sixth amino acid (glycine) by D-amino acids, and the C-terminal glycinamide residue was also replaced by a variety of substituents. The resulting compounds, in addition to being more stable to enzymatic degradation, are more lipophilic due to the introduction of a side chain at the sixth residue and have higher affinity for their receptor. Among these agonists, leuprorelin (leuprolide, leuprolide acetate, Lupron®, Prostap®), buserelin (Metralef®, Suprefact®), histrelin (Vantas®), triptorelin (Decapentptyl SR®, Trelstar®), and goserelin (Zoladex®), whose structures are summarized in Figure 3.36, are used for prostate cancer and, in some cases, for the treatment of endometriosis. They are administered parenterally, in subcutaneous formulations, or by inhalation to avoid their degradation in the gastrointestinal tract. Goserelin has also proved useful in premenopausal women with ER-positive early breast cancer, providing an alternative to chemotherapeutic regimens and avoiding the need for surgical ovariectomy. This means that younger women, when they finish their goserelin treatment, can recover their bone loss before they reach menopause. Histrelin has also proven to be very efficient for the treatment of central precocious puberty.

7.3 GnRH (LHRH) ANTAGONISTS

When administered to patients with prostatic cancer, GnRH antagonists act by direct inhibition of GnRH receptors in the pituitary gland and therefore block the release of LH and FSH secretion, leading to a faster onset of action (hours instead of days) and avoiding the initial rise of testosterone levels induced by GnRH receptor agonists.

GnRH antagonists currently in clinical use are peptidomimetics obtained by extensive modification of the natural GnRH hormone. The main problems of the first compounds to be overcome were the tendency to induce the release of histamine and their low solubility and propension to form gels, which

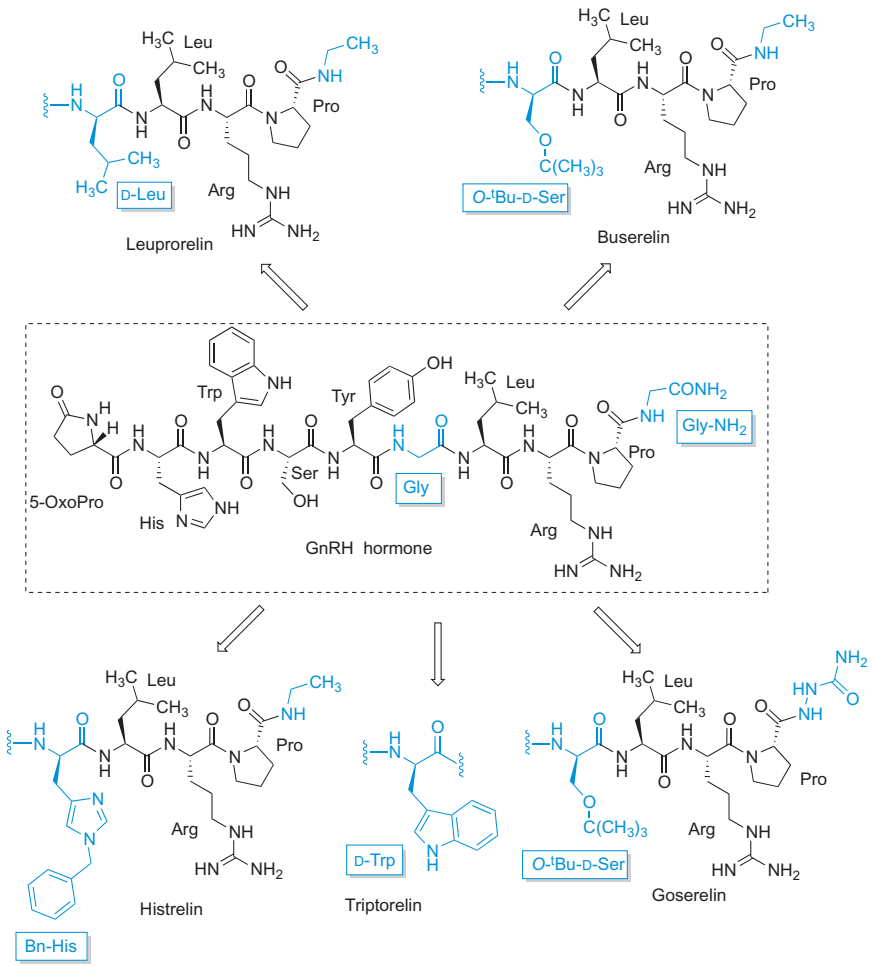


FIGURE 3.36

Analogs of the GnRH hormone.

severely limits their formulation.[78] The following are the main modifications that have been explored (Figures 3.37 and 3.38):

1. Replacement of the first three amino acids by D-amino acids with unnatural side chains. The most widely employed replacements are *N*-acetyl-D-(β-naphthyl)alanine for the first residue, D-(4-chloro)phenylalanine for the second, and D-(2-pyridyl)alanine or D-Trp for the third residue.
2. Modification of the sixth amino acid, which normally bears chains with amide, urea, or guanidine substituents. The purpose of this substitution is to increase the hydrogen bond-formation capabilities of the molecules and improve their solubilities.

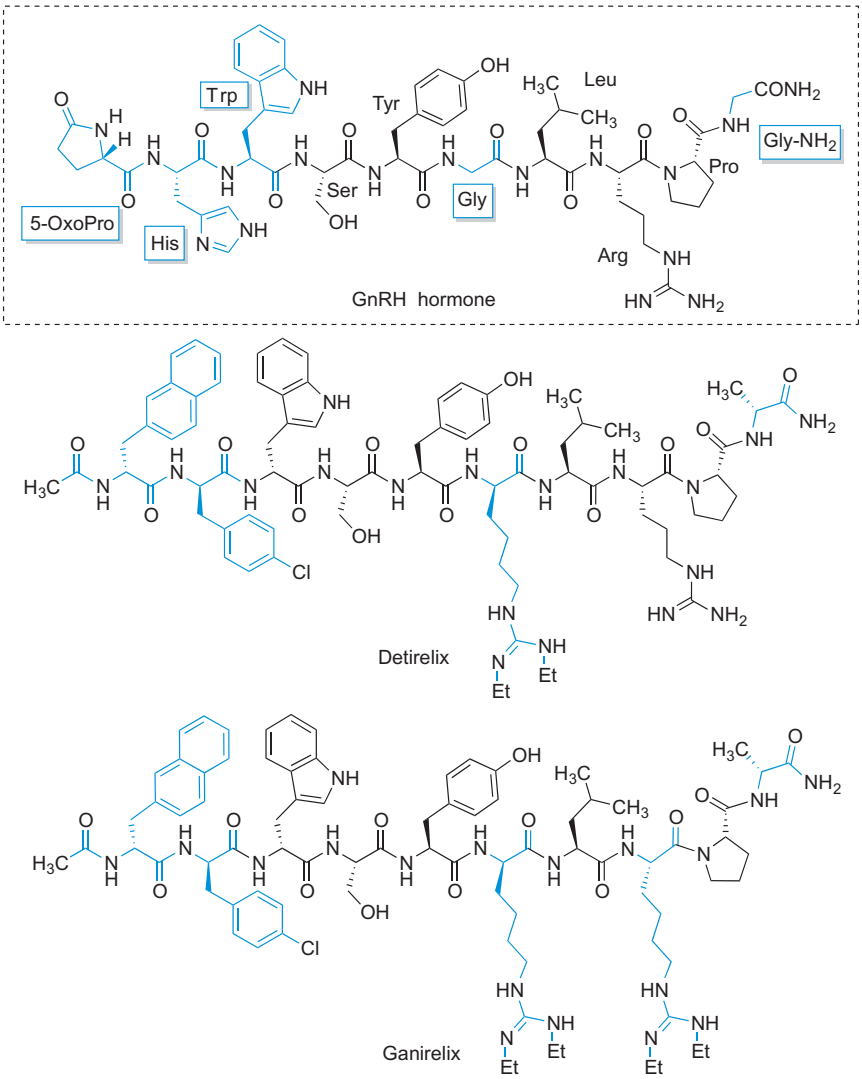


FIGURE 3.37

Additional analogs of the GnRH hormone.

3. Modifications of the side chain in the eighth amino acid.
4. Replacement of the C-terminal glycinamide by D-alaninamide.

The first long-term clinical studies were carried out with cetrorelix and ganirelix, but the first GnRH antagonist to achieve clinical use as an antitumor agent was abarelix (Plenaxis®),[79] which was approved by the FDA in November 2003. It is used as an intramuscular injection for the palliative treatment of advanced symptomatic prostate cancer in patients in whom LHRH agonist therapy and surgical

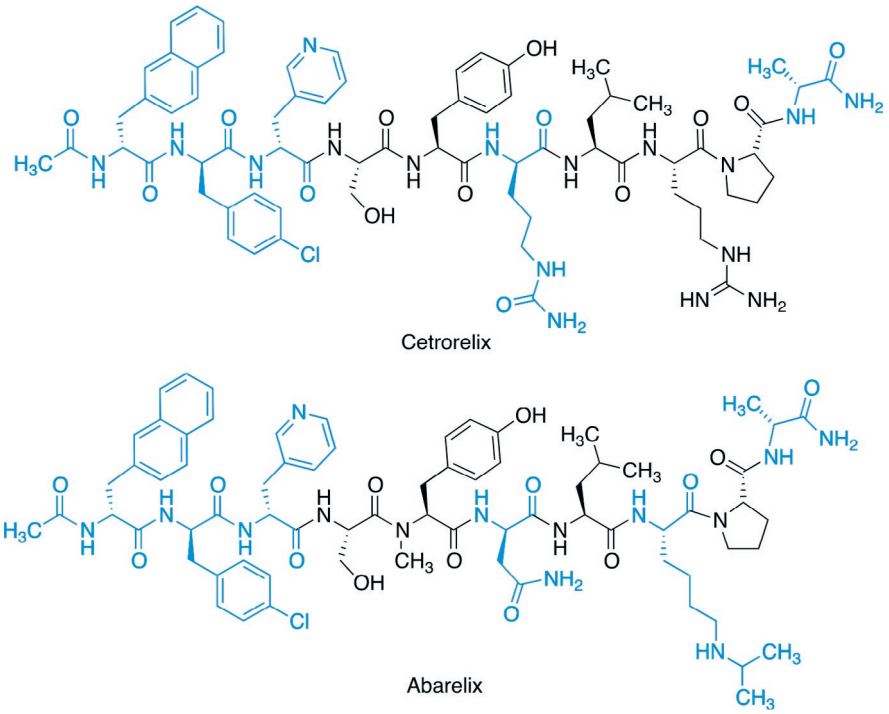


FIGURE 3.38

Analogs of the GNRH hormone (*continued*).

castration (orchidectomy) are not appropriate. Clinical studies have also shown the usefulness of some of these antagonists, such as cetrorelix, in ovarian, endometrial, and breast cancers.[80] Degarelix acetate (Firmagon®) was approved in 2008 (FDA) and 2009 (EMA) for use in adult male patients with advanced, hormone-dependent prostate cancer.[81]

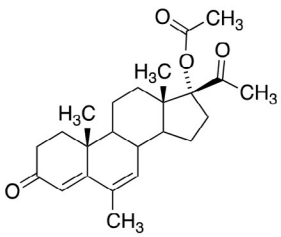
GnRH antagonists have a number of indications other than cancer treatment.[82] For instance, they are employed in assisted reproduction techniques[83] to prevent LH surge in women undergoing controlled ovary stimulation, allowing the follicles to mature for planned oocyte collection. The rationale for this treatment is that one of the physiological roles of LH is the initiation of ovulation during the menstrual cycle. When women are undergoing hormone treatment in assisted reproduction techniques, sometimes premature ovulation can occur, leading to the release of eggs that are not ready for fertilization.

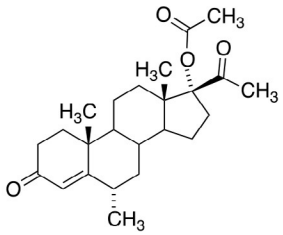
8 MISCELLANEOUS STEROID HORMONE-RELATED ANTICANCER THERAPY

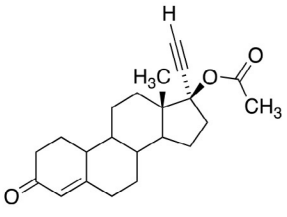
8.1 GESTAGENS AS ANTITUMOR AGENTS

Agonists of the gestagen receptor, such as medroxyprogesterone acetate (Provera® and Farlutal®), norethisterone acetate (Aygestin®), and megestrol acetate (Megace®), normally used in combination with other steroids as oral contraceptives, are licensed in some countries for the treatment of endometrial

carcinomas, some types of breast carcinoma, and other hormone-dependent cancers, in which they are able to induce apoptosis by binding to progesterone receptors.


Megestrol acetate

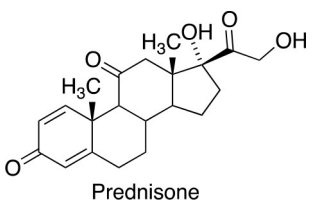

Medroxyprogesterone acetate


Norethisterone acetate

8.2 GLUCOCORTICOIDS AND INHIBITORS OF THEIR BIOSYNTHESIS AS ANTITUMOR AGENTS

Inflammation has been traditionally considered as a localized protective reaction of tissues to irritation, injury, or infection. This concept is probably accurate for the case of acute inflammation, which can be regarded as a part of this defense response, but chronic inflammation can lead to a wide variety of diseases, being a risk factor for most types of cancers.[84] Pro-inflammatory entities, as well as their products, are involved in cancer events such as suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Consequently, anti-inflammatory agents that suppress these products, including glucocorticoids, should have potential in the prevention and treatment of cancer.[85]

The anti-inflammatory and immunosuppressive activities of the glucocorticoids are well-known. They exert an influence in human lymphoid tissue, in which they can modify the homing of lymphocytes into lymphoid organs. For this reason, they are often useful in the treatment of acute lymphoblastic leukemia and other chronic and acute leukemias. The glucocorticoid prednisone is normally employed for this purpose, usually in association with other types of chemotherapy. Because of their anti-inflammatory action, corticosteroids are often also included in antitumor regimens to alleviate cancer pain.[86]


Prednisone

Mitotane (*o*,*p*′-DDD, Lysodren®), an analog of the insecticide DDT initially used for treatment of canine Cushing's disease because of its cortex-selective adrenalytic activity, was FDA approved for use in the treatment of adrenal cancer in 1970. Because of its high toxicity, it was later designated an orphan drug for use in the treatment of human inoperable cancer of the adrenal gland (adrenocortical cancer). Adrenal tissue is capable of metabolizing mitotane by action of a novel, nonsteroidogenic P450-type enzyme that catalyzes hydroxylation at the position adjacent to the two chlorine atoms. Subsequent dehydrohalogenation of this intermediate leads to a highly electrophilic acyl chloride, which has been shown to react with

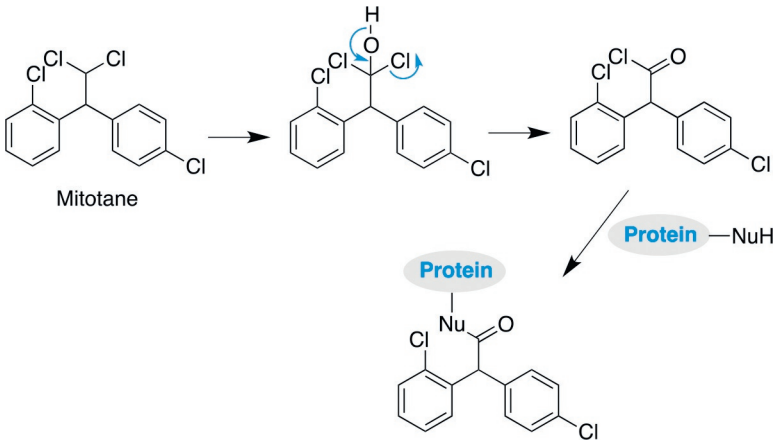


FIGURE 3.39

Biotransformations of mitotane.

proteins, leading to direct necrosis and atrophy of the adrenal cortex and, hence, inhibition of glucocorticoids, mineralocorticoids, and adrenal gland-produced sex hormone synthesis (Figure 3.39). Another possible mechanism is oxidative damage through the production of free radicals.[87]

9 COMPOUNDS ACTING ON OTHER PROTEINS OF THE NUCLEAR RECEPTOR SUPERFAMILY: RETINOIDS

Vitamin A and its analogs, collectively known as retinoids, have profound effects on cell growth and differentiation, and the loss of retinoid function is linked to carcinogenesis. Several retinoids have shown promising activity as antitumor and cancer chemopreventive agents by inhibiting carcinogenesis at the initiation, promotion, and progression stages.[88] The anticancer activity of the retinoids is mainly due to their binding to nuclear receptors that act as hormone receptors activating target genes. They are classified as classical retinoic acid receptors (RARs) and nonclassical retinoid X receptors (RXRs), each of which has three isoforms (α, β, and γ). Because they have different ligand-binding domains, they can be targeted separately. The diet-derived all-*trans* retinoic acid (ATRA, tretinoin), which is the main retinoid in humans, selectively activates the RARs, whereas 9-*cis*-retinoic acid can activate both RARs and RXRs (Figure 3.40). Retinoids with selectivity for RXRs are known as rexinoids. RARs can heterodimerize with RXRs, and the latter can also form heterodimers with other nuclear receptors, including the vitamin D receptors, thyroid hormone receptors, and peroxysome proliferator-activating receptors (PPARγ).

The RAR–RXR heterodimers bind to specific DNA sequences, known as retinoic acid response elements. In the absence of ligands, the heterodimer–DNA complex is linked to corepressors and histone deacetylases, inducing chromatin compaction and silencing the promoter region of the target genes (gene repression). However, the binding of ligands to the heterodimers induces a conformational change that destabilizes the interaction with corepressors and allows the union to coactivators, leading to gene transcription (Figure 3.41).

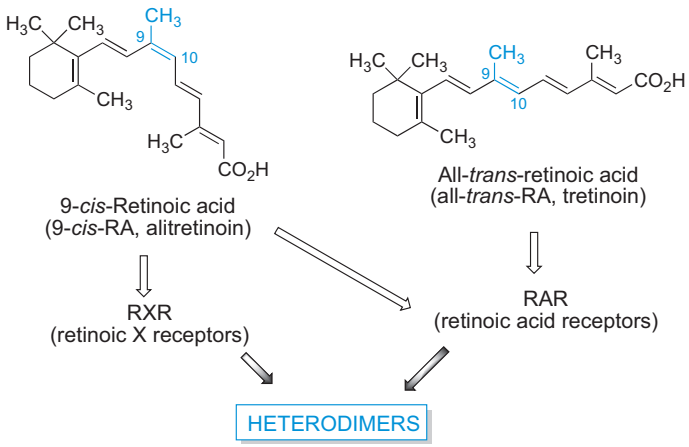


FIGURE 3.40

Retinoids and their receptors.

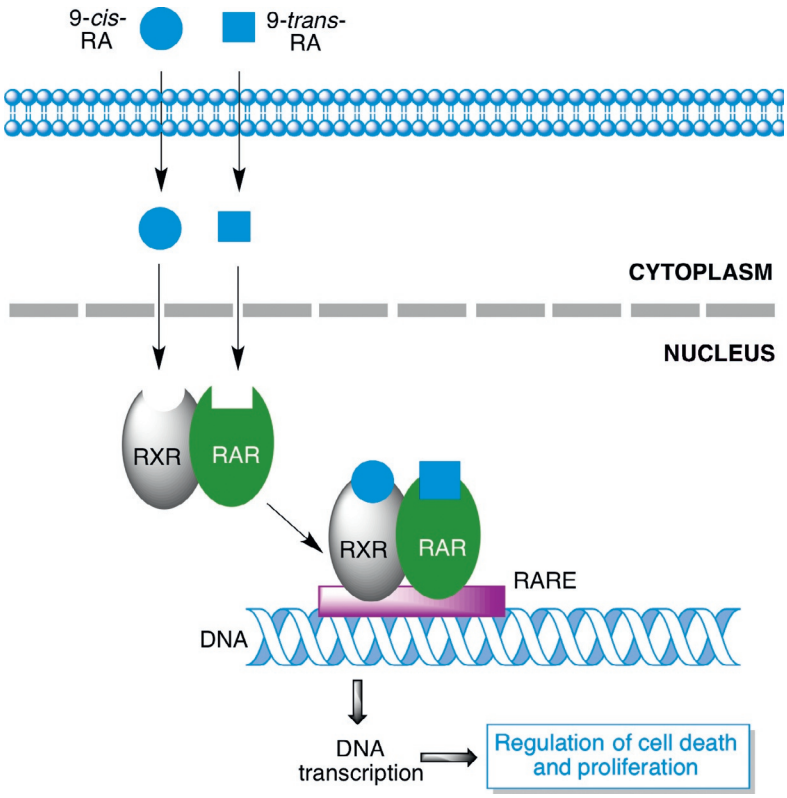
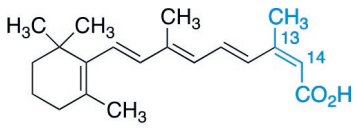


FIGURE 3.41

Binding of ligand-coupled retinoid receptors to DNA.

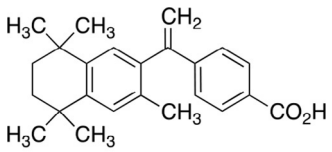
Some retinoids and their analogs are currently in use or under clinical trials for several types of cancer.[89] The most relevant success of retinoids in this field has been achieved in the therapy of acute promyelocytic leukemia (APL), the most malignant form of acute myeloid leukemia (AML), a term that refers to a group of hematopoietic neoplasms involving cells committed to the myeloid lineage. APL arises from a chromosomal translocation that produces a chimeric protein between RAR-α and promyelocyte leukemia protein (PML). This process interferes with the normal function of both proteins, resulting in the arrest of cell maturation at the stage of promyelocytes. Oral administration of tretinoin induces differentiation of these cells to produce mature neutrophils with a high rate of therapeutic success, and a combination of tretinoin with anthracycline and ara-C has become the standard therapy for this disease.[90] The mechanism of action of tretinoin in this tumor is not fully understood, although it has been shown that it induces the cleavage of the PML portion from the chimeric protein and its degradation.[91]

9-*cis*-Retinoic acid (alitretinoin, Panretin®) was approved for the topical treatment of cutaneous lesions of AIDS-associated Kaposi's sarcoma in combination with interferon,[92] and 13-*cis*-retinoic acid (isotretinoin, Accutane®) is mostly used for cystic acne under severe prescribing restrictions due to its potentially severe side effects. Oral forms of isotretinoin have shown great efficacy in the chemoprevention of squamous cell carcinoma[93] and oral cancer,[94] but among other important side effects, isotretinoin is a possible cause of inflammatory bowel disease, including Crohn's disease and ulcerative colitis. For this reason, after several jury trials awarded large compensations for injuries attributed to prescribed isotreonine, Roche Pharmaceuticals discontinued the manufacture and distribution of Accutane® in the United States.



13-*Cis*-retinoic acid
(isotretinoin)

ATRA is highly susceptible to isomerization when in solution, which can influence its effective concentration and, subsequently, its biological activity. To address this source of variability, synthetic retinoid analogs have been designed to retain stability and biological function during use,[95] and the synthesis and biological evaluation of these compounds is currently an active area of research.[96] Thus, bexarotene (Targretin®) is a synthetic rexinoid (selective ligand for RXRs) that has been approved by the FDA for cutaneous T-cell lymphoma.[97] In combination with chemotherapeutic agents such as cisplatin and vinorelbine, it has demonstrated encouraging results in patients with advanced non-small cell lung cancer.[98,99] In 2012, it was announced that bexarotene reduced amyloid plaque and improved mental functioning in a small sample of mice engineered to exhibit the symptoms of Alzheimer's disease, but full replication of the initial results proved impossible.



Bexarotene

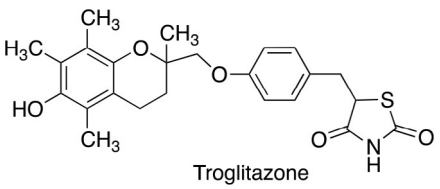
Despite the previously mentioned successes, the full potential of retinoids as anticancer agents has not yet been realized because of their potential toxicity and the problem of intrinsic or acquired resistances. Strategies to overcome this problem include their combination with other chemotherapeutic agents acting by related mechanisms and the use of nonclassical retinoids.[100] Compared with classical retinoids, the nonclassical retinoids might have lower toxicity and be able to induce apoptosis in RA-resistant cells. Retinoids are also relevant in the prevention of several cancers, including oral cavity, head and neck, breast, skin, and liver cancer.

10 PPAR LIGANDS AS ANTITUMOR AGENTS

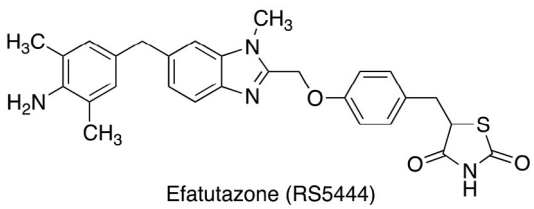
PPARs are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily. As previously mentioned, they heterodimerize with RXRs and bind to specific regions of DNA target genes known as peroxisome proliferator hormone response elements (PPREs). Their main endogenous ligands are eicosanoids and free fatty acids, and among other functions, they play essential roles in cellular differentiation and tumorigenesis. PPARα and PPARγ are the molecular targets of a number of marketed drugs, including the fibrates and the antidiabetic thiazolidinediones.

The antitumor activity of PPAR ligands against a variety of human cancers is associated with transcriptional activation of PPARγ, which could act as a tumor suppressor in several cancers. Among the PPARγ ligands studied as antitumor agents, promising results were obtained in initial clinical trials for liposarcoma and prostate cancers with troglitazone (Rezulin®). This compound was initially approved as an oral antidiabetic, but it was withdrawn from the market because of its liver toxicity. Although studies in colorectal and breast cancers have been disappointing,[101] troglitazone has shown its ability to inhibit human prostate cancer cell growth through inactivation of NF-κB via suppression of GSK-3β expression.[102]

PPARγ is also involved in some thyroid cancers. A fusion protein of PPARγ1 and the thyroid transcription factor PAX8 is present in approximately one-third of follicular thyroid carcinomas,[103] and PPARγ activation by the agonist efatutazone (RS5444) inhibits anaplastic thyroid cancer (ATC) growth. In fact, this compound is under phase I clinical testing for ATC, in combination with paclitaxel.[104] A phase II trial has been also carried out with efatutazone in patients with refractory non-small cell lung cancer, leading to the conclusion that it does not improve the efficacy of erlotinib.[105]



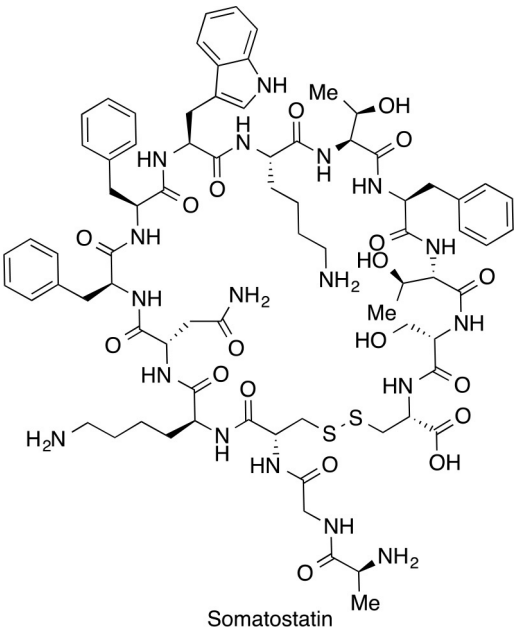
Troglitazone



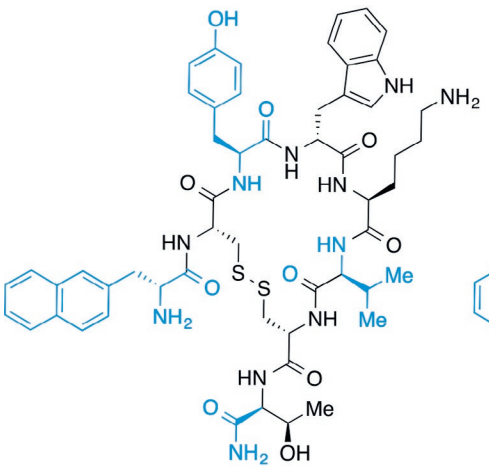
Efatutazone (RS5444)

11 SOMATOSTATIN ANALOGS IN NEUROENDOCRINE TUMORS

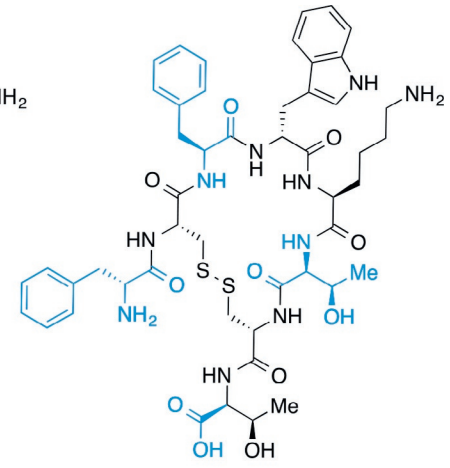
Somatostatin is a major endocrine hormone with multiple physiological actions modulated by one or more of the five somatostatin receptors (SSTr).



Although the biological role and the cellular distribution of each receptor subtype are far from being completely understood, numerous somatostatin analogs with druglike properties and agonist activity are currently being used in the clinic to manage a number of pathophysiological conditions and as ligands for diagnosis or radiotherapy. Octeotride (Sandostatin®) and lanreotide (Somatuline®) control the clinical signals related to hypersecretion in SST_2 and SST_5-positive neuroendocrine tumors (NETs) and exert some antiproliferative activities.[106] Octeotride, the most widely used somatostatin analog, has been registered in most countries for the control of hormonal symptoms in patients with gastrointestinal and pancreatic NETs, as well as in patients with acromegaly. Both drugs can be administered by multiple or continuous subcutaneous injections, intravenously, or by slow-release intramuscular formulations (Sandostatin LAR® and Somatuline LAR®). A randomized phase III trial showed that long-acting octreotide has an antitumor effect in midgut NETs.[107] On the other hand, radiolabeled octapeptide analogs can be used to visualize tumors and metastases that bear SST_2 or SST_5, and also as radiotherapeutic agents.[108]

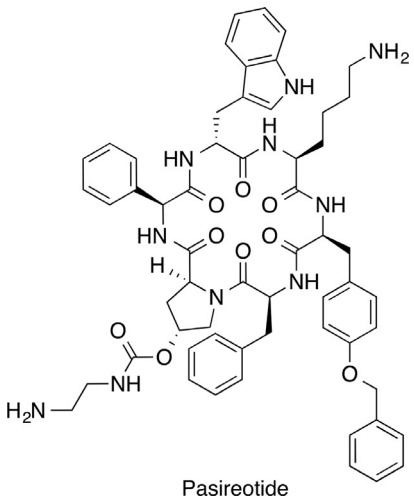


Lanreotide



Octeotride

Cyclic peptides with affinities to other SSTr subtypes are currently undergoing clinical testing.[109] Pasireotide (SOM230, Signifor®), a somatostatin analog with a high affinity to SSTr 1, 2, 3, and 5, was an orphan drug approved in Europe and the United States for the treatment of Cushing's disease. It entered phase III studies for the control of symptoms in advanced neuroendocrine tumors that are refractory or resistant to octeotride,[110] and it was approved by the FDA for this indication in 2012.



Pasireotide

REFERENCES

1 Liehr JG. *Endocr Rev* 2000;**21**:40.
2 Yager JD, Davidson NE. *N Engl J Med* 2006;**354**:270.
3 Santen RJ, Yue W, Wang JP. *Breast Cancer Res* 2005;**7**(Suppl. 8).
4 Bolton JL. *Adv Mol Toxicol* 2006;**1**:1.
5 Zahid M, Kohli E, Saeed M, Rogan E, Cavalieri E. *Chem Res Toxicol* 2006;**19**:164.
6 Watts CKW, Sutherland RL. *Mol Pharmacol* 1987;**31**:541.
7 Jordan VC. *Nature Rev Drug Discov* 2003;**2**:205.
8 Desta Z, Ward BA, Soukhova NV, Flockhart DA. *J Pharmacol Exp Ther* 2004;**310**:1062.
9 De Médina P, Favre G, Poirot M. *Curr Med Chem Anticancer Agents* 2004;**4**:491.
10 Howell SJ, Johnston SR, Howell A. *Best Pract Res Clin Endocrinol Metab* 2004;**18**:47.
11 Pagani O, Gelber S, Simoncini E, Castiglione-Gertsch M, Price K, Zahrieh D, et al. *Ann Oncol* 2004;**15**:1749.
12 Buzdar A, Hayes D, El-Khoudary A, Yan S, Lønning P, Lichinitser M, et al. *Breast Cancer Res Treatment* 2002;**73**:161.
13 Arpino G, Krishnan MN, Dinesh CD, Bardou VJ, Clark GM, Elledge RM. *Ann Oncol* 2003;**14**:233.
14 Gennari L, Merlotti D, De Paola V, Nuti R. *Expert Opin Drug Saf*. 2008;**7**:259.
15 Wardell SE, Nelson ER, Chao CA, McDonnell DP. *Clin Cancer Res* 2013;**19**:2420.
16 LaCroix AZ, Powles T, Osborne CK, Wolter K, Thompson JR, Thompson DD, et al. *J Natl Cancer Inst* 2010;**102**:1706.
17 Brzozwski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engström O, et al. *Nature* 1997;**389**:753.
18 Dardes RC, Jordan VC. *Br Med Bull* 2000;**56**:773.
19 https://clinicaltrials.gov/show/NCT00853996.
20 Vergote I, Robertson JFR. *Br J Cancer* 2004;**90**(Suppl. 1):S11.
21 Review: Croxtall JD, McKeage K. *Drugs* 2011;**71**:363.
22 Osborne CK, Wakeling A, Nicholson RI. *Br J Cancer* 2004;**90**(Suppl. 1):S2.
23 Witton J, Reeves JR, Going JJ, Cooke TG, Bartlett JMS. *J Pathol* 2003;**200**:290.
24 Lake DE, Hudis C. *Cancer Control* 2002;**9**:490.
25 Puddefoot JR, Barker S, Glover HR, Malouitre SDM, Vinson GP. *Int J Cancer* 2002;**101**:17.
26 Puddefoot JR, Barker S, Vinson GP. *Expert Opin Pharmacother* 2006;**7**:2413.
27 Grubjesic S, Moriarty RM, Pezzuto JM. *Expert Opin Ther Patents* 2002;**12**:1647.
28 Chen S. *Frontiers Biosci* 1998;**3**:922.
29 Santen RJ. *Steroids* 2003;**68**:539.
30 Osborne C, Tripathy D. *Annu Rev Med* 2005;**56**:103.
31 Ito K, Blinder VS, Elkin EB. *J Clin Oncol* 2012;**30**:1468.
32 Brueggemeier RW. *J Enz Inh* 1990;**4**:101.
33 Vaz ADN. In: Lee JS, Obach RS, Fisher MB, editors. *Drug metabolizing enzymes: Cytochrome P450 and other enzymes in drug discovery and development*. Boca Raton, FL: Informa Healthcare; 2003 [chapter 1].
34 Brueggermeier RW. *Breast Cancer Res Treat* 1994;**30**:31.
35 Narashimamurty J, Rao ARR, Sastry GN. *Curr Med Chem Anticancer Agents* 2004;**4**:523.
36 Reddy P. *J Clin Pharm Ther* 1998;**23**:81.
37 Brodie AM, Njar VC. *Steroids* 2000;**65**:171.
38 Dixon JM. *Expert Rev Anticancer Ther* 2002;**2**:267.
39 Zilembo N, Noberasco C, Bajetta E. *Br J Cancer* 1995;**72**:1007.
40 Ghosh D, Griswold J, Erman M, Pangborn W. *Nature* 2009;**457**:219.
41a Recanatini M, Cavalli A, Valenti P. *Med Res Rev* 2002;**22**:282.
41b Suvannang N, Nantasenamat C, Isarankura-Na-Ayudhya C, Prachayasittikul V. *Molecules* 2011;**16**:3597.

42 Johnston SRD, Dowsett M. *Nature Rev Cancer* 2003;**3**:821.
43 Ellis MJ, Ding L, Shen D, Luo J, Suman V, Wallis JW, et al. *Nature* 2012;**486**:353.
44 Purohit A, Woo LWL, Chander SK, Newman SP, Ireson C, Ho Y, et al. *Steroid Biochem Mol Biol* 2003;**86**:423.
45 Woo LWL, Purohit A, Potter BVL. *Mol Cell Endocrinol* 2011;**340**:175.
46 Maltais R, Poirier D. *Steroids* 2011;**76**:929.
47 Stanway SJ, Purohit A, Woo LWL, Sufi S, Vigushin D, Ward R, et al. *Clin Cancer Res* 2006;**12**:1585.
48 Lloyd MD, Pederick RL, Natesh R, Woo LWL, Purohit A, Reed MJ, et al. *Biochem J* 2005;**385**:715.
49 Woo LWL, Ganeshapillal D, Thomas MP, Sutcliffe OB, Malini B, Mahon MF, et al. *ChemMedChem* 2011;**6**:2019.
50 Woo LWL, Fischer DS, Sharland CM, Trusselle M, Foster PA, Chander SK, et al. *Mol Cancer Ther* 2008;**7**:2435.
51 Palmieri C, Januszewski A, Stanway S, Coombes RC. *Expert Rev Anticancer Ther* 2011;**11**:179.
52 Meunier B. *Acc Chem Res* 2008;**41**:69.
53 Woo PM, Woo LWL, Thomas MP, Mahon MF, Purohit A, Potter BVL. *ChemMedChem* 2011;**6**:1423.
54 Purohit A, Foster PA. *J Endocrinol* 2012;**212**:99.
55 Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, et al. *Proc Natl Acad Sci U S A* 2001;**98**:4904.
56 For overviews of therapies for castration-resistant prostate cancer, see. (a) Leibowitz-Amit R, Joshua AM. *Curr Oncol* 2012;**19**:S22; (b) Yin L, Hu Q, Hartmann RW. *Int J Mol Sci* 2013;**14**:13958.
57 Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. *N Engl J Med* 2014;**37**:1028.
58 For a review, see Attard G, Richards J, de Bono JS. *Clin Cancer Res* 2011;**17**:1649.
59 Selected reviews: (a) Chen Y, Clegg NJ, Scher HI. *Lancet Oncol* 2009;**10**:981; (b) Mohler ML, Coss CC, Duke CB, Patil SA, Miller DD, Dalton JT. *Expert Opin Ther Patents* 2012;**22**:541.
60 Koreckij TD, Trauger RJ, Montgomery RB, Pitts TEM, Coleman I, Nguyen H, et al. *Neoplasia* 2009;**11**:1216.
61 Bhasin S, Calof OM, Storer TW, Lee ML, Mazer NA, Jasuja R, et al. *Nat Clin Pract Endocrinol* 2006;**2**:146.
62 Jung ME, Ouk S, Yoo D, Sawyers CL, Chen C, Tran C, et al. *J Med Chem* 2010;**53**:2779.
63 Beer TM, Armstrong AJ, Rathkopf DE, Loriot Y, Sternberg CN, Higano CS, et al. *N Engl J Med* 2014;**371**:424.
64 Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, et al. *Cancer Res* 2012;**72**:1494.
65 https://clinicaltrials.gov/ct2/show/NCT01317641.
66 Ryan CJ, Eisenberger M. *J Clin Oncol* 2005;**23**:8242.
67 DeVore NM, Scott EE. *Nature* 2012;**482**:116.
68 O'Donnell A, Judson I, Dowsett M, Raynaud F, Dearnaley D, Mason M, et al. *Br J Cancer* 2004;**90**:2317.
69 Madan RA, Arlen PM. *IDrugs* 2006;**9**:49.
70 Massard Ch. *Clin Cancer Res* 2011;**17**:3876.
71 Brawer MK. *Rev Urol* 2010;**10**:294.
72 DeVore NM, Scott EE. *Nature* 2012;**482**:116.
73 Dreicer R, Agus DB, MacVicar GR, Wang J, MacLean D, Stadler WM. *J Clin Oncol* 2010;**28**:3084.
74 Bull HG, García-Calvo M, Andersson S, Baginsky WF, Chan HK, Ellsworth DE, et al. *J Am Chem Soc* 1996;**118**:2359.
75 Andriole G, Bostwick D, Brawley O, Gomella L, Marberger M, Tindall D, et al. *J Urol* 2004;**172**:1314.
76 Denmeade SR, Isaacs JT. *Nature Rev Cancer* 2002;**2**:389.
77 Lepor H, Shire ND. *Rev Urol* 2012;**14**:1.
78 Jiang G, Stalewski J, Galyean R, Dykert J, Schteingart C, Broqua P, et al. *J Med Chem* 2001;**44**:433.
79 Hedge S, Schmidt M. *Ann Rep Med Chem* 2004;**40**:443.
80 Emons G, Grundker C, Gunthert AR, Westphalen S, Kavanagh J, Verschraegen C. *Endocr Relat Cancer* 2003;**10**:291.

81 Princivalle M, Broqua P, White R, et al. *J Pharmacol Exp Ther* 2007;**320**:1113.
82 Huirne JAF, Lambalk CB. *Lancet* 2001;**358**:1793.
83 Hernández ER. *Hum Reprod* 2000;**15**:1211.
84 Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. *Biochem Pharmacol* 2006;**72**:1605.
85 Mantovani A, Marchesi F, Portal C, Allavena P, Sica A. *Adv Exp Med Biol* 2008;**610**:112.
86 Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, et al. *N Engl J Med* 2004;**351**:1502.
87 Schteingart DE, Braz J. *Med Biol Res* 2000;**33**:1197.
88 Okuno M, Kojima S, Matsushima-Nishigaki R, Tsurumi H, Muto Y, Friedman SL, et al. *Curr Cancer Drug Targets* 2004;**4**:285.
89 Altucci L, Gronemeyer H. *Nature Rev Cancer* 2001;**1**:181.
90 Fenaux P, Wang ZZ, Degos L. *Curr Top Microbiol Immunol* 2007;**313**:102.
91 Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, et al. *Nature* 1998;**319**:815.
92 Von Roenn JH, Cianfrocca M. *Cancer Treat Res* 2001;**104**:127.
93 Jones E, Korzenko A, Kiegel D. *J Drugs Dermatol* 2004;**3**:498.
94 Lee JJ, Hong WK, Hittelman WN, Mao L, Lotan R, Shin DM, et al. *Clin Cancer Res* 2000;**6**:1702.
95 Christie VB, Barnard JH, Batsanov AS, et al. *Org Biomol Chem* 2008;**6**:3497.
96 Gluyas JBG, Burschka Ch, Dörrich S, et al. *Org Biomol Chem* 2012;**10**:6914.
97 Hurst RE. *Curr Opin Invest Drugs* 2001;**1**:514.
98 Rigas JR, Dragnev K. *Oncologist* 2005;**10**:22.
99 Dragnev KH, Petty WJ, Shah SJ, Lewis LD, Black CC, Memoli V, et al. *Clin Cancer Res* 2007;**13**:1794.
100 Freemantle SJ, Spinella MJ, Dmitrovsky E. *Oncogene* 2003;**22**:7305.
101 Rumi MAK, Ishihara S, Kazumori H, Kadowaki Y, Kinoshita Y. *Curr Med Chem Anticancer Agents* 2004;**4**:465.
102 Ban JO, Oh JH, Son SM, Won D, Song HS, Han SB, et al. *Cancer Biol Ther* 2011;**12**:288.
103 Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, et al. *Science* 2000;**289**:1357.
104 Smallridge RC, Copland JA, Brose MS, Wadsworth JT, Houvras Y, Menefee ME, et al. *J Clin Endocrinol Metab* 2013;**98**:2392.
105 http://www.sciencedaily.com/releases/2014/04/140408122131.htm.
106 Sbojamanesh H, Gibril F, Louic A, et al. *Cancer* 2002;**94**:331.
107 Sidéris L, Dubé P, Rinke A. *Oncologist* 2012;**17**:747.
108 Öberg K, Kvols L, Caplin M, et al. *Ann Oncol* 2004;**15**:965.
109 Lamberts SW, van der Lely AJ, Hofland LJ. *Eur J Endocrinol* 2002;**146**:701.
110 Kvols LK, Oberg KE, O'Dorisio TM, Mohideen P, de Herder WW, Arnold R, et al. *Endocr Relat Cancer* 2012;**19**:657.

CHAPTER

4

ANTICANCER DRUGS ACTING VIA RADICAL SPECIES

RADIOTHERAPY AND PHOTODYNAMIC THERAPY OF CANCER

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00004-1

1 INTRODUCTION: RADICALS AND OTHER REACTIVE OXYGEN SPECIES

A radical (sometimes called *free radical*) is a chemical species that contains one or more unpaired electrons and is sufficiently stable for independent existence. Molecular oxygen is the main promoter of the formation of radicals within cells because ground state oxygen contains two unpaired electrons, each one in a different π^* antibonding orbital, and hence it can be considered as a biradical. Both electrons have the same spin quantum number, and therefore oxygen tends to accept electrons one at a time. A pair of electrons in an atomic or molecular orbital will have opposite spin numbers, in accordance with Pauli's principle, and for this reason it will not be able to form two pairs of electrons with antiparallel spins by combination with the oxygen electrons, which have parallel spins. Singlet oxygen species, on the other hand, do not have this restriction because the two electrons of the π^* antibonding orbitals have opposite spins, and this explains why they are more potent oxidants than ground state oxygen. Addition of one electron to oxygen gives the superoxide radical anion, and incorporation of a new electron to the latter leads to the peroxide dianion (Figure 4.1).

Another oxidizing species found in biological systems is hydrogen peroxide (H_2O_2), arising from diprotonation of peroxide (O_2^{2-}). The main source of peroxide is the enzyme superoxide dismutase (SOD), which catalyzes the one-electron transfer between two superoxides. Because the second

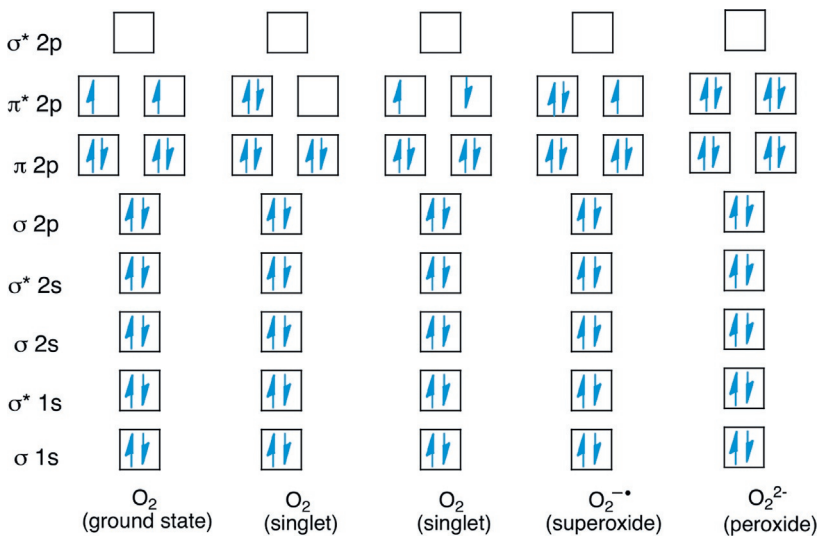


FIGURE 4.1

Electronic configuration of molecular oxygen species.

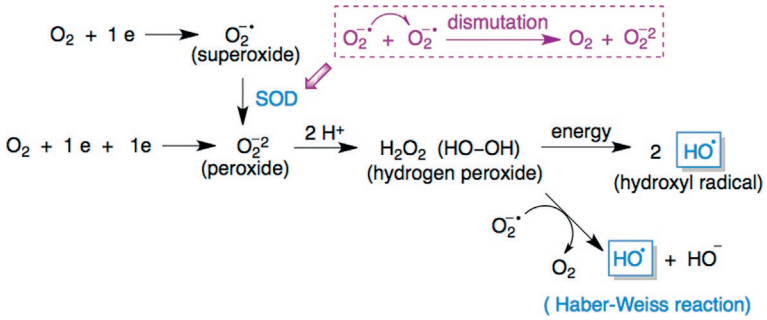


FIGURE 4.2

Redox processes involving molecular oxygen.

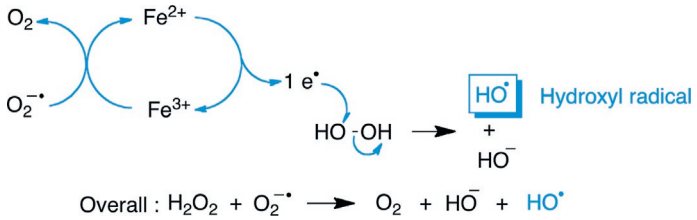


FIGURE 4.3

The Fenton reaction.

electron is added to an antibonding orbital, the O–O bond in peroxide or hydrogen peroxide is weak and can be homolyzed under certain conditions (e.g., exposure to ultraviolet radiation), leading to two hydroxyl radicals. Alternatively, electron transfer from superoxide to hydrogen peroxide gives a hydroxide anion and a hydroxyl radical (Haber–Weiss reaction; Figure 4.2).

The production of hydroxyl radical through the mechanisms in Figure 4.2 is very slow, but it can be catalyzed by the presence of certain relatively common cations, such as Fe^{2+} or Cu^{+}. For instance, Fe^{2+} can decompose hydrogen peroxide to a hydroxyl radical and a hydroxy anion in the so-called Fenton reaction, which is coupled to the regeneration of Fe^{2+} by one-electron reduction of Fe^{3+} by superoxide radical (Figure 4.3). The extremely reactive hydroxyl radical cannot diffuse from its site of formation, and therefore drugs that act through this radical must generate it very close to the target biomolecule.

Oxygen radicals such as superoxide and hydroxyl radicals, as well as some highly reactive nonradical derivatives of oxygen such as hydrogen peroxide, are collectively known as "reactive oxygen species" (ROS). They can react with NO, leading to the generation of reactive nitrogen species.

2 BIOLOGICAL EFFECTS OF REACTIVE OXYGEN SPECIES

Reactive oxygen species arise as a consequence of oxidative metabolism in mitochondria and have roles in normal cell signaling and homeostasis, for example, by regulating the duration of the vascular response to NO. Beyond these normal effects, excessive production of ROS can happen as a response to

stress situations derived from exposure to toxic agents, damage due to radiation, and a variety of diseases, resulting in adaptive responses associated with local oxidative stress.

Oxidative stress can be defined as a situation of imbalance between the production of radical species and antioxidant defense systems in the cell. Oxidative stress can cause damage to all kinds of biomolecules, including lipids, proteins, and DNA. For this reason, the mechanism of action of several kinds of antitumor agents is based, at least partly, on the production of hydroxyl radicals and other ROS and the subsequent damages that they cause on biological molecules by a number of mechanisms that are summarized in this section.[1,2] Most of these mechanisms have been discovered during the course of studies on the anthracyclines.[3] On the other hand, there is an increasing body of evidence showing that ROS can directly interact with crucial signaling molecules essential for cell proliferation and survival and can therefore be viewed as critical for cellular signaling.[4]

2.1 MEMBRANE PHOSPHOLIPID PEROXIDATION

Cell membranes are one of the biological structures more sensitive to damage by radicals because of the presence in them of polyunsaturated fatty acids (PUFAs) containing methylene groups that are simultaneously adjacent to two double bonds. The C–H units in these methylenes are particularly suitable points of attack by hydroxyl and other radicals because of the stabilization of the resulting carbon radical by double resonance (Figure 4.4).

The reaction of these polyunsaturated side chains with oxygen radicals leads to *phospholipid peroxidation* and subsequent membrane injury. This process is initiated by the attack of a hydroxyl radical to one of the previously mentioned bis-allylic positions existing in the fatty acid side chains, leading to the generation of an alkyl radical **4.1**. Superoxide radical is not sufficiently reactive to initiate lipid peroxidation, and in any case its negative charge precludes its transport across the highly lipophillic cell membrane. Carbon radical **4.1** reacts rapidly with a molecule of oxygen, which is sufficiently hydrophobic to access the interior of the membranes, generating a peroxyl radical (R–O–O$^{\cdot}$, **4.2**), which can abstract a new hydrogen atom from a doubly allylic C–H bond in the adjacent fatty acid side chain. This leads to a hydroperoxide **4.3** and a new radical **4.1**, allowing a self-maintained radical process that extends to an expanding area of the membrane, as long as there is sufficient oxygen (propagation

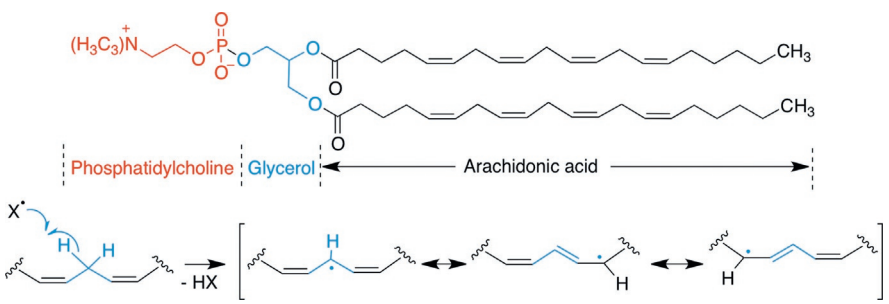


FIGURE 4.4

Radical generation in membrane phospholipids.

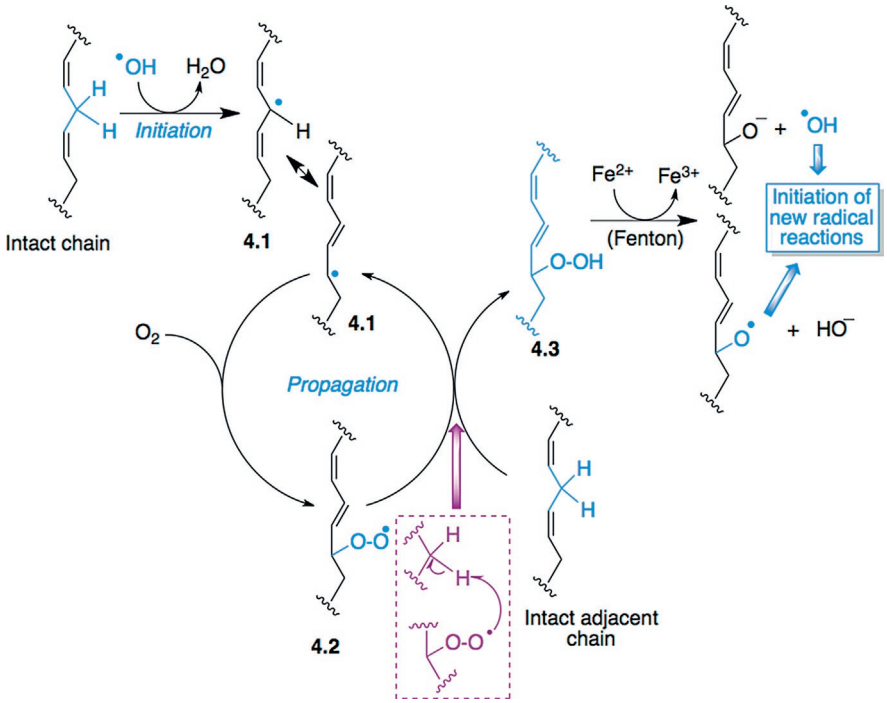


FIGURE 4.5

Phospholipid peroxidation by hydroxyl radicals.

phase). If traces of cations such as Fe^{2+} are present, they can generate new oxygen radicals ($RO^{\cdot}$ and $HO^{\cdot}$) from hydroperoxides **4.3** through Fenton chemistry, contributing to the extension of the peroxidation process (Figure 4.5).

2.2 MALONDIALDEHYDE GENERATION AND ITS CONSEQUENCES

Peroxyl radicals **4.2** can also evolve to cyclic endoperoxides by attack onto a neighboring carbon–carbon double bond in the same chain in a process resembling the one catalyzed by cyclooxygenase, as shown in Figure 4.6 for the case of a molecule of arachidonic acid. Peroxyl radicals **4.2** may lead to lipid peroxidation, as previously mentioned (see Section 2.1). Alternatively, they can cyclize to radical **4.4**, which then undergoes a new cyclization, coupled with the addition a second oxygen molecule and subsequent reduction of the hydroperoxyl radical thus generated, to give **4.5**. Together with other products, these intermediates generate malondialdehyde (MDA) through a retro Diels–Alder mechanism.

MDA can link covalently to amino groups in proteins, especially at Lys residues, resulting in intra- and intermolecular protein cross-links (Figure 4.7a). It may also react with DNA bases and cause mutagenic lesions, consisting of large insertions and deletions at GC base pairs, by reaction with guanine amino groups to give the oxopropenyl derivatives **4.6**, which are finally cyclized to

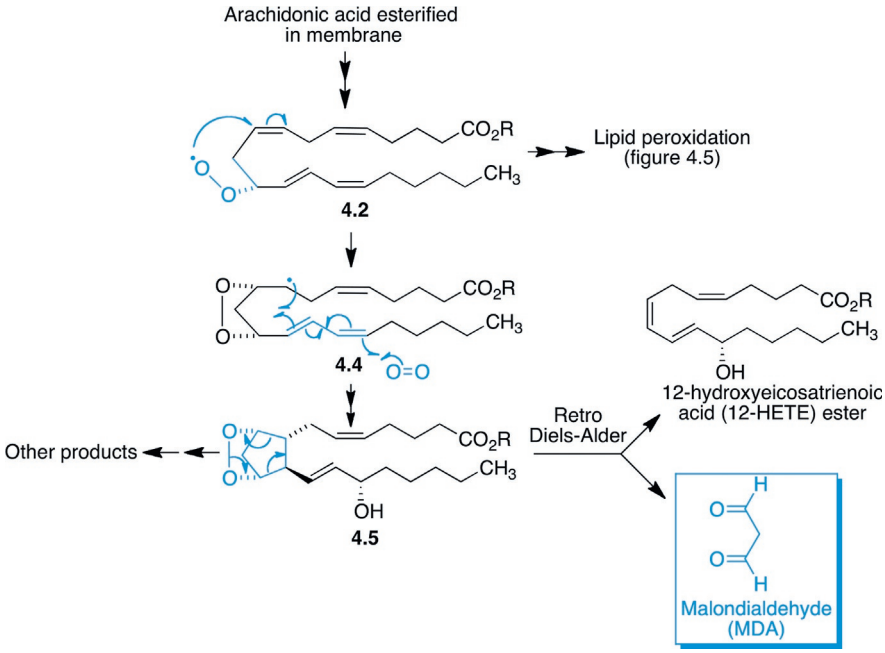


FIGURE 4.6

Generation of malondialdehyde from arachidonic acid.

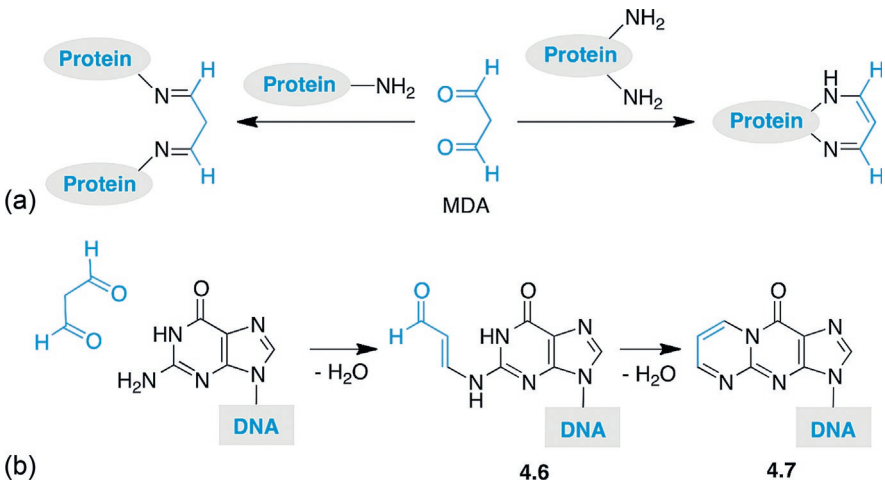


FIGURE 4.7

Covalent derivatives from malondialdehyde and proteins (a) or DNA (b).

pyrimidopurine derivatives **4.7**, known as M_1dG adducts (Figure 4.7b).[5] The implication of MDA-induced DNA damage in the antitumor effects of radical-generating drugs such as the anthracyclines has been clearly established.[6] In proliferating cells, the formation of M_1dG adducts is accompanied by cell cycle arrest and inhibition of cyclin-associated kinase activities. It has been proven that antitumor compounds of the anthracyclin group, at low concentrations, increase MDA-dependent DNA oxopropenylation several-fold,[7] establishing a potential link between antitumor drug-dependent generation of ROS, induction of lipid peroxidation, and DNA damage.[8]

In addition to MDA, a number of acrolein derivatives are generated by homolytic cleavage of hydroperoxides derived from PUFAs. These electrophilic species also give DNA adducts, with potential mutagenic consequences.[9]

2.3 DNA STRAND CLEAVAGE

In mammalian cells, the DNA both in the nucleus and in the mitochondria has to be maintained throughout the entire life of the cell. These genomes and their precursor nucleotides are highly exposed to ROS, which are inevitably generated as a result of the respiratory function in mitochondria. Oxidative stress by hydroxyl radical causes direct DNA damage, mainly by strand cleavage, and also causes damage by oxidation of pyrimidine and purine bases.[10]

Because of the negative charge of its phosphate groups, DNA acts as an anion and is therefore capable of binding many cations, including those required for Fenton chemistry such as Fe^{2+} and Cu^{+}. In addition, deoxyribose also has good iron binding properties. This allows "site-specific" hydroxyl radical generation that cannot be countered by radical scavengers. Perhaps for this reason, antitumor compounds that act by DNA strand cleavage are also normally chelating agents.

The main products of DNA strand scission, which have been studied mainly in connection with the mechanism of action of the antitumor drug bleomycin, are free DNA bases and *N*-(3-oxopropenyl) bases, which are accompanied by 5′-phosphate-modified DNA fragments and 3′-phosphoglycolate DNA derivatives (see Figure 4.9). The formation of *N*-(3-oxopropenyl) bases requires additional oxygen,[11] whereas that of free bases does not,[12] as shown by isotope studies with $^{18}O_2$ and $H_2{}^{18}O$.

This process starts by the radical-induced abstraction of a proton from any position of the deoxyribose moiety and can lead to a large number of products. For instance, oxidation at C-4 leads to carbon radical **4.8**, stabilized by resonance with the ring oxygen. Addition of an oxygen molecule gives the sugar peroxyl radicals **4.9**, which are transformed into hydroperoxide **4.10** by incorporation of one proton and one electron. If their source is the desoxyribose unit of another DNA molecule, the radical process becomes self-maintained, as shown in Figure 4.8.

One possible degradation pathway for the hydroperoxides that explains some of the products observed in the presence of additional oxygen involves a ring expansion through a modified Criegee rearrangement, where isotope studies with $^{18}O_2$ and $H_2{}^{18}O$ prove that hydroxide is released from **4.10**.[7] The stabilized cation **4.11** resulting from the rearrangement undergoes an elimination reaction to **4.12**, which is subsequently decomposed to the observed fragments **4.13**, **4.16**, and **4.17**. The last two species come from **4.14** and **4.15** by the mechanism shown in Figure 4.9.

Fragments **4.17** are known as base propenals and can serve as precursors to the mutagenic M_1dG adducts previously discussed as arising from MDA, as shown in Figure 4.10.[13]

The liberation of DNA bases in this pathway can be explained by the mechanism shown in Figure 4.11, in which the 4′-radical **4.8** evolves to the oxonium cation **4.18** by one-electron oxidation.

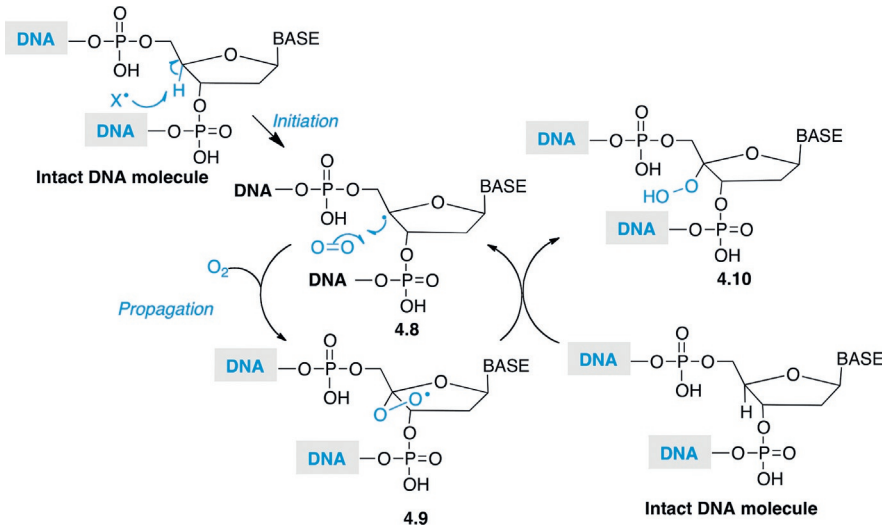


FIGURE 4.8

DNA peroxidation by hydroxyl radicals.

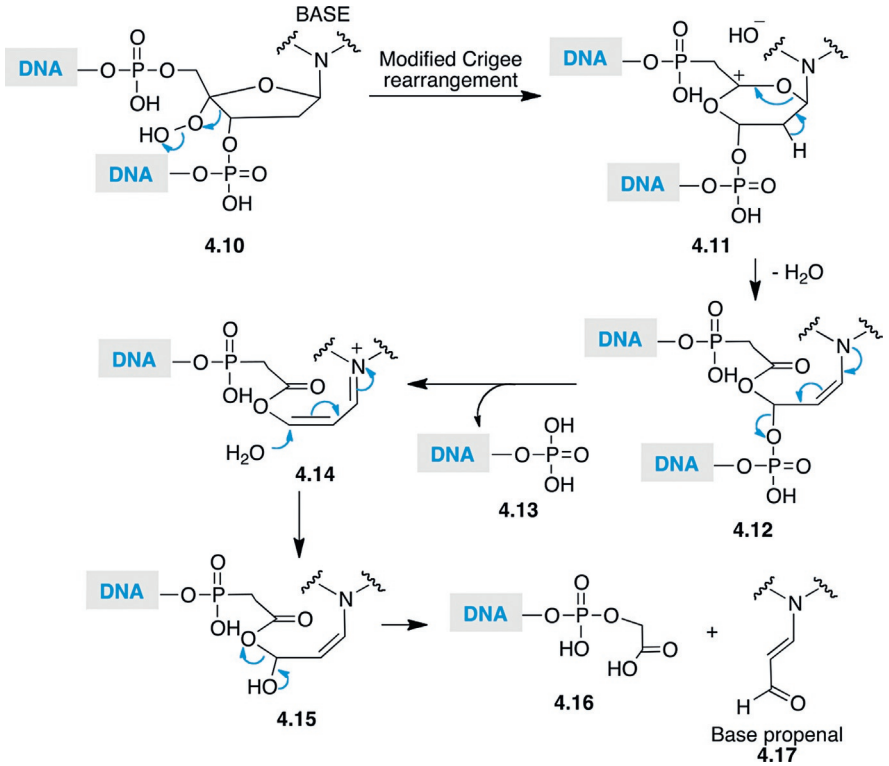


FIGURE 4.9

Further reaction in the degradation of DNA hydroperoxide **4.10**.

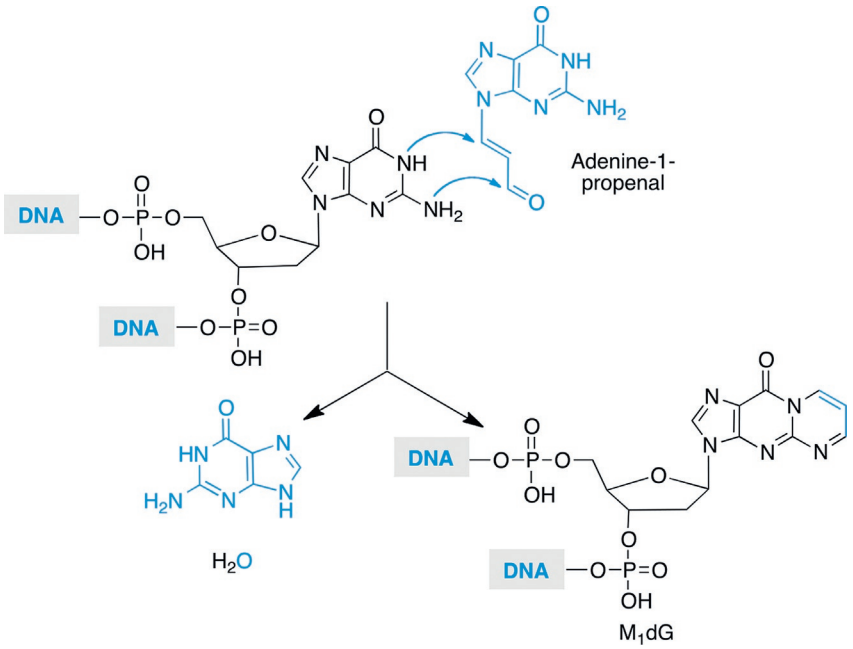


FIGURE 4.10

Generation of M_1dG adducts from base propenals.

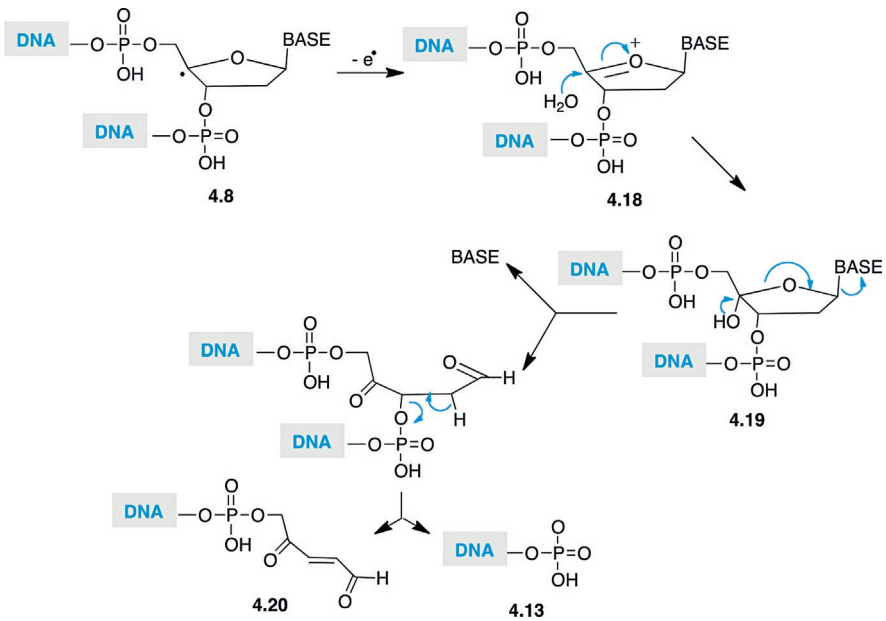


FIGURE 4.11

Scission of DNA bases following attack by radicals.

Nucleophilic attack by a water molecule gives the hydroxy derivative **4.19**, which then decomposes to the free base and finally to fragments **4.13** and **4.20**. This mechanism predominates in oxygen-limited environments.

Interestingly, RNA is less susceptible to oxidative strand cleavage than DNA. This observation can be understood broadly in terms of the oxidative cleavage pathways shown previously. The 2-hydroxy group that distinguishes RNA from DNA has the potential to destabilize cationic intermediates such as those generated following abstraction of both the 1- and 4-hydrogen atoms (see Figure 4.8).

2.4 OXIDATION OF DNA BASES

Attack of hydroxyl radicals to purine or pyrimidine bases produces other DNA damages. The structures of the degradation products arising from this reaction have been established mainly from studies with ionizing radiation,[14] but many of them were similarly isolated from patients receiving anthracyclines for the treatment of breast cancer.[15]

The main site for the reaction of hydroxyl radical with pyrimidines is the 5,6-double bond. For instance, thymine is transformed into the hydroxy hydroperoxides **4.21**, which can be reduced to give thymine glycols (ThyGly) or be degradated to 5′-hydroxyhydantoin (5′-OH-Hyd) through the intermediacy of open intermediate **4.22**. Thymine can also suffer hydrogen abstraction from its methyl group, giving 5-(hydroxymethyl)uracyl (5-OH-MeUra) after coupling with a hydroxyl radical (Figure 4.12).

Among other reactions, hydroxyl radicals can add to the guanine C-8 position to give radical **4.23** that can be reduced by addition of one electron and one proton to the unstable intermediate **4.24**, which finally gives the ring-opened product known as FapyGua. Alternatively, **4.23** can undergo one-electron oxidation to 8-hydroxyguanine (8-OHGua). A very similar process transforms adenine into the ring-opened derivative FapyAde (Figure 4.13).

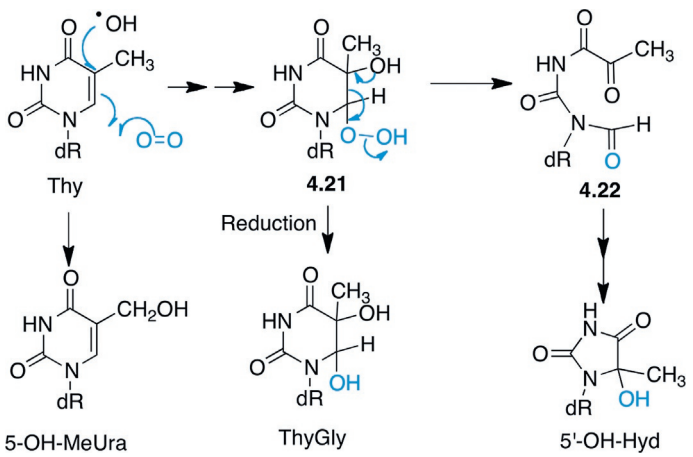


FIGURE 4.12

Oxidation products from thymidine.

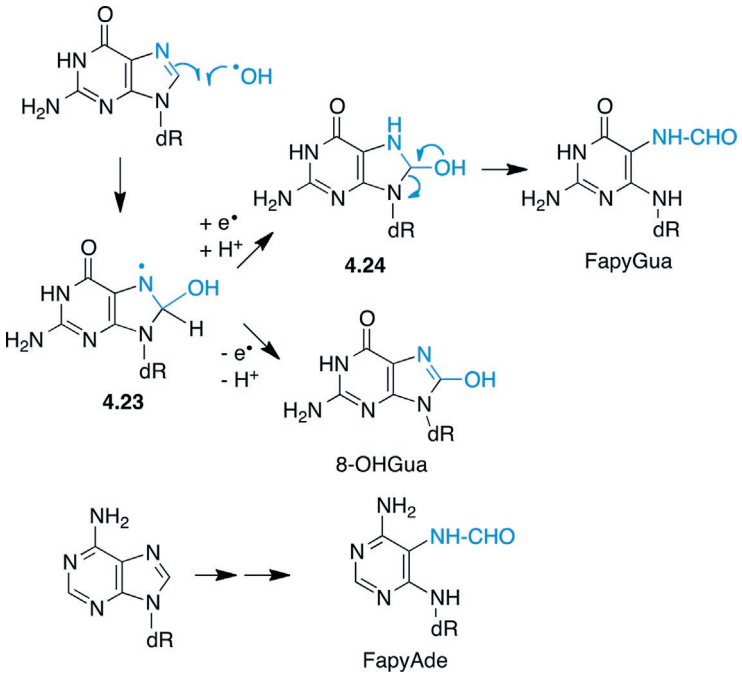


FIGURE 4.13

Oxidation products from purine nucleosides.

The mutagenic potential of some of these degradation products has been clearly established. Thus, FapyGua and ThyGly block DNA replication or increase reading error frequencies by DNA polymerase, resulting in mutations.[16] This polymerase dysfunction may also be due to oxidation-induced conformational changes in DNA. Nuclear proteins can also be attacked by radicals, especially at Tyr residues, and the resulting protein-derived radicals can cross-link to base-derived radicals that interfere with chromatin unfolding and DNA repair, reparation, and transcription.[17] Furthermore, oxidized bases can induce mutations via the generation of mismatched base pairs. For instance, the trinucleotide 2′-deoxy-8-oxoguanosine triphosphate (8-oxo-dGTP), arising from the triphosphorylation of 8-oxo-dG, can be misincorporated into the DNA and, if unrepaired, gives a mismatched pairing with adenine that causes G → T and C → A substitutions in the genome (Figure 4.14). Similarly, FapyGua induces GC → CG transversions.

To counteract oxidative damage in nucleic acids, cells are equipped with several defense mechanisms. In addition to the base excision repair (BER) system, which is initiated by the excision of damaged bases by specific DNA glycosylases (see Chapter 14, Section 4.2), oxidized nucleotides in the nucleotide pools are hydrolyzed by a variety of hydrolases, including MTH1, MUTYH, and OGG1. The protein MTH1 (MutT homolog 1), a member of the nudix hydrolase superfamily that is located in the cytoplasm, mitochondria, and nucleus, hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP, 8-oxo-dATP, and 2-hydroxy (OH)-dATP, to the corresponding monophosphates, thus avoiding their incorporation into DNA. OGG1 is a DNA glycosylase that excises 8-oxoG in DNA

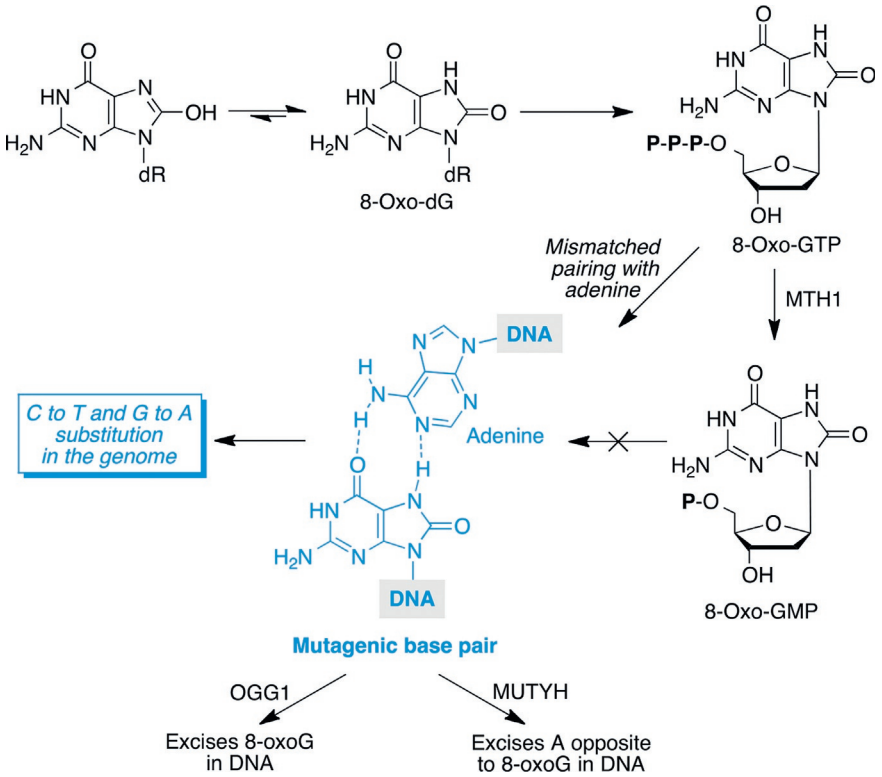


FIGURE 4.14

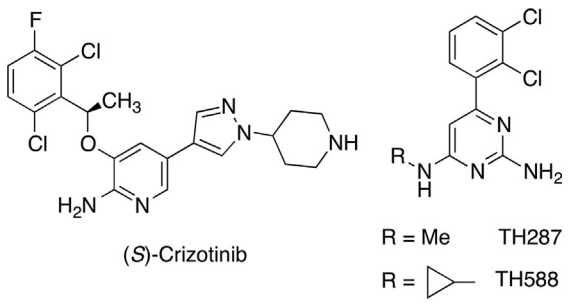
Defense against oxidative damage of nucleic acids by the MTH1 protein.

and thus minimizes the accumulation of 8-oxoG in the cellular genomes. MUTYH is an adenine/2-hydroxyadenine DNA glycosylase that excises adenine opposite 8-oxoG and thus suppresses 8-oxoG-induced mutagenesis (see Figure 4.14). An increased susceptibility to spontaneous carcinogenesis in MTH1-, OGG1-, and MUTYH-deficient mice has been observed.[18]

These defense mechanisms also play an important role in neuroprotection. 8-Oxoguanine is accumulated in nuclear and mitochondrial genomes during aging, and it increases dramatically in nigrostriatal dopaminergic neurons of patients with Parkinson's disease (PD), showing that oxidative damage in nucleic acids is a major risk factor for PD.[19]

These enzymes play important roles in mammalian cells, avoiding an accumulation of oxidative DNA damage, in both nuclear and mitochondrial genomes, and thereby suppressing carcinogenesis and cell death. Nevertheless, they can be regarded as nonessential in normal cells that have a regulated metabolism preventing damage of nucleotide building blocks, whereas some cancer cells require them for survival due to their altered metabolism. In this context, MTH1 has been recently validated as an anticancer target.[20] Interestingly, (*S*)-crizotinib (Xalkori®), an anticancer drug acting as an ALK (anaplastic lymphoma kinase) and ROS1 (c-ros oncogene 1) inhibitor that is discussed in Section 4.10 of Chapter 10, has been shown to be a nanomolar inhibitor of MTH1.[21] Some additional small molecules,

such as TH287 and TH588, have been identified as members of the nudix hydrolase family of inhibitors that potently and selectively inhibit the MTH1 protein.[16]



2.5 FORMALDEHYDE GENERATION

Another consequence of the formation of hydroxyl radicals can be the generation of formaldehyde by reaction with certain cell components such as spermine and lipids. This mechanism seems to be relevant only in the case of some anthracyclines and is discussed in connection with Figure 4.22.

2.6 ROS AS SIGNALING MOLECULES

Besides their role as mediators of oxidative modifications of cell constituents, ROS can also function as signaling molecules, even at very low concentrations.[22] Examples of this type of response are (1) ROS activation of neutral sphingomyelinase leading to ceramide formation and (2) modulation by ROS of several kinases or transcription factors controlling the cell cycle.[23,24] One mechanism by which ROS transmit signals is by oxidation of thiol residues in cysteines of the target proteins to sulfenic acids. This transformation serves as a chemical switch that can either activate or deactivate the protein function.

2.7 OXIDATIVE STRESS INDUCTION AS A STRATEGY IN CANCER TREATMENT

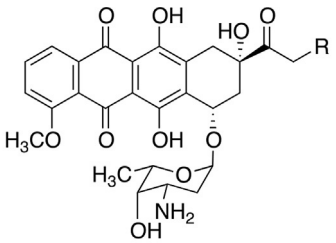
To summarize the previous sections, if the equilibrium between ROS formation and endogenous antioxidant defense mechanisms is disturbed, oxidative stress may be produced, resulting in damage of all the important cellular components (proteins, DNA, and membrane lipids) that can cause cell death. Mutations of the mitochondrial or nuclear DNA that affect components of the mitochondrial respiratory chain result in inefficient ATP production, ROS overproduction, and oxidative damage to mitochondria and other macromolecules, which favor chromosomal instability and carcinogenesis. However, oxidative stress is not always detrimental, and selective oxidative stress can be utilized therapeutically. Numerous drugs are utilized therapeutically that act by this mechanism, as discussed in the remainder of this chapter. New therapeutic strategies that take advantage of increased ROS or inhibition of endogenous antioxidant defense that produce a selective state of oxidative stress in cancer cells are gaining importance.

ROS are constantly being neutralized by antioxidative proteins such as glutathione, superoxide dismutase, catalase, and thioredoxin to prevent irreversible damage to DNA and proteins. The nuclear related factor 2 (Nrf2), a transcription factor that upregulates the synthesis of antioxidant proteins

and of glutathione, is itself upregulated by the cell growth and division-promoting *RAS*, *RAF*, and *MYC* oncogenes. This means that cancer cells that are largely driven by *RAS* and *MYC* oncogenes are among the most difficult to treat due to their high levels of ROS-destroying antioxidants. Cancer stem cells are apparently much more resistant to ROS-induced apoptotic killing than the more differentiated cells.[25]

3 ANTHRACYCLINES AND THEIR ANALOGS

Anthracyclines are a group of antibiotics characterized by the presence of a planar chromophore containing an anthraquinone fragment, attached to an amino sugar. Daunorubicin (DNR, daunomycin, Cerubidine®) and doxorubicin (DOX, Adriamycin®), were isolated from a *Streptomyces* species and were the first anthracycline antibiotics introduced in the clinic for cancer treatment. They are widely used for the treatment of human cancers, and despite its very similar structure, their antitumor spectra of activity differ widely.[26] Thus, DNR is effective in acute lymphocytic and myeloid leukemia, whereas DOX is an essential component of the chemotherapy of a large number of solid tumors, including breast cancer, childhood solid tumors, soft tissue sarcomas, and aggressive lymphomas. Despite their longstanding clinical utilization, their mechanism of action is still unclear and subject to controversy.[2,27] We deal here with the mechanisms related to the generation of radical species, whereas some other mechanisms (intercalation into DNA and consequent inhibition of macromolecular biosynthesis and inhibition of topoisomerase II), are studied in Section 6 of Chapter 7.



R = OH Doxorubicin (adriamycin)
R = H Daunorubicin (daunomycin)

The main drawback of anthracyclines is their ability to cause chronic cardiomyopathy, which is related to damages associated with ROS generation and consequent apoptosis induction. These damages are especially important in cardiac tissue because of the low levels of catalase and the easy inhibition of cardiac selenium-dependent glutathione peroxidase by the anthracyclines, both enzymes being key in the detoxification of hydrogen peroxide.[28] Apoptosis induction in cardiac tissue proceeds through activation of NF-κB. This is opposite to what is observed in cancer cells, in which NF-κB activation usually inhibits apoptosis induced by antracyclines; this action is still not fully understood.

The production of radical species by quinone-containing antibiotics was first demonstrated in 1975, and 2 years later, doxorubicin and daunorubicin were shown to generate free radicals through redox cycling.[29] Because of their ability to bind to nucleic acids, these drugs can be considered as site-specific free radical generators.

From a chemical standpoint, the generation of radicals from quinones is based on the captodative effect. Whereas cations are stabilized by electron-releasing groups and anions by electron-withdrawing groups, radicals are best stabilized by the simultaneous presence of both types of substituents

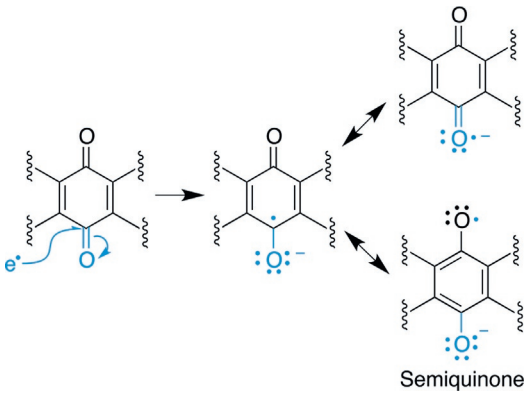


FIGURE 4.15

Captodative stabilization of semiquinone radicals.

(captodative effect). In the case of quinones, the ease of formation of the so-called semiquinone radicals by one-electron reduction is due to their stabilization through the captodative effect of the electron-releasing negatively charged oxygen atom and the electron-withdrawing carbonyl groups (Figure 4.15).

The reversibility of semiquinone formation allows these radicals to induce one-electron reduction of oxygen molecules to superoxide anions, leading to an overall increase in the electron flow to oxygen derived from the activity of enzymes such NADPH dehydrogenase, xanthine dehydrogenase, and the reductase domain of nitric oxide synthase (Figure 4.16).[30]

A competitive reaction of semiquinone **4.25** can take place, involving loss of daunosamine. Thus, two molecules of **4.25** can disproportionate to give the starting quinone and hydroquinone **4.26**, which is unstable and evolves by elimination of the sugar moiety to give the anthracycline aglycon (Figure 4.17).[31] Because of their relatively high lipophillicity with regard to the glycosides, these

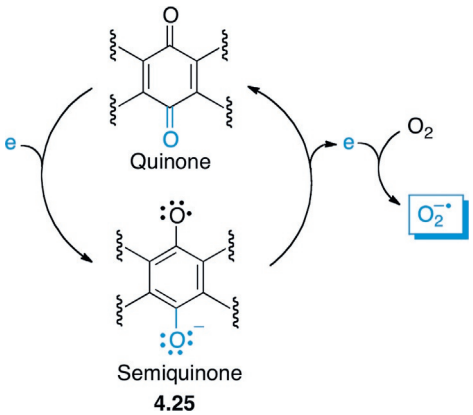


FIGURE 4.16

Electron flow from semiquinone radicals to oxygen molecules.

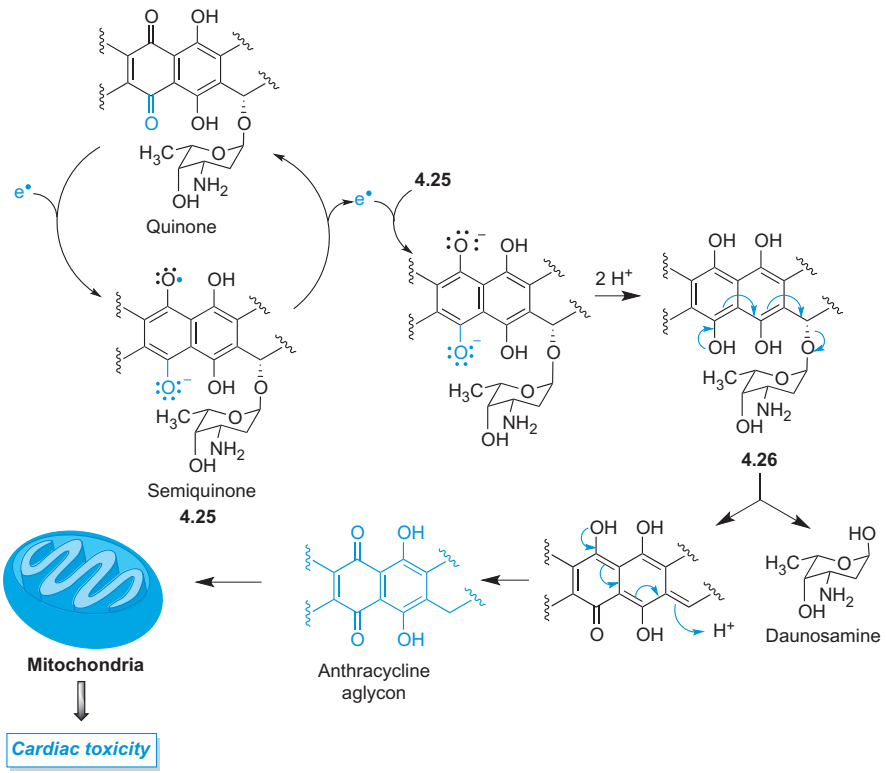


FIGURE 4.17

Elimination of the sugar moiety from anthracyclines and its role in anthracycline cardiotoxicity.

aglycons tend to accumulate in the inner mitochondrial membrane. The oxidative deterioration of mitochondrial functions due to the formation of radicals from these aglycons is one of the factors responsible for the cardiomyopathy associated with the use of anthacyclines.[20]

Another important chemical property of the anthracyclines relevant to their antitumor activity is their chelating ability, due to the presence of β-hydroxycarbonyl moieties in their structure, especially at the C-11 and C-12 positions.[32] Probably due to ionic interactions with the phosphate groups, the anthracycline–Fe^{3+} chelate binds to DNA much more tightly than the anthracycline itself and can then generate Fe^{2+} by reaction with superoxide anion. As previously mentioned, Fe^{2+} cations thus generated *in situ* can form hydroxyl radicals through their Fenton reaction with hydrogen peroxide (Figure 4.18). The high efficiency of DNA fragmentation by these hydroxyl radicals is reflected in the routine use of the Fenton reaction in DNA footprinting, a technique that fragments DNA indiscriminately and allows the determination of where DNA–protein interactions take place.[33]

Anthracyclines also induce a severe dysregulation of iron homeostasis, possibly mediated by the release of iron from intracellular stores. This helps to explain why the Fenton reaction takes place despite the fact that cells normally have very little or no free iron available,[34] and it is also very important in explaining the cumulative cardiotoxicity of the anthracyclines. The main target responsible for this dysregulation of iron homeostasis by the anthracyclines seems to be aconitase, a Krebs cycle enzyme

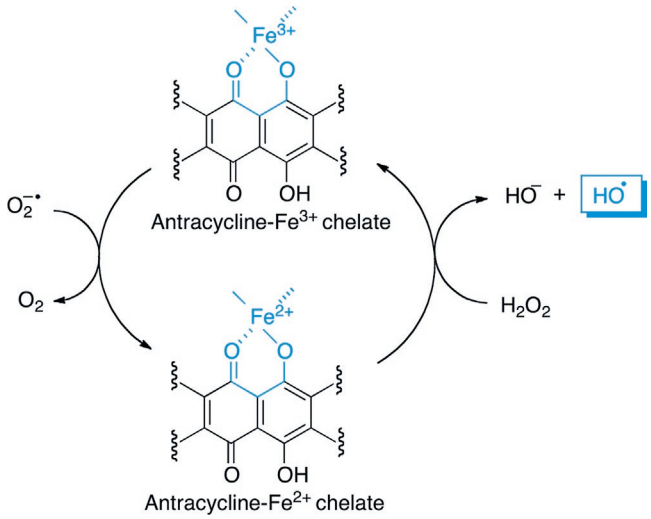


FIGURE 4.18

Anthracycline-mediated Fenton reaction.

that reversibly isomerizes citrate to isocitrate and is characterized by a catalytic [4Fe–4S] cluster. The anthracycline-mediated release of one of the four Fe atoms from this cluster leads to loss of aconitase activity and converts the enzyme into an iron regulatory protein called IRP-1. This protein has a high affinity for mRNAs corresponding to transferrin receptor and ferritin, an iron storage protein, resulting in an increased synthesis of the former and decreased synthesis of the latter. The overall effect leads to an increase of iron uptake upon iron sequestration and, therefore, to an increase in available iron (Figure 4.19).

Regarding the mechanism of anthracycline-mediated loss of iron from the aconitase [4Fe–4S] cluster, it has been shown that the secondary alcohols doxorubicinol (DOXol) and daunorubicinol (DNRol); Figure 4.19 derived from two-electron reduction of the C-13 carbonyl in anthracyclines by NADPH-dependent cytoplasmic reductases are more reactive than superoxide or hydrogen peroxide toward artificially generated mimics of the cluster.[35] Further research using intact tumor cells has shown, however, that the effects of anthracyclines on IRP–RNA binding activity are not due to DOXol or free generation of free radicals but, rather, to formation of Fe complexes with DOX.[36]

In summary, whereas the oxidant activity of anthracycline aglycons seems to be responsible for the acute toxicity of anthracyclines, the alterations in iron homeostasis have been proposed to be responsible for their life-threatening chronic toxicity.[37]

Because ROS-associated toxicity is iron dependent, the association of anthracyclines with chelating agents such as dexrazoxane (ICRF-159) prevents anthracycline-induced cardiotoxicity without seriously compromising antitumor activity. This compound is a prodrug that can enter the cells easily and is then hydrolyzed in two stages to give the iron chelator ADR-925, an EDTA analog (Figure 4.20).[38] After its approval by the U.S. Food and Drug Administration (FDA) and other regulating agencies for patients receiving anthracyclines and its introduction in the clinic,[39] it has been proven that, in addition to their cardioprotecting activity, dexrazoxane and other bis(dioxopiperazines) have antitumor activity in themselves, due to topoisomerase II inhibition (see Chapter 7, Section 7.3).[40]

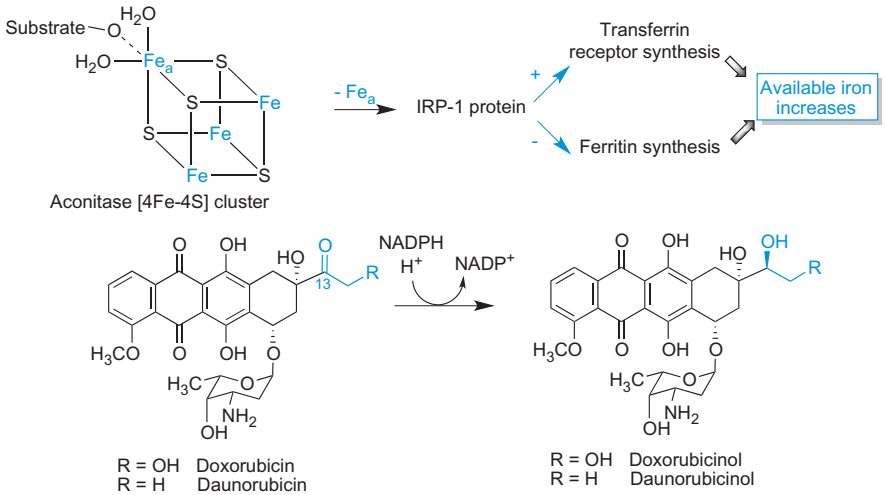


FIGURE 4.19

Effects of anthracyclines on iron regulation and biosynthesis of doxorubicinol and daunorubicinol.

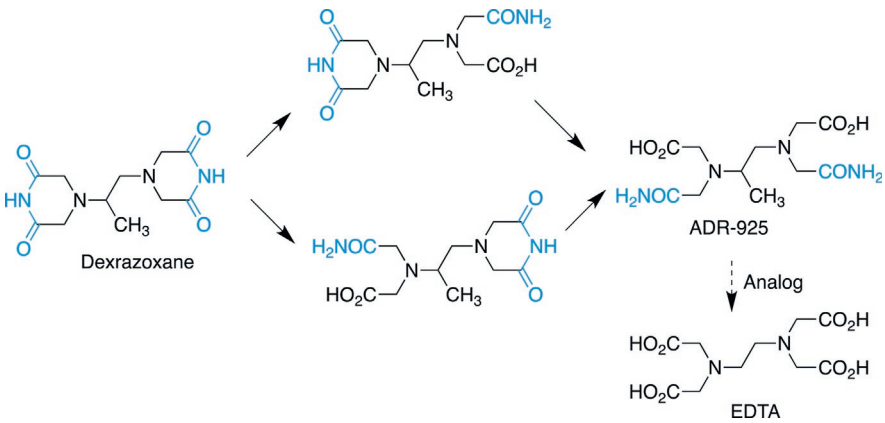


FIGURE 4.20

Bioactivation of dexrazoxane.

Anthracyclines have been long known to form unstable drug–DNA cross-links at 5′-GC-3′ sequences after redox activation in the presence of iron, leading to transcription blockade.[41] Kinetic studies showed the presence of two bonds with different half-lives, and a model was proposed involving a more labile covalent bond to an isolated G base on one strand of DNA and a less labile one involving cross-linking of both strands by the drug aglycon.[42] However, during studies of the less labile complex, negative ion electrospray mass spectrometric studies showed the presence of an additional carbon atom for each cross-linked drug molecule, the source of the extra carbon being a molecule of formaldehyde generated from the Tris buffer employed in the experiments. These mass spectral data were consistent with the X-ray structure of a complex formed from $(CG)_3$, daunorubicin, and formaldehyde, which in

this case was present as an impurity of the crystallization solvent.[43] Further experiments proved that several biomolecules such as spermine and lipids are able to yield formaldehyde in the presence of anthracycline-induced ROS,[44] which provided a link between these *in vitro* experiments and the mode of action of the anthracyclines *in vivo*. It was shown later that daunorubycin and doxorubicin react with formaldehyde to yield dimeric oxazolidine structures (doxoform (DOXF) and daunoform (DAUF) in Figure 4.21) that would liberate by hydrolysis the monomeric structures **4.27** predicted to be the active metabolites of the anthracyclines. Coadministration of doxorubicin and known formaldehyde precursors such as pivaloyloxymethyl butyrate or hexamethylenetetraamine (HMTA) increases the

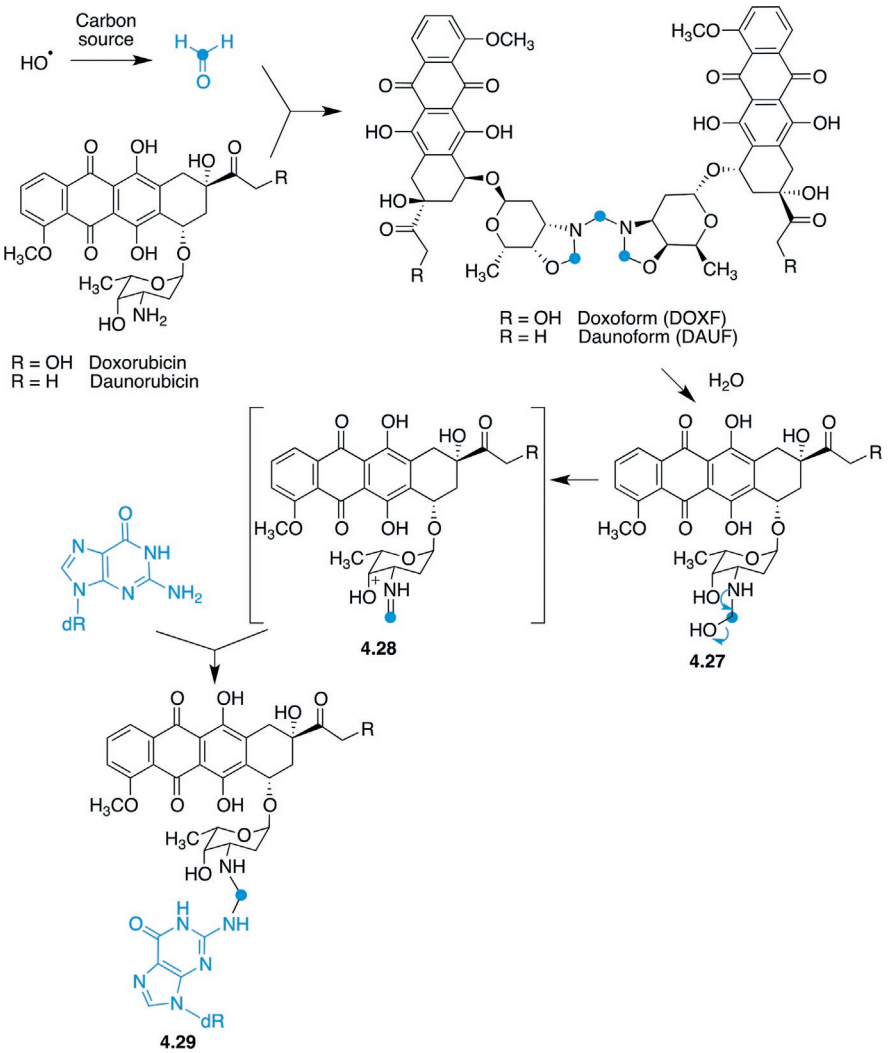


FIGURE 4.21

Generation of formaldehyde adducts of anthracyclines.

levels of anthracycline–DNA adducts, which can be considered another proof of the role of formaldehyde in this process.[45]

Formaldehyde adducts such as DAUF and DOXF are responsible for the formation of a covalent bond with the 2-amino group of a guanine in the DNA minor groove through the formation of an intermediate Schiff base **4.28**. The formation of species **4.29** by this mechanism is accompanied by intercalation of the anthracycline aglycon, as shown in Figure 4.21. Formation of hydrogen bonds with the G base in the opposite strands completes the formation of the anthracyclin–DNA complex (Figure 4.22). This combination of intercalation, covalent bond, and hydrogen bonding is known as *virtual cross-linking*.

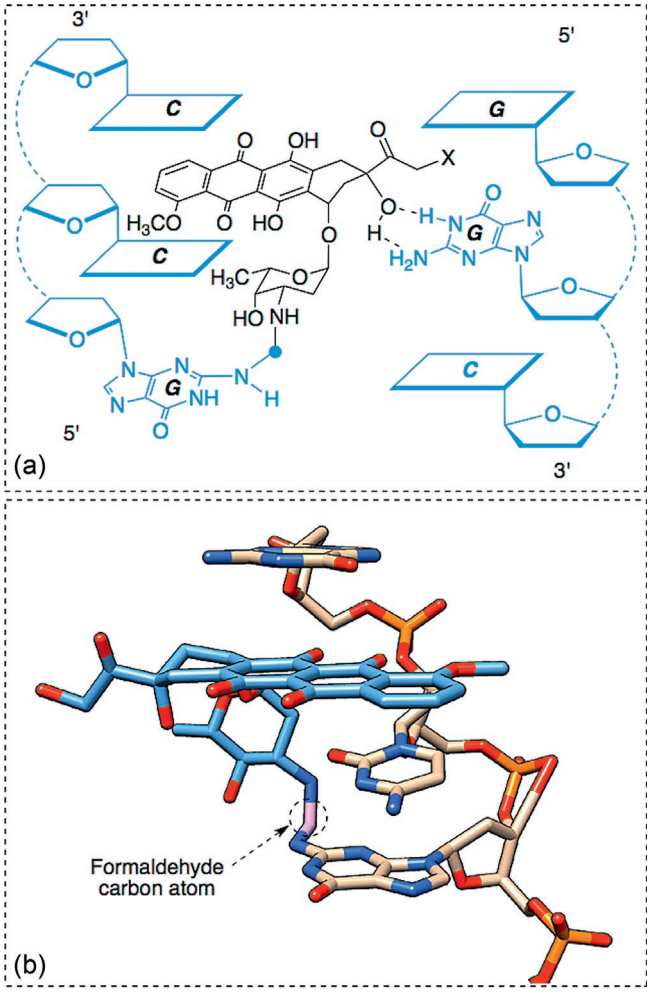
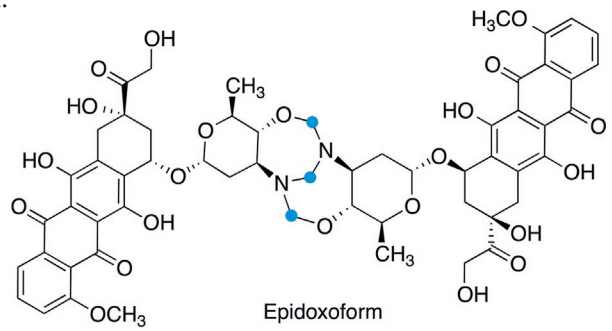


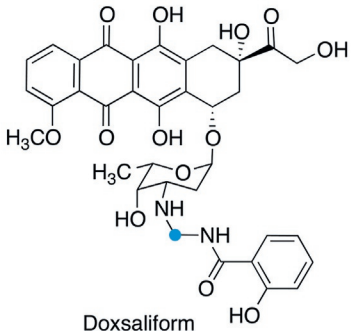
FIGURE 4.22

Interaction of anthracycline–formaldehyde adducts with DNA. The three-dimensional structure was generated from Protein Data Bank reference 1QDA and displayed with Chimera 1.8.1.

The existence of this mechanism has prompted the synthesis of anthracycline–formaldehyde conjugates as novel drugs potentially less cardiotoxic and active than the parent compounds against resistant cancer cells.[46,47] In addition to the previously mentioned doxoform and daunoform, which can be considered as anthracycline prodrugs and which are more active than the parent drugs in some cell lines, other important examples of alkylating anthracyclines belonging to this family include epidoxoform and doxsaliform.



Epidoxoform

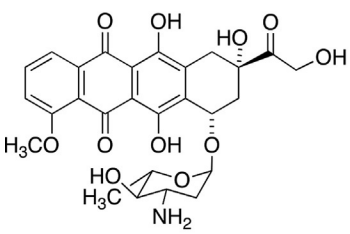


Doxsaliform

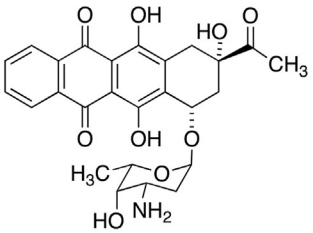
Among other cardiovascular effects,[48] anthracyclines are thought to be modulators of angiogenesis, a key process in tumor growth and metastasis, inducing a breakdown of tumor vasculature. This is partly due to their effects on NO production by inhibition of endothelial NO synthase and inhibition of the expression of inducible NO synthase.[49]

Because anthracyclines are among the most widely employed antitumor drugs, intensive research has been performed during the past two decades trying to find a "better anthracycline" lacking cumulative cardiotoxicity and susceptibility to cell efflux pumps responsible for resistance, such as P-gp (see Chapter 14), but all studies indicate that this goal is yet to be achieved. More than 300 new compounds have been discovered through biosynthetic studies, and more than 2000 analogs have been obtained from structural modifications of natural compounds or from total synthesis, but only a few of them have been submitted to clinical studies and even fewer have reached approval.[50,51] Among them, epirubicin (EPI, Ellence®)[52] and idarubicin (IDA, Idamycin®)[53] can be mentioned as useful alternatives to DOX or DNR, respectively. Epirubicin is an epimer of DOX at the daunosamine C(4′) position that induces pharmacokinetic and metabolic changes related to the increased 4′-*O*-glucuronidation and increased elimination. Despite this finding, clinical studies have shown that replacing DOX with EPI does not eliminate the risk of chronic cardiotoxicity. IDA, an analog of DNR obtained by removal of the methoxy group, has a broader spectrum of activity. This is probably related to its increased

lipophilicity, which facilitates the cellular uptake and contributes to stabilize the ternary complex that forms the drug with DNA and topoisomerase II (see Chapter 7). The last effect is important because a major mechanism of anthracycline activity depends on formation of this complex. However, the cardiac safety of IDA has not been clearly established.

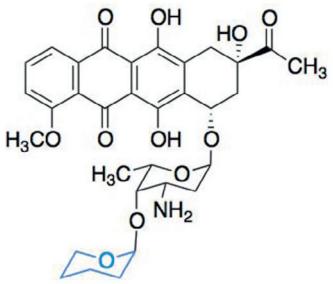


Epirubicin

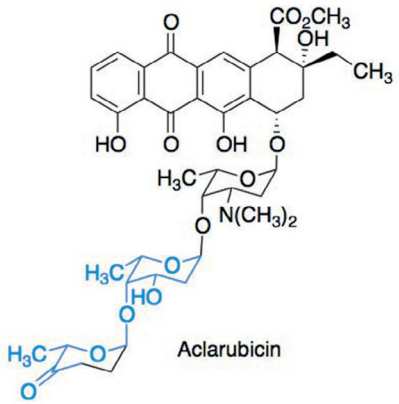


Idarubicin

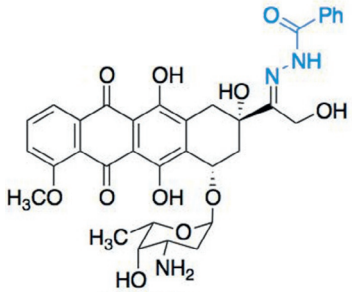
Pirarubicin, a 4′-tetrahydropyranyl doxorubicin,[54] and aclarubicin (aclacinomycin A), a trisaccharide anthracycline,[55] showed only modest improvement over DOX and DNR in terms of drug resistance without relevant cardiotoxic safety. Zorubicin, the benzoylhydrazone of daunorubicin,[56] and valrubicin (Valstar®)[57] are additional anthracyclines that have undergone advanced clinical testing or reached the market. The latter compound is indicated for *in situ* intravesical therapy[58] of BCG-refractory carcinomas of the urinary bladder.[59]



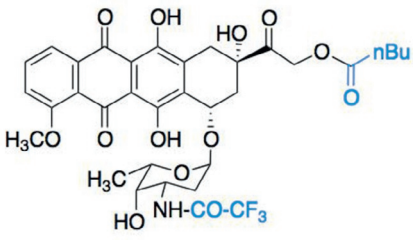
Pirarubicin



Aclarubicin

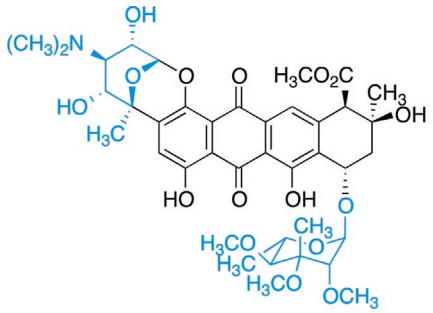


Zorubicin

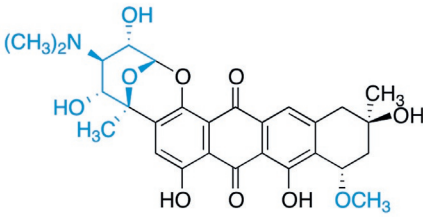


Valrubicin

Among other analogs that have reached clinical trials, we mention nogalamycin, which was first isolated in 1968 and featured a second amino sugar molecule attached to the D-ring through a glycosidic linkage and a C–C– bond. This compound was later modified to give menogaril, where the first amino sugar structural fragment was replaced by a methoxy group (TUT-7).[60] Menogaril is active against several human lymphomas and has been advanced to phase II clinical trials in patients with previously treated multiple myeloma or chronic lymphocytic leukemia.[61]

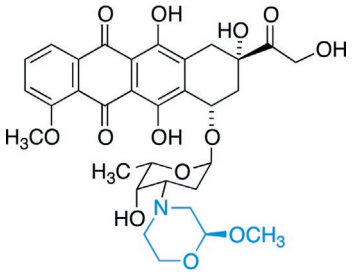


Nogalamycin



Menogaril

Another interesting compound is nemorubicin,[62] a doxorubicin analog bearing a 2(*S*)-methoxy-4-morpholinyl chain that differs from the parent compound in many respects, including its spectrum of antitumor activity, pharmacokinetics, metabolism, and toxicity. Its characteristic methoxymorpholinyl group is responsible for a high lipophilicity that leads to improved cell penetration and higher intracellular levels in comparison to those achieved with doxorubicin. Faster cell extravascular diffusion and cell uptake and low cardiotoxic effects have also been claimed for this compound,[63] which is in phase III clinical studies.



Nemorubicin (MMRA)

Nemorubicin is extremely potent *in vivo*, reaching similar effects as doxorubicin with concentrations approximately 100 times lower. The fact that *in vitro* experiments did not show this major difference in potency suggests that nemorubicin is transformed into a very potent metabolite. This compound was eventually identified as PNU-159682, arising from the oxidative cyclization of the amino sugar fragment of nemorubicin by a single isoform of CYP3A (Figure 4.23).[64] Interestingly, it has been shown that nemorubicin exerts its antitumor action through a mechanism different from that of the other anthracyclines, involving the inhibition of the DNA nucleotide excision repair (NER) mechanism (see Chapter 7, Section 9).

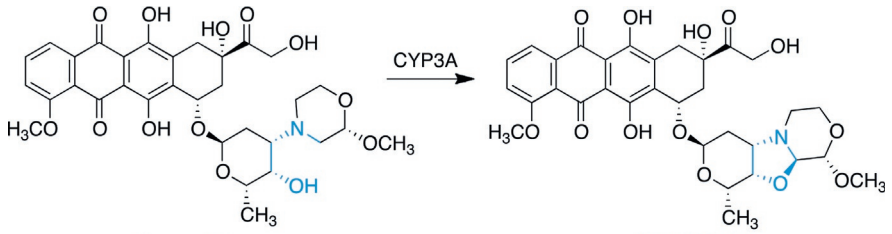
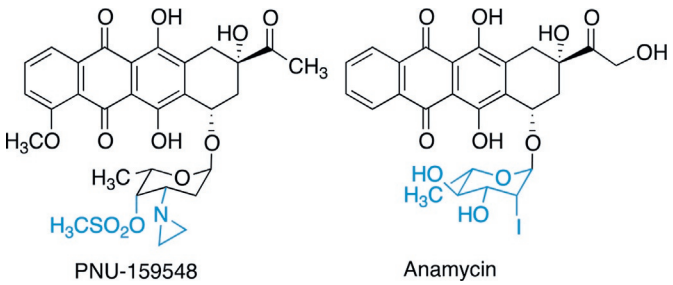


FIGURE 4.23

Bioactivation of nemorubicin into PNU-159682.

Based on the concept of drug hybridization, several anthracycline-based molecules with both DNA alkylating and intercalation properties, such as PNU-159548, a 4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulfonyl daunorubicin,[65] have been studied. Due to its high lipophilicity, PNU-159548 crosses the blood–brain barrier and is effective against intracranial tumors. Another approach for designing more effective anthracyclines was directed at the preparation of less basic compounds having a more stable glycosidic bond. One of them is anamycin,[66] which, after having been incorporated into liposomes, went into clinical trials. To increase the topoisomerase II-mediated DNA cleavage, some groups have prepared 8- and 10-fluoroderivatives.[67] Anthracyclines that have been designed primarily as topoisomerase inhibitors (e.g., sabarubicin) are discussed in Chapter 7.



Finally, note that drug carrier technology, implying either specific recognition or simply preferential drug distribution, has been widely employed for targeting anthracyclines to tumors in the past several years, as discussed in Chapter 13.

4 MITOXANTRONE AND RELATED QUINONES

Mitoxantrone, an anthraquinone derivative bearing polyamine side chains, can be considered as a partial analog of the anthracyclines, including the hydroxyquinone function but not the amino sugar fragment. This compound was obtained as an analog of ametantrone, which was initially prepared as a component of ballpoint pen ink, but a routine screening by NCI led to recognition of its antitumor activity. The reasoning that led to the design of mitoxantrone[68] was based on the observation that

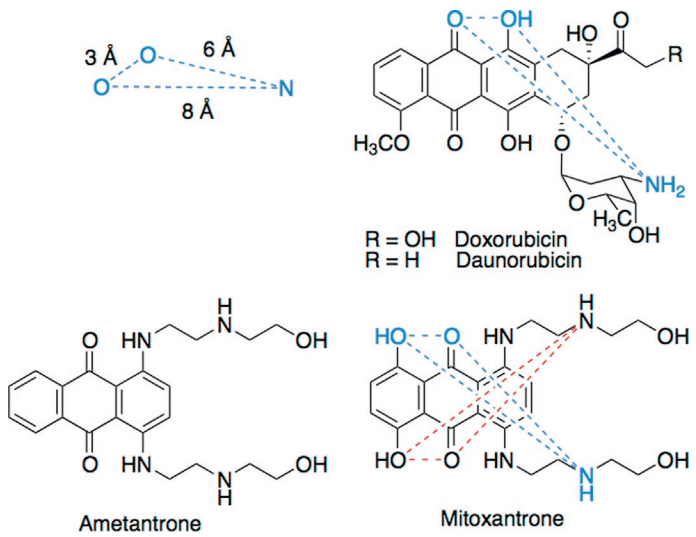


FIGURE 4.24

Similarities between the anthracyclines, ametantrone, and mitoxantrone.

a large number of antileukemic agents shared a common N–O–O triangular pharmacophore, which was also present in the anthracyclines and involved the daunosamine amino group (Figure 4.24). The introduction of the two phenolic hydroxy groups in ametantrone allowed to envision two sets of N–O–O triangles and had the advantage of allowing the elimination of the daunosamine amino group, which was considered to have some influence in the cardiotoxicity of the anthracyclines.[69]

Mitoxantrone is active in breast cancer, acute promyelocytic or myelogenous leukemias, and androgen-independent prostate cancer. Although early reports seemed to indicate that its cardiotoxicity was lower than that of the anthracyclines,[70] this claim has been subsequently challenged.[71] Mitoxantrone has been approved for treatment of secondary progressive multiple sclerosis (MS).[72] The rationale for this application stems from the fact that MS is considered to be an autoimmune disease in which a heightened immune action results in the destruction of the myelin of the central nervous system, causing nerve impulses to be slowed or halted and leading to the symptoms of MS. Because chemotherapeutic agents diminish the numbers of white blood cells, it should slow down or halt this autoimmune destruction.

The mechanism of action of mitoxantrone has not been fully elucidated. As discussed in Chapter 7, this drug is a classic intercalating agent that acts as a topoisomerase II poison. Mitoxantrone can also be oxidatively activated to bind DNA; although the mechanism and binding properties have not been resolved, peroxidase-mediated free radical formation suggested that a mitoxantrone reactive intermediate may be involved in the observed DNA strand damage.[73] Later, it was found that mitoxantrone can be activated by formaldehyde and is able, like adriamycin, to form adducts that stabilize double-stranded DNA, blocking the progression of RNA polymerase during transcription and producing truncated RNA transcripts.[74] This explains why mitoxantrone is particularly active in myeloid tumors, which are known to have increased levels of formaldehyde, formed from

spermine and other polyamines by neutrophile-generated ROS.[75] Although mitoxantrone can be reductively activated to a semiquinone free radical, this process has a low efficiency and the compound undergoes less redox cycling *in vitro* than the anthracyclines.[76] The formation of adducts of formaldehyde-activated mitoxantrone occurs preferently at CpG and CpA sequences, and it is stimulated by cytosine methylation.[77] Thus, the reaction of mitoxantrone with formaldehyde leads to the hydroxymethyl derivative **4.30**, which forms a covalent bond with a guanine amino group to give the covalent adduct **4.32**, presumably through iminium cation **4.31** as an intermediate. The involvement of a single covalent bond has been proved by mass spectrometry, and further stabilization of the complex by hydrogen bonding has been suggested on the basis of molecular modeling studies (Figure 4.25).[78] PIM1 kinase has been identified as a new target for mitoxanthrone that might contribute to its anticancer activity.[79]

Heteroanalogs of mitoxantrone, such as pixantrone, act primarily as topoisomerase II inhibitors and are discussed in Chapter 7.

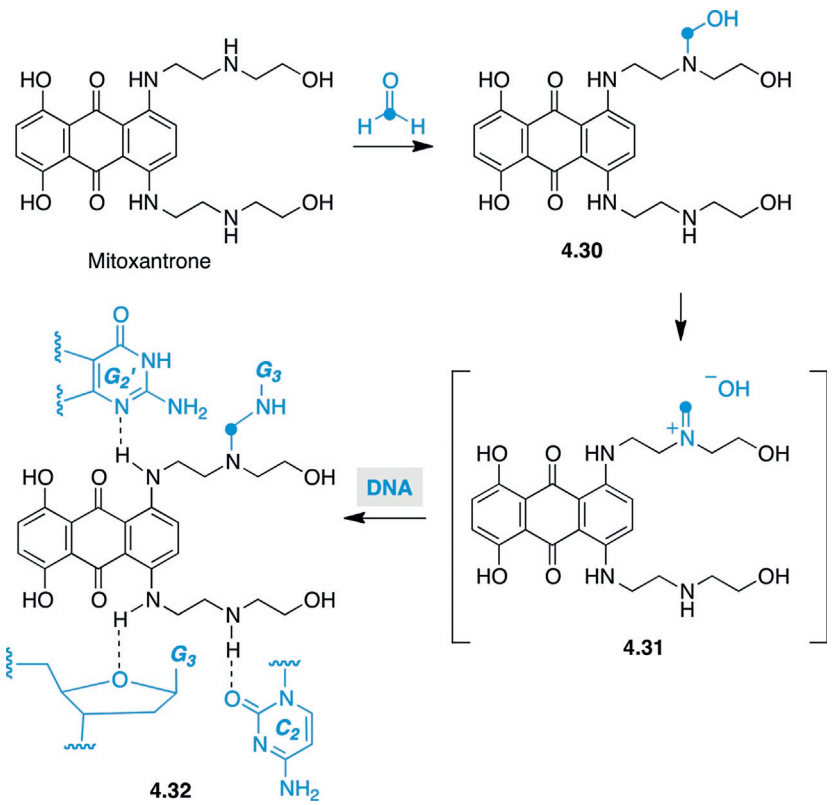
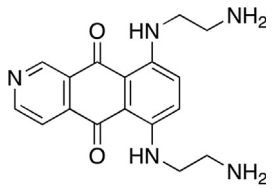


FIGURE 4.25

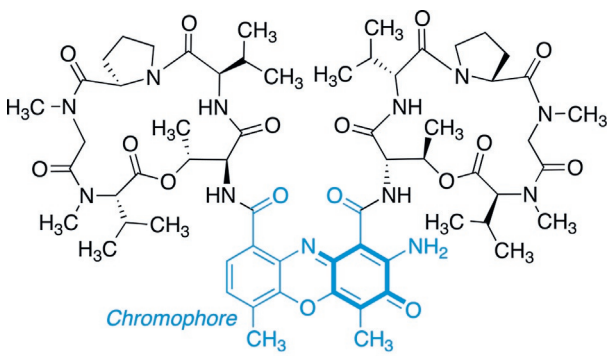
Adducts of DNA with formaldehyde-activated mitoxantrone.



Pixantrone

5 ACTINOMYCIN D

Actinomycin D (dactinomycin, Cosmogen®) is a natural chromopeptide composed of a heterocyclic chromophore and two cyclic pentapeptide lactone rings. The heterocyclic fragment is a phenoxazine derivative, containing a quinonimine portion, and is responsible for the color of the compound and its intercalative ability. Actinomycin D, one of the oldest anticancer drugs and the first antibiotic that showed anticancer activity, is administered intravenously and used in the treatment of gestational trophoblastic disease, Wilm's tumor, rhabdomyosarcoma, Ewing's sarcoma, and others.



Actinomycin D

The ability of actinomycin D to generate superoxide radicals was first reported in 1978,[80] and it is probably due to the mechanism summarized in Figure 4.26. However, the clinical relevance of this mechanism is very doubtful because the concentration required to generate free radicals is approximately 10^{-4} M, whereas actinomycin concentrations as low as 10^{-8} M are sufficient to inhibit RNA transcription.[81] For further discussion of DNA intercalation by the actinomycins, see Chapter 7, Section 2.2.

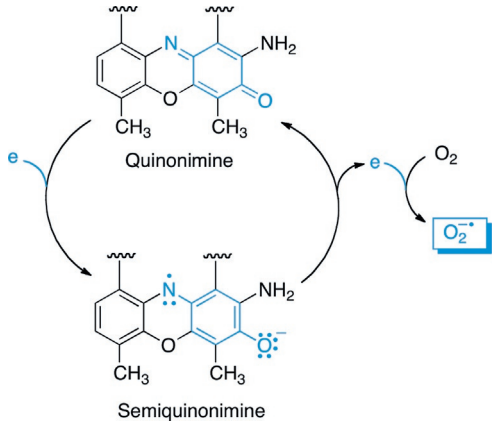
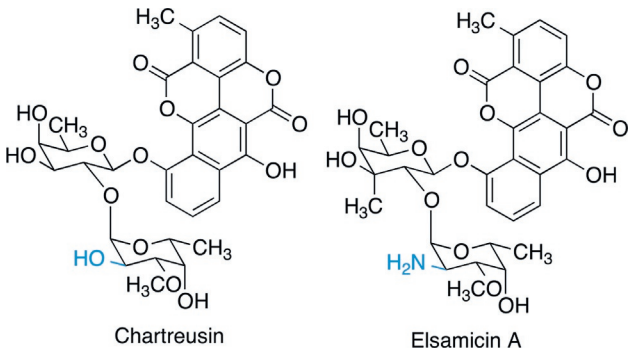


FIGURE 4.26

Generation of superoxide radicals from the actinomycin D chromophore.

6 CHARTREUSIN, ELSAMICIN A, AND RELATED COMPOUNDS

Chartreusin and elsamicin A are structurally related antibiotics with antitumor activity.



Chartreusin (NSC 5159) and elsamicin A cause single-strand scission of DNA in the presence of reducing agents via the formation of free radicals. Electron spin resonance spin-trapping experiments showed that the elsamicin A–iron complex produces hydroperoxyl radicals in the presence of dithiothreitol as reducing agent.[82] The most likely mechanism involves reduction of either carbonyl group followed by reoxidation by oxygen (Figure 4.27).

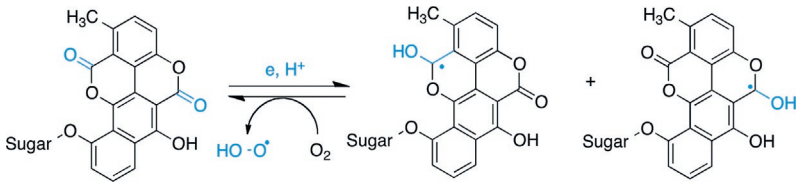


FIGURE 4.27

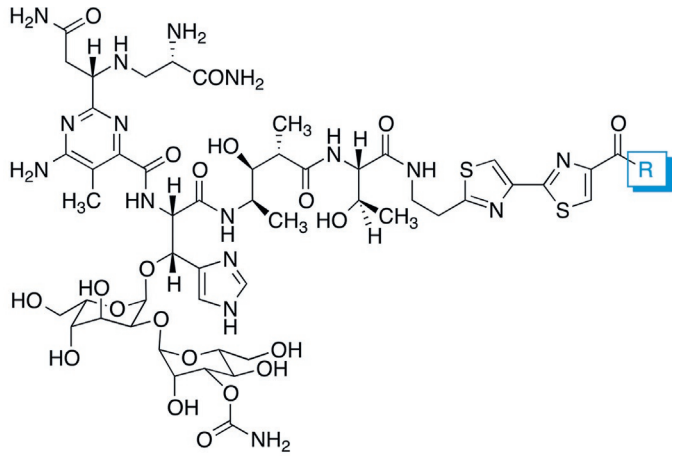
Generation of peroxide radicals by chartreusin and elsamicin A.

Because elsamicin A is an extremely potent inhibitor of topoisomerase II, these compounds are further discussed in Chapter 7.

7 BLEOMYCINS

The bleomycins are a family of natural glycopeptidic antibiotics produced by *Streptomyces verticillus* with clinical efficacy against several types of tumors, especially squamous cell carcinomas, testicular carcinoma, and malignant limphomas.[83] The anticancer drug blenoxane is a mixture of compounds, consisting primarily of the bleomycins A_2 (~60%) and B_2 (~30%). Bleomycins differ from other chemotherapeutic agents in that they produce very little bone marrow depression and are routinely used in cancer chemotherapy, mostly in combination with radiotherapy or other chemotherapeutic agents. Their most serious side effect is a dose-dependent induction of interstitial pneumonitis in approximately 45% of patients, with 3% developing fatal lung fibrosis;[84] this lung toxicity is probably unrelated to their toxicity to tumor cells. Bleomycin A_2 is the most thoroughly studied of the DNA-cleaving reagents.

The structure of the bleomycins is complex and is shown here. A large number of semisynthetic bleomycins, most notably BAPP and liblomycin, have been prepared by addition of alkylamines to the fermentation media.[85]



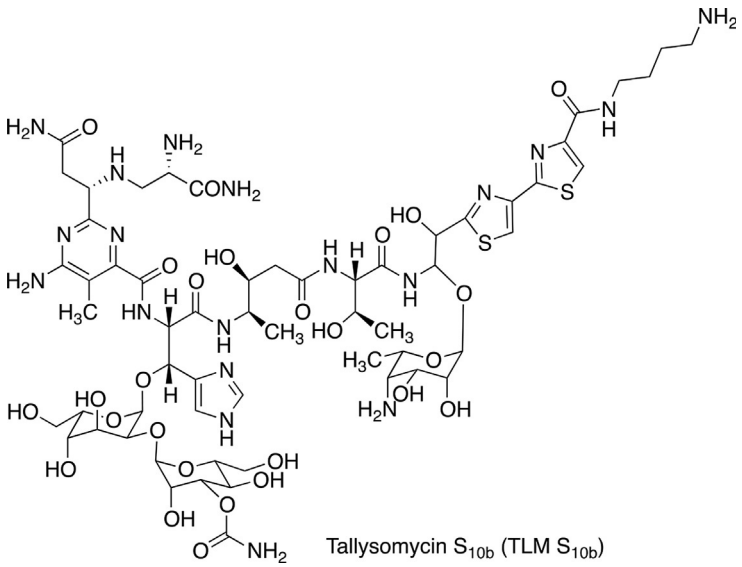
Bleomycin A_2: R = HN $\overset{+}{S}(CH_3)_2$ X^-

Bleomycin B_2: R = HN NH$_2$ NH

BAPP: R = HN N H N H CH_3

Liblomycin: R = HN N CH_3 N OBn OBn BnO OBn

Tallysomycin S_{10b} (TLM S_{10b}) is another member of the bleomycin family that has reached clinical trials in patients with advanced head and neck tumors, showing a response similar to that of bleomycin A2.[86] Its high toxicity prompted the development of immunoconjugates for intracellular targeting (see Chapter 13, Section 4.6).



Tallysomycin S_{10b} (TLM S_{10b})

The bleomycins require a reduced transition metal, Fe(II) or perhaps Cu(I), oxygen, and a one-electron reductant to generate an "activated" bleomycin. The primary mechanism of action of the bleomycins is the generation of single- and double-strand DNA breaks and is initiated by the abstraction of a deoxyribose 4′-hydrogen. The species directly responsible for the removal of this hydrogen atom is an "activated" bleomycin complex **4.35**, arising from one-electron reduction of the bleomycin–Fe(II)–oxygen ternary complex. The electron may come from external reductors such as ascorbic acid or thiols or from another molecule of **4.34**, which would then be transformed into an inactive Fe(III) species **4.36**. Reaction of **4.35** with DNA involves abstraction of the ribose 4′-hydrogen and proceeds as previously discussed (see Figure 4.8). Finally, **4.33** can be regenerated from **4.36** by an NADH-dependent enzyme system in the nucleus or by reduction of external thiols, creating a cyclic process. This redox cycling (Figure 4.28) is important for bleomycin activity because only very small amounts of the drug enter the tumor cells due to its low lipophilicity.

A mechanism explaining the chemical details of the activation of the bleomycin ternary complex is shown in Figure 4.29. Addition of one electron and one proton to the bleomycin–Fe(II)–oxygen ternary complex **4.34** gives an Fe(III) hydroperoxo complex **4.37**, which has been detected experimentally by a variety of techniques.[87] One possible mechanism explaining the formation of the activated bleomycin species **4.35**, which is analogous to the one postulated for the case of heme-dependent enzymes such as cytochrome P450, involves the heterolytic cleavage of the O–O bond, initiated by a protonation step. This reaction gives a bleomycin–Fe(V)=O species **4.35** or its alternative Fe(IV) resonating form, which can abstract a hydrogen atom from DNA, initiating the series of events that culminate in strand cleavage.

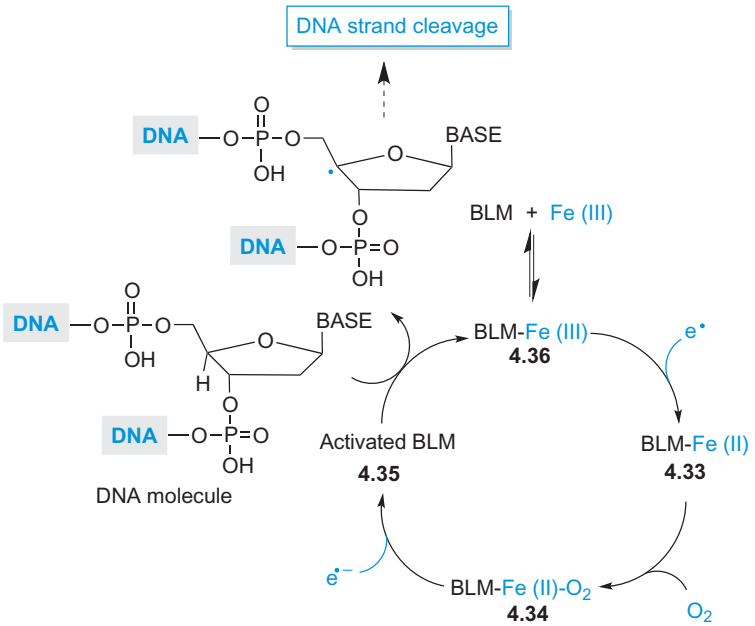


FIGURE 4.28

Redox cycling of bleomycin.

Alternatively, the O–O bond in **4.37** could be homolytically cleaved, giving the bleomycin–Fe(IV)=O species **4.39** and a hydroxyl radical, any of which can abstract the DNA 4′-hydrogen (Figure 4.30). A concerted reaction of **4.37** with DNA with concomitant O–O bond homolysis to give **4.39** is also possible.[88]

The bleomycin molecule can be viewed as finely tuned for its function, and its various structural portions act synergistically to effect efficient DNA cleavage, with the roles summarized in Figure 4.31.[89] The bleomycin–iron complex is formed at the metal binding domain, comprising the β-aminoalanine–pyrimidine–β-hydroxyhistidine moiety. This portion of the molecule contains five nitrogen atoms at a distance suitable to form a stable chelate with Fe(II), leaving a sixth coordination valence available for a molecule of oxygen. The mode of interaction of the bleomycins with DNA involves two types of interactions, one of which is electrostatic binding of the cationic or protonated amino side chain with DNA phosphate groups, as proven by the observation that non-basic side chains, although more easily transported into the cells, are much less active. The role of the bisthiazole system in DNA interaction has also been thoroughly studied, and two binding modes seem possible, namely intercalation and binding into the minor groove. Because DNA strand scission starts by abstraction of the deoxyribose 4′-hydrogen, which lies in the minor groove, it seems likely that bleomycin binds there, but intercalation has also been proven by the lengthening of linear DNA or the uncoiling of circular DNA.[90] Bleomycin shows selectivity toward 5′-GC-3′ and 5′-GT-3′ sequences because of hydrogen bonding recognition of either the bithiazole unit or the aminopyrimidine function.[91,92] Finally, the sugar moiety may be responsible for the uptake of the drug into

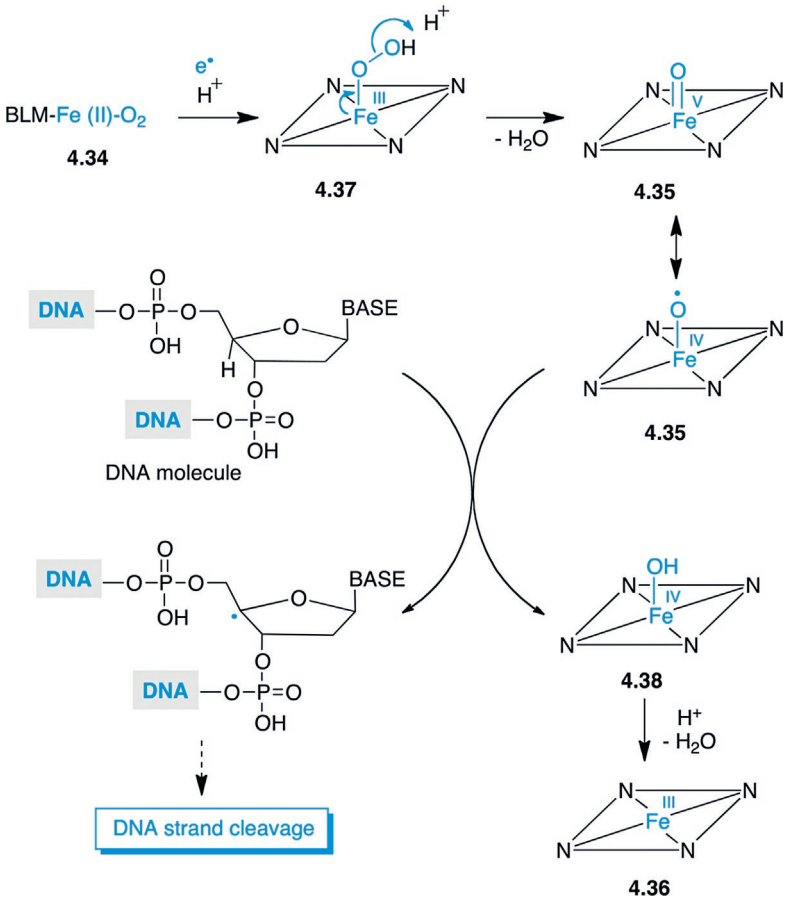


FIGURE 4.29

Activation of the bleomycin–Fe(II)–oxygen complex.

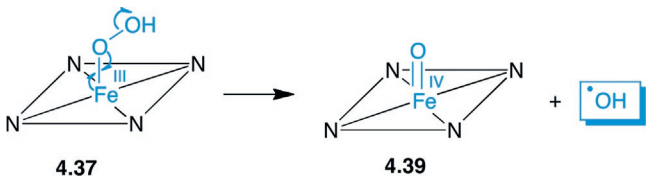


FIGURE 4.30

Homolytic cleavage of the Fe(III) hydroperoxy complex.

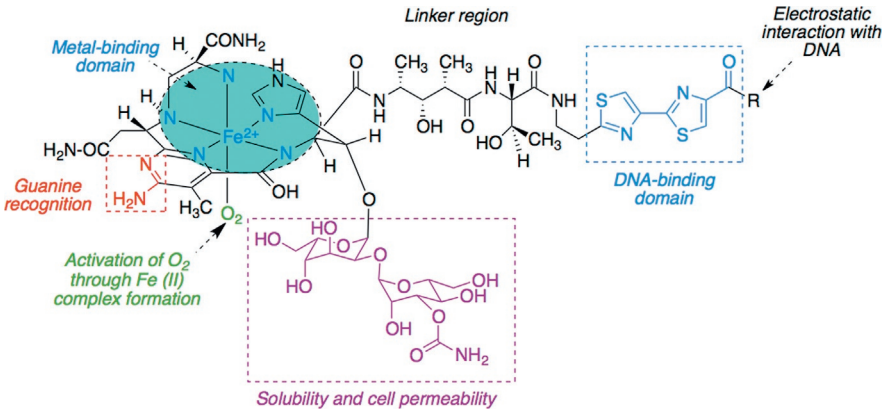


FIGURE 4.31

Roles of several bleomycin structural fragments.

cells, but it does not seem to be involved in DNA cleavage, although it has been proposed that it has a role in the capability of the bleomycins to accommodate oxygen.[93] The linker region is also essential for activity because it is responsible for the preorganization and stabilization of a compact conformation implicated in DNA cleavage.[74]

Bleomycins are large molecules (~1.5 kDa), and therefore they are probably unable to diffuse through cell membranes. It has been proposed that after administration, they bind rapid and irreversibly to Cu(II) in plasma. It is believed that both the free bleomycin and the bleomycin–Cu(II) complex are transported into the cells. The Cu(II) complex is intracellularly reduced to bleomycin–Cu(I), which can react with oxygen to initiate a series of reactions leading to DNA strand scission. On the other hand, this complex is less stable than the one from Cu(II), and it can dissociate, allowing the formation of the bleomycin–Fe(II) complex and its transformation into the activated bleomycin species (Figure 4.32).[80] Bleomycin transport is probably critical to the success of chemotherapy, and the use of internalizing antibodies for this purpose is under study (see Chapter 13).[94]

8 ENEDIYNE ANTIBIOTICS

This family of antitumor antibiotics contains as a common structural feature a macrocyclic ring with a conjugated system containing at least one double and two triple bonds.[95] Some members of the group are neocarzinostatin (zinostatin), the oldest of them, isolated from various microorganisms; the esperamicins/calicheamicins, from a *Micromonospora echinospora* ssp. *calichensis*; and dynemicin A, from *Micromonospora chersina*, which combines the structural features of the anthracyclines and the enediynes. In their natural environments, most of these compounds are embedded in a protein that stabilizes them. This protein has selective proteolytic activity on basic proteins such as the histones, which are responsible for DNA packaging and ordering into nucleosomes, and therefore it facilitates access of the enediynes to its target.

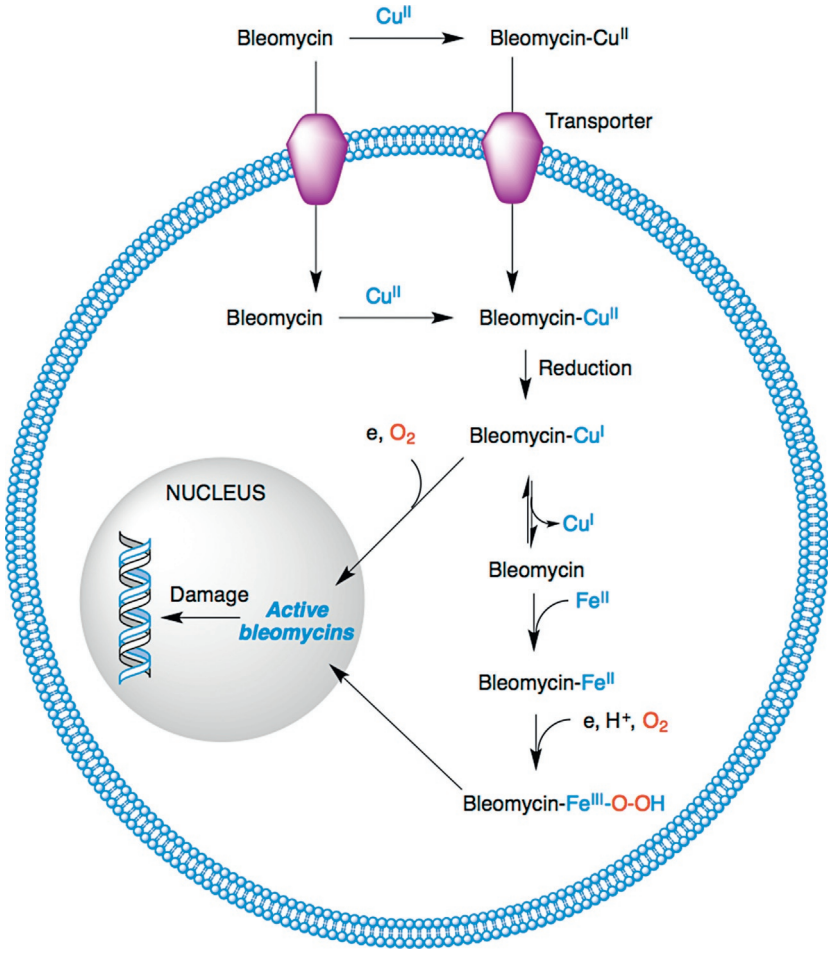
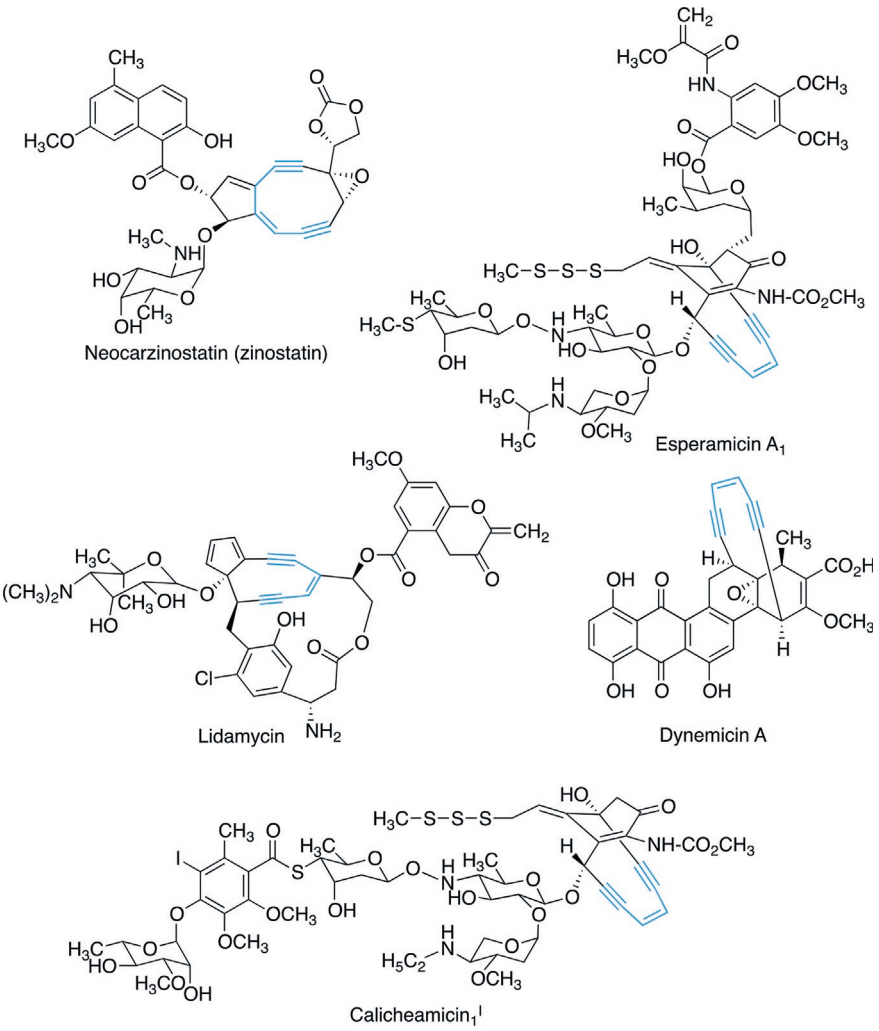


FIGURE 4.32

Bleomycin transport and bioactivation.

In general, the enediynes are too toxic for clinical use, and only a few of them have found application. For instance, a chemical conjugate of a synthetic copolymer of styrene maleic acid (SMA) and neocarzinostatin (NCS), known as SMANCS, has been proposed for the treatment of hepatocellular carcinoma.[96] A conjugate of a calicheamicin derivative with an antibody for the CD33 antigen, known as gemtuzumab ozogamicin (Mylotarg®), was approved in 2000 by the FDA for the treatment of acute myeloid leukemia, which is the most common type of leukemia in adults,[97] but withdrawn 10 years later (see Chapter 13, Section 4.6). Furthermore, some nonconjugated enediynes are or have been under clinical investigation, including dynemicin A, the esperamicins, and lidamycin. The latter compound displays an extremely potent cytotoxicity (~1000-fold more potent than adriamycin in human hepatoma cells) and has several mechanisms of action besides DNA damage.[98]



Enediynes have a double mechanism of action that involves binding to DNA by interaction of parts of the molecule with the minor groove[99] and activation to DNA-cleaving biradical species, either by reaction with thiols or by reduction.

The chemical basis for enediyne activation is the Bergmann reaction,[100] through which enediyne systems **4.40** undergo cycloaromatization to benzene derivatives, with the intermediacy of the highly reactive 1,4-benzenoid biradical species **4.41** (Figure 4.33a). In the related Myers–Saito reaction, one of the triple bonds can be replaced by an allene unit (**4.42**), leading to biradical **4.43** (Figure 4.33b). These processes do not take place in the natural products because their spacial arrangement prevents coplanarity of the three bonds involved in Bergmann-type chemistry, and therefore an activation reaction or cascade of reactions that alters the compound geometry is necessary.

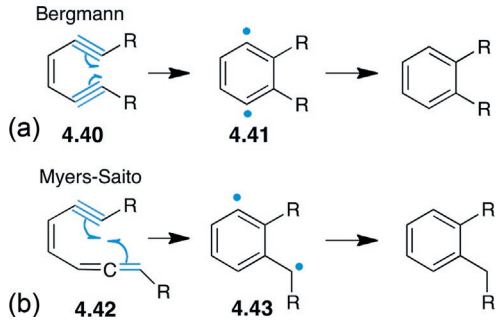


FIGURE 4.33

Radical generation from enediyne antibiotics through the Bergmann (a) and Myers–Saito (b) reactions.

In the case of neocarzinostatin, conjugate nucleophilic addition of a thiol results in epoxide opening and formation of a highly strained cumulene **4.45**, which has the correct geometry to undergo a Myers cycloaromatization to biradical **4.46** (Figure 4.34a).[101] In the absence of thiols, a base-catalyzed intramolecular addition reaction takes place, leading to cumulene **4.47** and subsequently to biradical **4.48** (Figure 4.34b).[102]

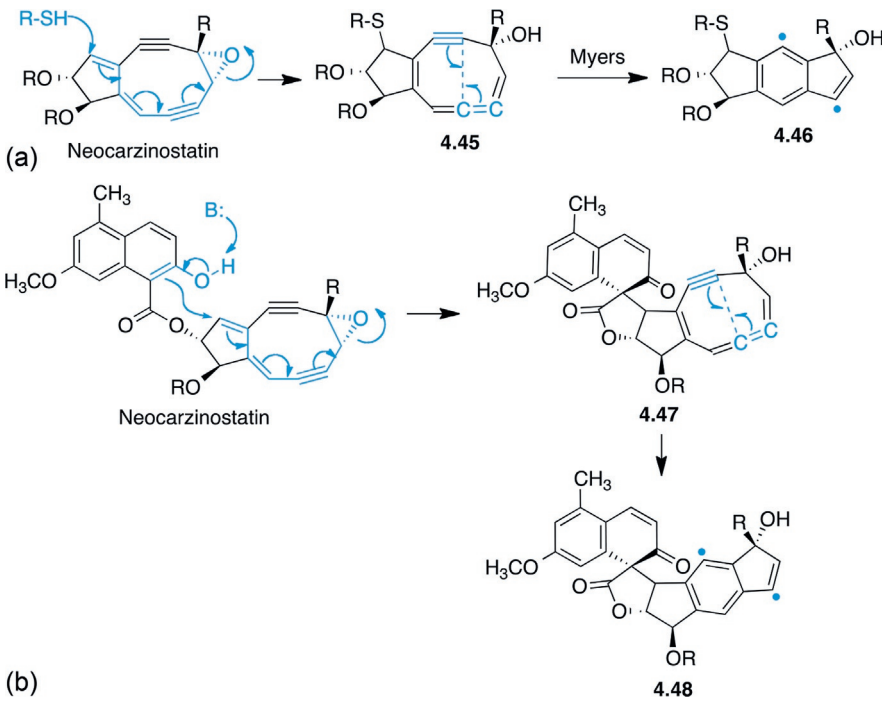


FIGURE 4.34

Activation of enediynes through a thiol addition (a) or an intramolecular addition (b).

The calicheamicins and esperamicins are also activated by attack of a thiol, in this case to the trisulfide portion, giving the thiolate **4.49**, which undergoes a Michael addition to the bridgehead α,β-unsaturated ketone to give the dihydrothiophene derivative **4.50**. The accompanying change in hybridation of the bridgehead atom triggers a Bergmann cyclization to biradical **4.51** (Figure 4.35).

In the case of dynemicin A, its rigid structure keeps the alkynes separate, preventing the Bergmann cyclization. The anthraquinone part intercalates into the minor groove, and subsequent activation may involve nucleophilic attack by a thiol or a reductive mechanism mediated by NADPH. In the first case, epoxide opening gives the highly electrophilic quinonimine methide **4.52**. Addition of a thiol gives **4.53**, the precursor of biradical **4.54** through a Bergmann reaction (Figure 4.36).

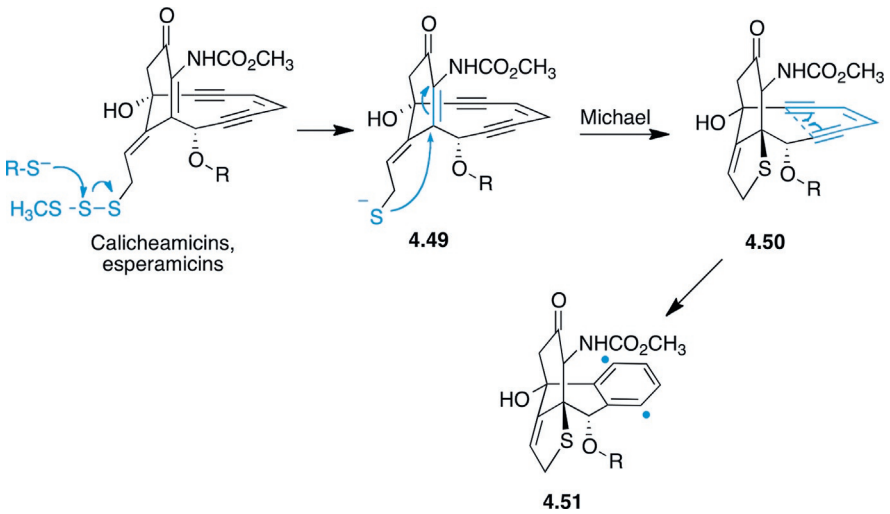


FIGURE 4.35

Generation of radicals from calicheamicins and esperamicins.

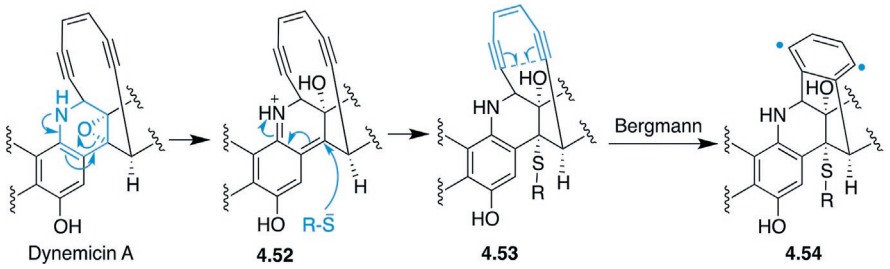


FIGURE 4.36

Generation of a diradical from dynemicin A through a thiol addition.

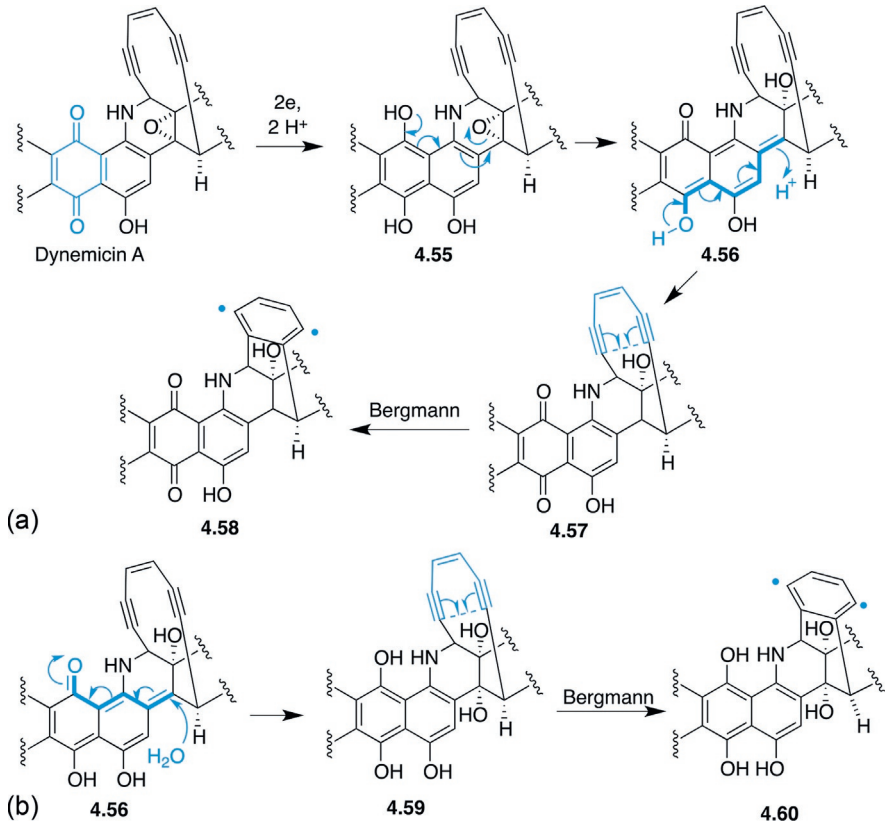


FIGURE 4.37

NAD-mediated generation of a diradical from a dynemicin A extended quinone methide acting as a nucleophile (a) or as an electrophile (b).

In the NAD-mediated mechanism, formation of hydroquinone **4.55** is followed by epoxide ring opening with formation of an extended quinone methide **4.56**. This intermediate can behave as a nucleophile (Figure 4.37a) or as an electrophile (Figure 4.37b).[103] In the first case, protonation leads to **4.57,** and in the second case trapping by water gives bishydroquinone **4.59**, with both compounds being suitable biradical precursors by Bergmann chemistry.

DNA strand scission by the enediyne biradicals involves hydrogen abstraction from DNA molecules. Both H-4′ and H-5′ of DNA desoxyribose residues in the minor groove are accessible to the biradicals. In the case of H-4′ abstraction, a mechanism similar to that shown in Figure 4.8 operates, but ~80% of DNA lesions correspond to the abstraction of H-5′ from thymidine or deoxyadenosine residues. As shown in Figure 4.38, radical **4.61** formed in this reaction consumes a molecule of oxygen, one electron, and one proton to give hydroperoxide **4.62**. Fragmentation of **4.62** by nucleophilic attack from a thiol leads to the 3′-phosphate portion **4.63** and the nucleoside-5′-aldehyde **4.64**.

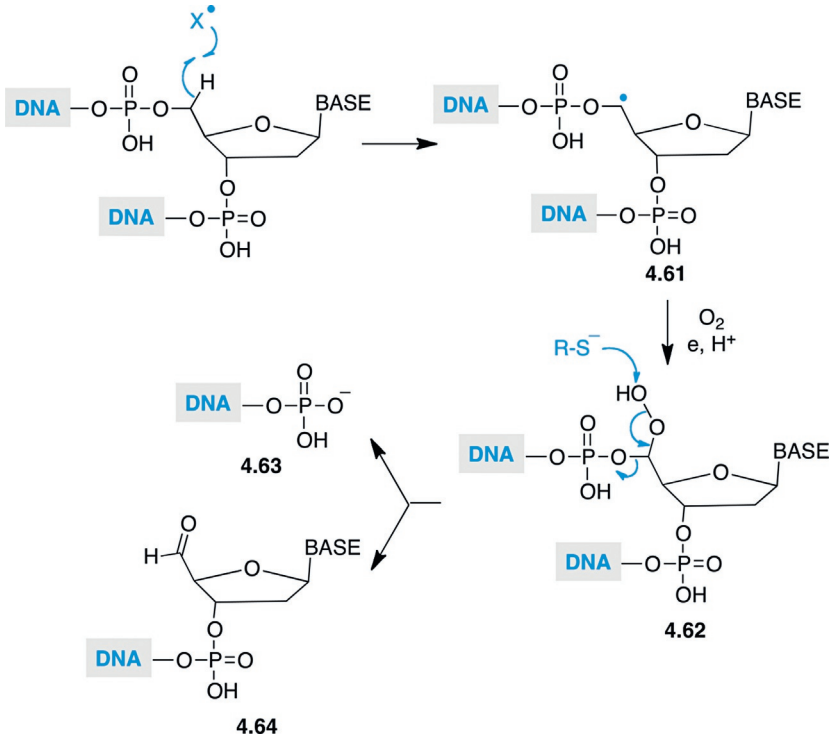


FIGURE 4.38

DNA strand scission induced by enediyne radicals.

9 TIRAPAZAMINE

Tirapazamine (TPZ) is the lead compound in the benzotriazine di-*N*-oxide class of hypoxic cytotoxins that selectively act in hypoxic tumor cells through bioreductive mechanisms (see Chapter 13, Section 2.2.1).[104] The mechanism for the selective toxicity toward hypoxic cells is the result of a one-electron reduction of the parent molecule to a free radical species that interacts with DNA to produce single- and double-strand breaks. It has also shown activity when combined with some chemotherapy agents, particularly cisplatin and carboplatin, or radiotherapy, whose efficacy it enhances under hypoxic conditions.[105] Several clinical studies have been undertaken to study the effectiveness of these combinations in non-small cell lung cancer and other refractory solid tumors.[106,107] It has also been shown that the use of electric pulses combined with TPZ and radiotherapy (electroradiochemotherapy) is more efficient than radiochemotherapy (TPZ and radiation) alone.[108]

One-electron reduction of TPZ by NADPH-dependent cytochrome 450 reductase (P450R) leads to the formation of the TPZ radical, which is rapidly destroyed by oxygen in normal cells, leading to superoxide radical, which is thought to be responsible for the muscle cramps seen in patients given the drug. Under hypoxic conditions, the TPZ radical can undergo homolytic cleavage to the reduced

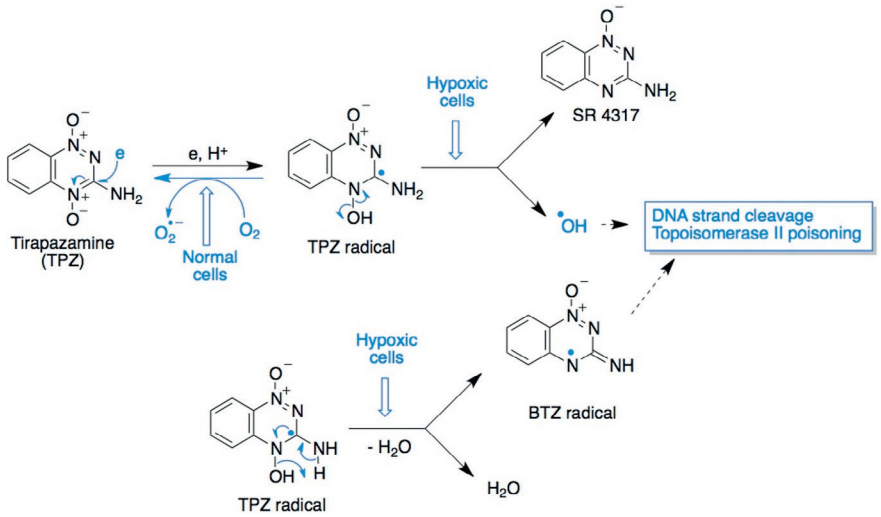


FIGURE 4.39

Radical species derived from tirapazamine metabolism.

species SR 4317 and a hydroxyl radical. Both radicals can react with DNA, but damage caused by TPZ can be detected both at the DNA backbone and the heterocyclic bases and can therefore be considered as typical of hydroxyl radicals.[109] Another DNA-damaging species generated in the metabolism of TPZ is the benzotriazinyl (BTZ) radical,[110] formed by loss of water (Figure 4.39). Double-strand breaks are caused, at least partially, by poisoning of topoisomerase II, either by direct damage from the radical species derived from TPZ or from radicals generated on the DNA molecules, which are the topoisomerase II substrates.[111]

In addition to its ability to generate DNA-damaging radicals, TPZcan also react with DNA radicals arising from these reactions, playing a role similar to the oxygen molecule. This dual role helps to explain the very high efficiency of TPZ in hypoxic cells. Thus, the reaction of DNA radical **4.65** with a molecule of unactivated TPZ gives intermediates **4.66** and **4.67**, leading to the hydroxylation of the DNA molecule after further reduction and protonation. Evolution of **4.68** leads finally to strand breaks, as shown in Figure 4.40.

10 PENCLOMEDINE

Penclomedine, a 2-trichloromethylpyridine derivative, entered clinical trials for solid malignancies[112] after initial observations of its strong antitumor properties in animal brain tumor models. It has been shown to be a DNA monoalkylating agent, and it has been proposed that its alkylating properties stem for the homolytic cleavage of one of the C–Cl bonds by reductive microsomal metabolism (Figure 4.41).

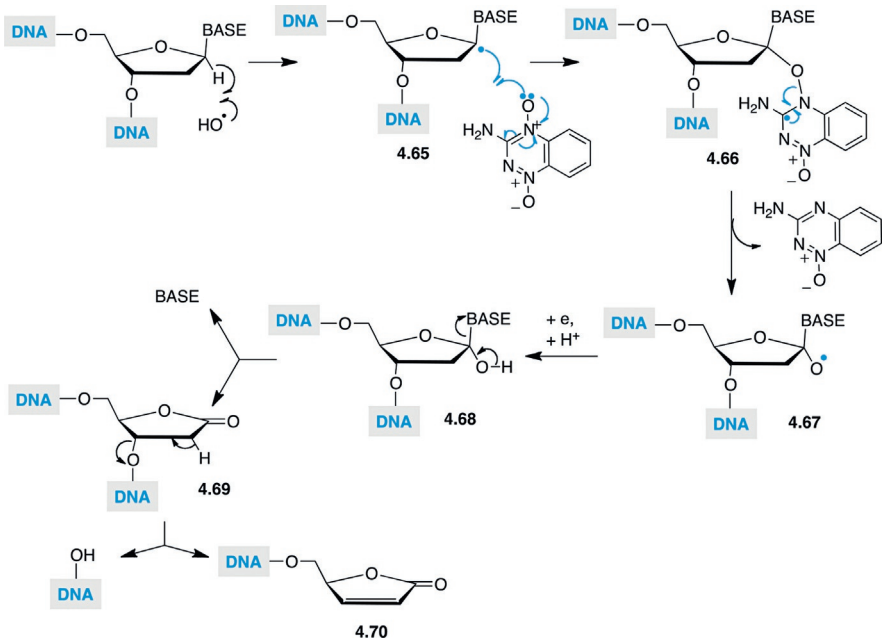


FIGURE 4.40

Addition of tirapazamine to DNA radicals.

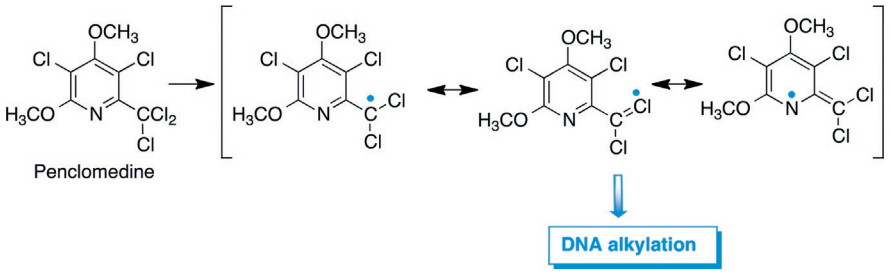


FIGURE 4.41

Generation of radicals from penclomedine.

11 RADIOTHERAPY AND RADIOSENSITIZERS

11.1 RADIOTHERAPY

Radiotherapy is one of the major approaches to cancer therapy, and it has been estimated that approximately 50% of cancer patients will receive this treatment during the course of their disease.[113] In several solid tumors, including lung, head and neck, gastrointestinal, and brain tumors, radiation is combined with standard cytotoxic chemotherapeutic agents. These radiochemotherapy regimes provide local tumor

control induced by radiation, whereas chemotherapy is intended for metastases. Associated chemotherapy may also have a direct effect on the tumor cells by enhancing their radiosensitivity.

Radiotherapy can be defined as the medical use of ionizing radiation for the purpose of cancer treatment. It is based on the generation of hydroxyl radicals from homolytic fragmentation of water molecules upon local application of ionizing radiation. This fragmentation can be preceded by ionization of water molecules (Figure 4.42a) or by their excitation (Figure 4.42b).

As previously mentioned, the main mechanism of cytotoxicity of hydroxyl radicals is based on the generation of radicals from biomolecules (Figure 4.43a). Cellular defenses against this process are varied, but they are normally based on the reaction of these radicals with an antioxidant molecule such as glutathione, which reacts with the biomolecule radicals, repairing them and leading to a glutathione radical. The latter species is harmless despite being a radical because of its tendency to dimerize to a disulfide (Figure 4.43b).

Damage by ionizing radiation is enhanced by the presence of oxygen by a factor of 2- to 3.5-fold; therefore, oxygen can be considered to act as a very efficient radiosensitizer. In fact, hypoxic cells in tumors are resistant to radiotherapy, with increased resistance to ionizing radiation being observed at oxygen concentrations of less than 1% (8 torr).[114] For the same reason, patients with low hemoglobin levels often do not show a good response to radiotherapy. This so-called "oxygen effect" is due to the property of oxygen of reacting with biomolecule radicals to generate other radicals that cannot be repaired because their reaction with glutathione does not lead back to the biomolecule but, rather, to an oxidized derivative (Figure 4.44).

Radiotherapy can be applied using several methods, which are summarized here.

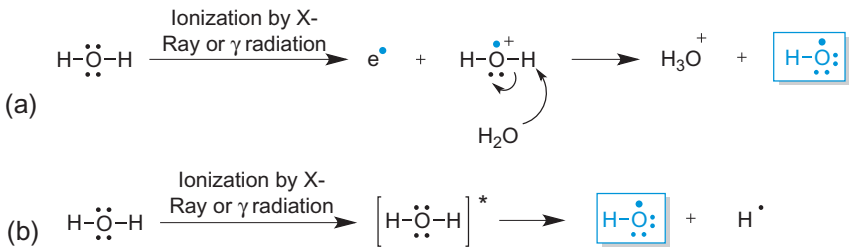


FIGURE 4.42

Generation of hydroxyl radicals from water during radiotherapy.

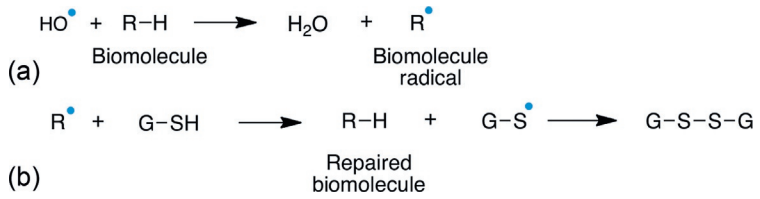


FIGURE 4.43

Repair of biomolecule radicals by glutathione.

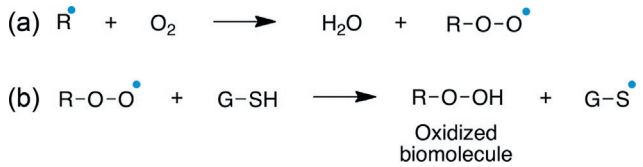


FIGURE 4.44

Enhancement of the effects of ionizing radiation by oxygen.

11.1.1 External Beam Radiotherapy

This group of methods, also known as *teletherapy*, are the most common form of radiotherapy and involve the use of an external source of ionizing radiation that is pointed at the part of the body of the patient where the tumor is located. This ionizing radiation is normally applied in the form of X-rays, which arise from the impact of accelerated electrons striking a target, usually tungsten. They are classified according to the voltage employed to produce the photon beam into "superficial" (from a kilovolt source), employed for skin cancer, or "deep" (from a megavolt source), employed for deep-seated tumors. Some modern variations of this general technique are in use, such as *radiosurgery*, which involves the use of a high dose of externally generated radiation directed by three-dimensional imaging techniques ("stereotactic radiosurgery") to eradicate tumors, normally in the brain or spine, without the need for conventional surgery. A detailed description of these and related techniques is outside the scope of this book.

11.1.2 Brachytherapy (Internal Radiation Therapy)

A radiation source is placed inside the body, at the site of the tumor, minimizing the exposure of healthy tissues to irradiation and allowing the use of high doses. In a variation known as *intraoperative radiotherapy*, the radiation source is applied to the tumor during surgery.

11.1.3 Radioisotope Therapy

This technique involves the systemic administration of radioisotopes, which need to be targeted to the tumor.[115] Radioisotopes employed in cancer therapy should have a relatively short half-life to avoid a prolonged effect on the patient, and they should emit radiation with a relatively short range. They normally belong to one of the following categories:

1. β^- emitters: Because β particles have spans of approximately 50 cell diameters, they are suitable for treating large or poorly vascularized tumors. On the other hand, their use to treat small tumors may damage nearby normal cells.
2. α emitters are suitable for small tumors because α particles have a very short range of approximately 10 cell diameters and are less harmful for surrounding tissue.
3. Nuclei emitting Auger electrons, which have an extremely short range of approximately one cell diameter and therefore require very precise targeting.

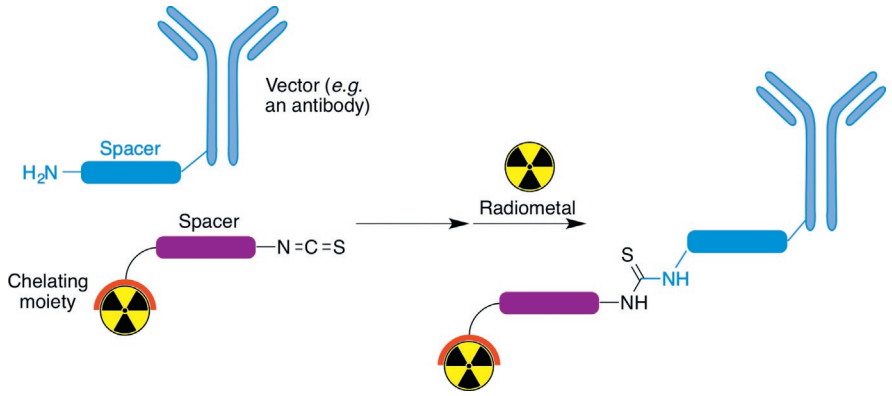


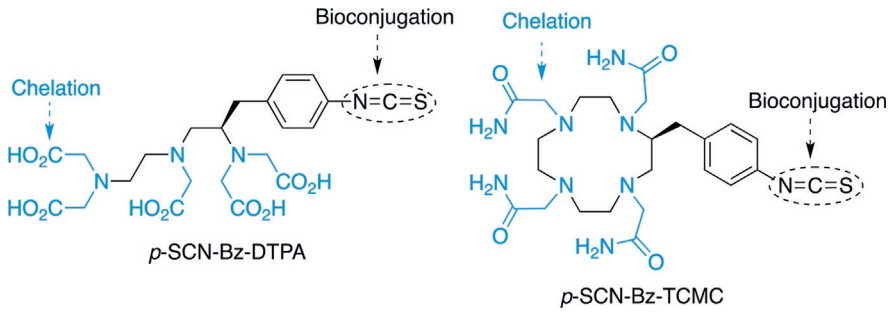
FIGURE 4.45

The BFC approach to radioisotope targeting.

Radioisotope targeting can sometimes be achieved easily due to the selective tissue concentration of certain elements; for instance, iodine is specifically absorbed by the thyroid gland, where it achieves a concentration approximately 1000 times higher than that in other organs, and this allows the use of the radioactive isotope ^{131}I to treat thyroid cancer. Similarly, radium, which can be viewed as a calcium mimic, has a tendency to accumulate in bone. For this reason, $^{233}RaCl_2$ (alpharadin, Xofigo®) was approved by the FDA in 2013 for the treatment of bone metastases found in 80–90% of patients with metastatic prostate cancer.[116] Alpharadin mainly emits α-rays with a track length shorter than 100 μm (~2–10 cell diameters), thus causing less damage to normal tissues, especially bone marrow, than other radiopharmaceuticals and other radiation therapies. It is the only radiopharmaceutical that has demonstrated improvement on overall survival.[117]

In most cases, however, more sophisticated targeting methods are required, involving the use of biological vectors. In the so-called bifunctional chelate (BFC) approach, the final radiopharmaceutical contains four components, namely a chelating moiety, the radiometal, the vector, and suitable spacer chains, acting as linkers (Figure 4.45).

The bifunctional chelating compound must contain a moiety able to bind the radiometal and also a functional group suitable for the attachment of the vector. Acyclic and macrocyclic chelators, such as *p*-SCN-Bz-DTPA and *p*-SCN-Bz-TCMC, are representative examples.



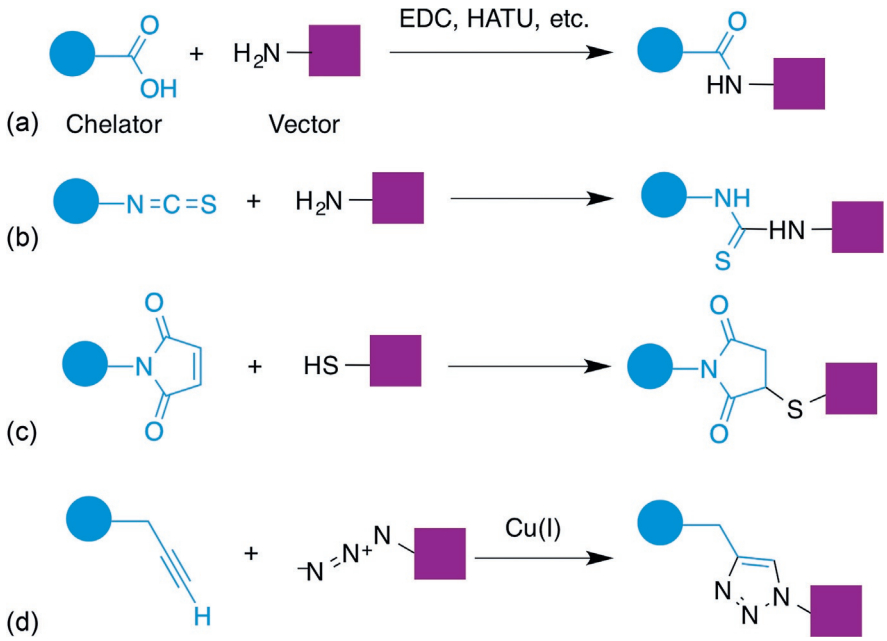


FIGURE 4.46

Main bioconjugation strategies for attaching chelator moieties to the biological vector.

Four major bioconjugation strategies are employed for attaching the chelator to the biological vector (Figure 4.46):

1. Peptide bond formation, which involves coupling one or more of the many carboxylic groups of the chelator to an amino group of the vector, using one of the many well-known coupling reagents employed in peptide synthesis (EDC, HATU, HOBt, etc.).
2. Thiourea formation, from nucleophilic addition of an amino group of the vector to an isocyanate in the chelator.
3. Thioether formation, from Michael addition of a thiol group from a cysteine in the peptide or protein acting as a vector and a maleimide unit in the chelator.
4. Use of the "click" reaction—that is, a Cu(I)-catalyzed Huisgens [3+2] dipolar cycloaddition between a terminal alkyne and an azide. The electron-rich nitrogen atoms of the resulting triazole ring may contribute to chelation.

The biological half-life of the vector must be adjusted to the radioactive half-life of the nuclide. In general, antibodies, which require long times to accumulate at the tumor site, are best matched to isotopes with long half-lives, whereas peptides are more suitable for short half-life isotopes. There are several radiolabeled monoclonal antibodies marketed for cancer therapy, including Bexxar® (^{131}I), Zevalin® (^{90}Y-labeled ibritumomab tiuxetan), and ProstaScintas® (^{111}In capromab pendetide),

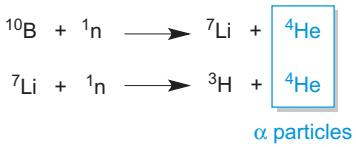


FIGURE 4.47

Generation of alpha particles in BNCT.

together with many others in clinical trials. Others, especially those based on ^{99}Tc, are useful for diagnostic purposes.

11.1.4 Neutron Irradiation

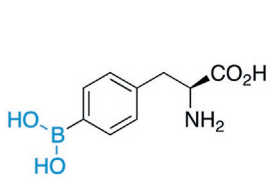
Bombardment of tumors with fast neutrons can generate radioisotopes *in situ*. This method has the advantage of not requiring the intervention of oxygen and therefore allowing radiotherapy in hypoxic tissues.[118]

In a variation known as *neutron capture therapy*, the patient is treated with a tumor-localizing drug that contains a nonradioactive element with a high cross section for neutron capture, which allows the use of slow neutrons. In practice, all clinical trials performed to date have been carried out with the ^{10}B isotope of boron (boron neutron capture therapy, BNCT). This is followed by irradiation with a neutron beam, which interacts with ^{10}B to produce α particles according to the nuclear reactions summarized in Figure 4.47. The nuclei thus generated do not damage surrounding tissues because they dissipate their kinetic energy in a very short distance, less than one cell diameter (5–9 μm).[119]

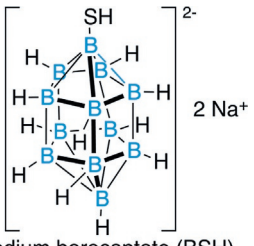
In addition to the problems associated with neutron generation, the main obstacle for the widespread use of neutron capture therapy is the scarcity of suitable boron delivery agents. This is due to the stringent requirements that they must satisfy, which include the following:

1. Low systemic toxicity
2. Selectivity for tumors with regard to other tissues, which ideally should be above 3:1
3. Ability to deliver levels of at least 20 μg of ^{10}B/g of tumor
4. Sufficient persistence in the tumor to allow the neutron capture process
5. Rapid elimination from normal tissue

Only two such compounds have been employed in clinical studies, namely boronophenylalanine (BPA)[120] and sodium borocaptate (BSH).[121]



Boronophenylalanine (BPA)



Sodium borocaptate (BSH)

11.2 DRUGS USED TO IMPROVE THE RESULTS OF RADIOTHERAPY

There are two complementary strategies to improve radiotherapy, involving increasing the damage to tumor cells and decreasing the damage to healthy tissues.[122] Following treatment with ionizing radiation, the so-called DNA damage response (DDR) comes into play. This complex process stops the cell cycle to prevent the transfer of DNA mutations to progeny, and this may happen in the G_1 to S phase transition, intra-S phase, or at the G_2/M transition. DDR also facilitates the DNA damage repair machinery and has a crucial role in the induction of apoptosis when repair mechanisms fail. Defects in DDR, and particularly a failure to stop the cell cycle upon DNA damage, are a feature found in many cancers.

The pharmacological strategies available to increase the efficiency of radiotherapy are summarized in Figure 4.48. We discuss radioprotectors and radiosensitizers here, whereas the combination of radiotherapy with drugs acting by different mechanisms to overcome radioresistance is discussed in Section 8 of Chapter 14. Another important aspect of the use of ionizing radiation in the treatment of cancer is the activation of prodrugs by therapeutic radiation, which is discussed in Section 2.2.5 of Chapter 13.

11.2.1 Radiosensitizers

For the reasons explained in Section 11.1, tumor hypoxia is associated with resistance to radiotherapy and also to some types of chemotherapy based on the generation of oxidizing species. In these hypoxic tumors, some types of chemical agents can play a similar role to oxygen, and therefore they can be used to increase the sensitivity toward radiotherapy. These compounds are known as radiosensitizers,[123] and they are being applied to an increasing number of human cancers, such as those of cervix, head and neck, and lung.[124] Another interesting application of some radiosensitizers is their use as hypoxia markers to accurately measure oxygen gradient at the cellular level.[125]

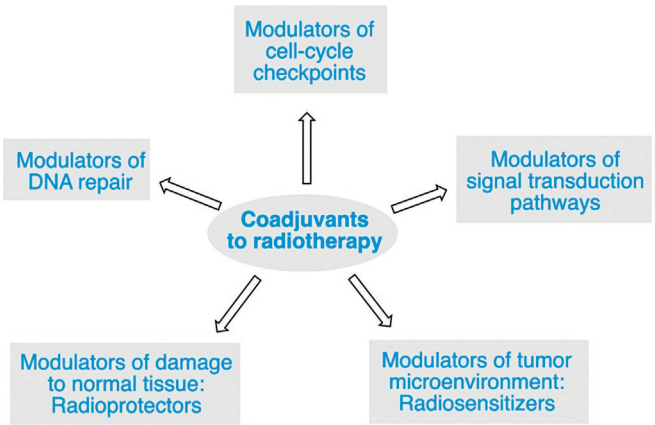


FIGURE 4.48

A summary of pharmacological strategies used to improve the results of radiotherapy.

The first compounds that were studied in clinical trials as hypoxic radiosensitizers were nitroimidazoles. The mechanism of hypoxic-cell sensitizing by the nitro derivatives is based on their ability to react with biomolecule radicals giving a radical adduct that cannot be repaired, thereby acting as oxygen surrogates (Figure 4.49a). Alternatively, addition of the biomolecule radical to the nitro group gives nitro radical anions (Figure 4.49b).

Nitro radical anions are cytotoxic in themselves in hypoxic environments, although normally only at doses too high to be achieved in clinical situations. However, this cytotoxicity is reinforced by the generation of other radical species, some of which are shown in Figure 4.50. It is interesting to mention in this context that the antibacterial and antiprotozoal activity of many nitroheterocycles is explained by one-electron reduction of the nitro group to nitro radical anions.

The first nitro compounds to be clinically studied as radiosensitizers, in the early 1970s, were metronidazole and especially misonidazole, which were studied in a large number of clinical assays.

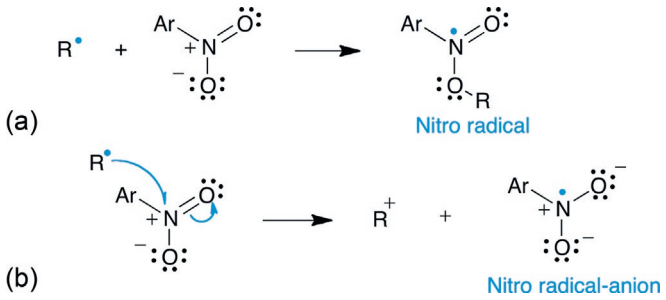


FIGURE 4.49

Generation of nitro radicals (a) and nitro radical anions (b).

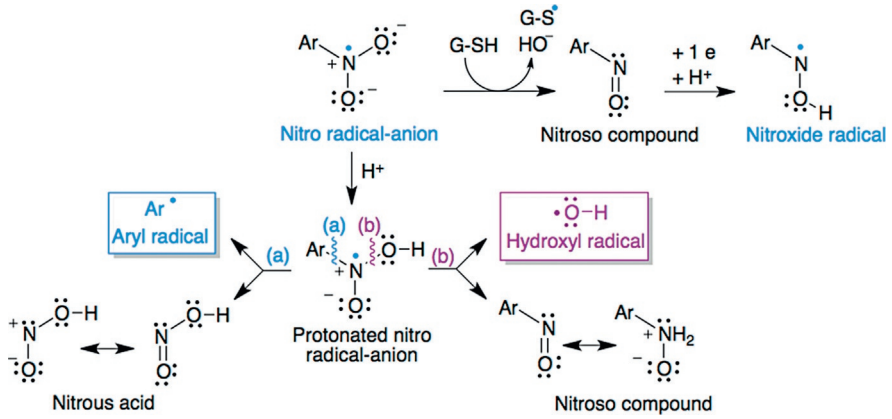
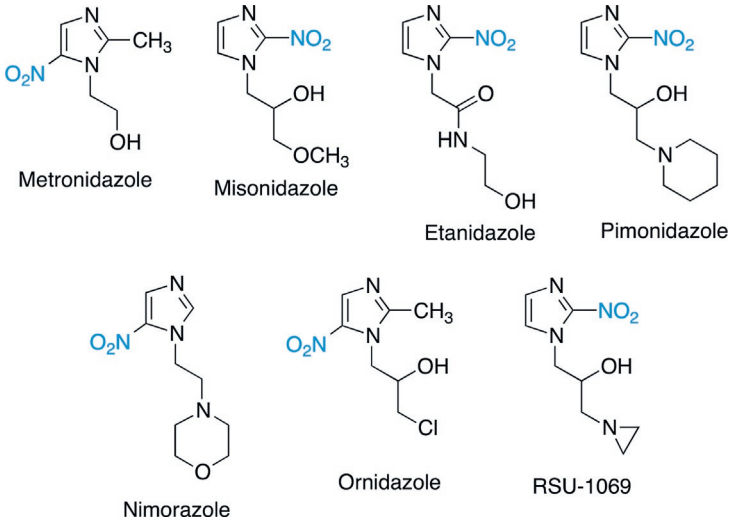


FIGURE 4.50

Cytotoxic radical species generated from nitro radical anions.

Despite initial promise, these clinical studies were disappointing, and the combination of misonidazole with radiotherapy failed to show significant benefits, with some studies reporting a significant neurotoxicity. In the 1980s, other 2-nitroimidazoles (etanidazole and pimonidazole) were studied as radiosensitizers. Because of their lower lipophilicity compared to that of misonidazole, both compounds showed lower penetration in the nervous system and a more rapid excretion, which result in lower neurotoxicity, but their clinical data did not demonstrate any benefit for radiotherapy. Subsequently, some of the newer 5-nitroimidazoles, such as ornidazole, entered clinical trials, with similarly discouraging results. On the other hand, nimorazole has shown good efficacy in sensitizing tumor cells to radiotherapy in phase III trials,[126] although its use is limited by supply problems.

In the case of RSU-1069, a high efficiency has been observed with certain tumors, such as the KHT sarcoma, but this effect seems to be due to cytotoxicity of the compound itself toward hypoxic cells rather than radiosensitization. Other bioreductive antitumor agents ("hypoxic cytotoxins"), particularly the previously mentioned porfiromycin and tirapazamine, have shown a great efficacy in combination with radiotherapy.[127]

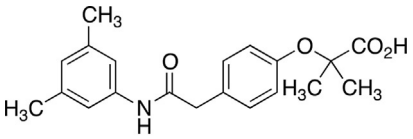


11.2.2 Oxygen Enhancement for Radiosensitization

Because oxygen is probably the most efficient known radiosensitizer, one simple approach to aid radiotherapy is increasing blood oxygen levels. Breathing carbogen, a mixture of oxygen (95–98%) and carbon dioxide (2–5%), before and during irradiation has been shown to enhance tumor radiosensitivity in clinical assays. The role of carbon dioxide is to activate physiological mechanisms against potential suffocation, thereby further decreasing tissue hypoxia. A combination of carbogen with nicotinamide, which increases blood flow, is known as accelerated radiotherapy with carbogen and nicotinamide (ARCON). This therapy is under clinical assays for larynx cancer and has been shown to improve the outcome of radiotherapy in anemic patients.[128]

In another approach, blood levels of oxygen can be increased by decreasing the affinity of hemoglobin for oxygen and thereby displacing oxygen from hemoglobin. One compound that achieves this

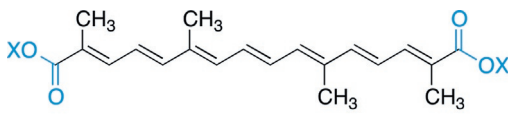
effect is efaproxiral (RSR13), a member of the clofibrate class of compounds that was initially developed as an analog of the cholesterol-lowering drug bezafibrate and acts as an allosteric modifier of hemoglobin. Efaproxiral gave positive results as a radiation sensitizer in a phase III, randomized clinical trial of patients with brain metastases, and it is being investigated for breast cancer.[129]



Efaproxiral

A third approach to improve the efficiency of radiotherapy involves improving the transport of oxygen to the tumors, with the main barrier to be overcome being diffusion through blood plasma to body tissue. *Trans*-sodium crocetinate (TSC) is the sodium salt of crocetin, a natural carotenoid showing a dicarboxylic acid structure that makes it significantly soluble in water, a property that is very unusual in the highly lipophilic carotenoid class of compounds.

TSC has antihypoxic and radiosensitizing properties that are due to the fact that it increases the diffusion rate of oxygen in aqueous solutions, including blood plasma. The lipophilic core of the compound exerts hydrophobic forces that result in a greater degree of hydrogen bonding among water molecules, which become thus more organized, allowing the formation of lattice-like structures that facilitate the diffusion of oxygen molecules.[130] TSC has been shown to increase the availability of oxygen during a variety of hypoxic and ischemic conditions (e.g., from hemorrhage) and also in the tumor microenvironment. In particular, it has been shown to make hypoxic tissues approximately three times more susceptible to radiation treatment.[131] For this reason, it gained orphan drug status from the FDA for the treatment of metastatic brain cancer together with standard radiotherapy.



X = H Crocetin
X = Na *Trans* sodium crocetinate (TSB)

11.2.3 Radioprotectors in Radiotherapy

To obtain optimum results from radiotherapy, the normal tissues surrounding the tumor must be protected against damage caused by radiation. Thus, radioprotective compounds are very important in clinical radiotherapy. Although there is a huge literature on this topic,[132] only a few compounds are used in clinical practice or have undergone clinical assays. The most important one is amifostine,[133] a prodrug that is hydrolyzed by alkaline phosphatase to furnish aminothiol WR-1065, the active metabolite. This compound exerts its action by trapping electrophilic species derived from the action of alkylating agents and scavenging free radicals (Figure 4.51).

Amifostine activation takes place selectively in normal tissues, which can contain a concentration of the free thiol up to 100-fold higher than tumors and are thus protected selectively. Several factors explain this selectivity, including the following: (1) Many tumors are characterized by a lower interstitial

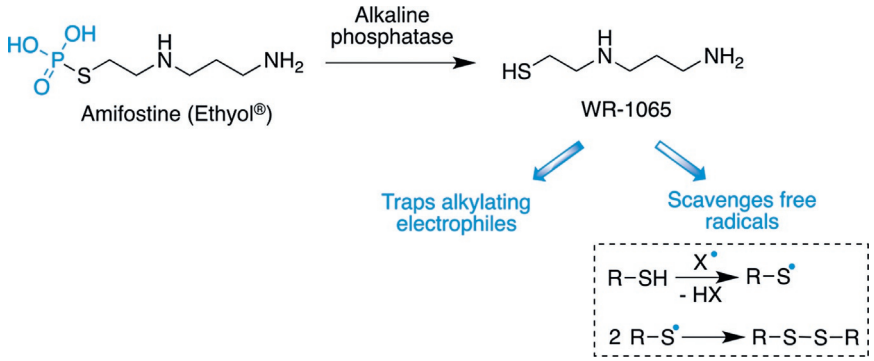


FIGURE 4.51

Amifostine as a radioprotector.

pH and oxygen content due to the fact that their lower vascularization makes them hypoxic compared with normal tissue, and (2) many malignant tissues show a reduced expression of alkaline phosphatase. Amifostine has been FDA-approved to reduce xerostomia—a chronic dry mouth condition—in patients undergoing postoperative radiation treatment for head and neck cancer, in which the radiation port includes a substantial portion of the parotid glands. Xerostomia is a severe and often irreversible side effect of radiation therapy caused by damage to the salivary glands.

12 PHOTODYNAMIC THERAPY OF CANCER

Photodynamic therapy of cancer (PDT) is based on the use of compounds that are able to absorb harmless visible light energy and transfer it efficiently to other molecules in their vicinity or, alternatively, use it for photochemical reactions with biomolecules.[134] These compounds are normally known as photosensitizers (PS). After irradiation with light of the suitable wavelength, the PS molecules are excited from the ground state PS(S_0) to a singlet excited state PS*(S_1) that can reverse to the ground state by nonradiative internal crossing (IC) or by fluorescent emission (F), the latter of which can be used for imaging and detection (photodynamic diagnosis, PDD). Alternatively, it may undergo an electronic rearrangement to the excited triplet state PS*(T_1) by intersystem crossing (ISC; Figure 4.52). Most reactions of relevance to photodynamic therapy take place in the triplet state, which must be sufficiently long-lived to give intermolecular reactions before its deactivation by emission of phosphorescence (P). In the Type 1 reactions, the PS triplet state reacts with an organic molecule (e.g., a component of the cell membrane) and transfers an electron to form a radical. These radicals may react further with oxygen, giving superoxide and other ROS. In Type 2 reactions, the PS triplet state transfers its energy directly to oxygen, leading to the formation of excited state singlet oxygen, a very potent oxidizer that is believed to be the main damaging agent acting by nonspecific oxidation of intracellular targets. The efficiency of these processes can be improved by increasing the stability of the triplet state, which can be achieved by spin-orbit coupling. In more familiar chemical terms, this involves the inclusion of heavy atoms in the structure of the photosensitizer—for example, by replacement of oxygen by sulfur, sulfur by selenium, or hydrogen by bromine or iodine.

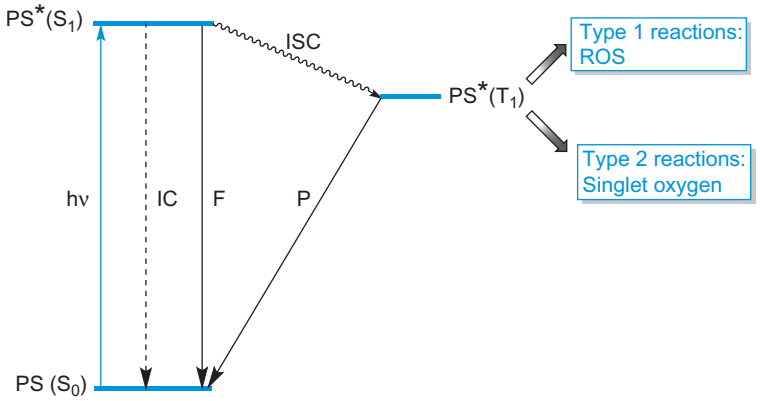


FIGURE 4.52

Jablonski diagram illustrating schematically the electronic transitions involved in photodynamic therapy.

The following are the main requirements that the ideal photosensitizer in cancer photodynamic therapy should satisfy:

1. Selectivity to tumor cells.
2. Biological stability, with no cytotoxicity in the absence of light.
3. Photostability—that is, low sensitivity to oxidation by singlet oxygen.
4. Strong absorption in the 600- to 800-nm region of the spectrum (red to near infrared).
5. Photochemical efficiency and long triplet excited state lifetime.
6. Good tissue penetration. This is better achieved if the absorption maximum is in the near-infrared (NIR) region, where tissue absorption is minimal. Thus, a tissue penetration depth of 4 mm can be obtained using a 763-nm light source, whereas a 630-nm radiation, corresponding to the absorption maximum of a typical porphyrin, has only 1.6-mm penetration.[135]

As shown in Figure 4.53, there are four main stages in the treatment of cancer by photodynamic therapy:

1. Delivery of the photosensitizer, which, with the exception of skin cancers, is normally done by intravenous injection (vascular targeted photodynamic therapy).
2. Ideally, the photosensitizer should be accumulated in the tumor. The lack of such selective accumulation of photoactivable molecules within tumor tissues is the main potential problem of PDT, and for this reason the development of targeted photosensitizers is an active research area.[136]
3. Approximately 24–72 hours after injection, selective irradiation of the target tissue is performed. This is normally achieved by use of a fiber-optic diffuser inserted through an endoscope, which leads to local activation and the generation of singlet oxygen and ROS.
4. Selective tumor destruction by these highly reactive species. Due to the low stability of the toxic species involved, diffusion to surrounding healthy tissues is not significant, and therefore the method is minimally invasive and is well tolerated. In addition to the direct killing of cancer cells, PDT can damage blood vessels in the tumor, preventing the access of necessary nutrients. It also may activate the immune system to attack the tumor cells.[137]

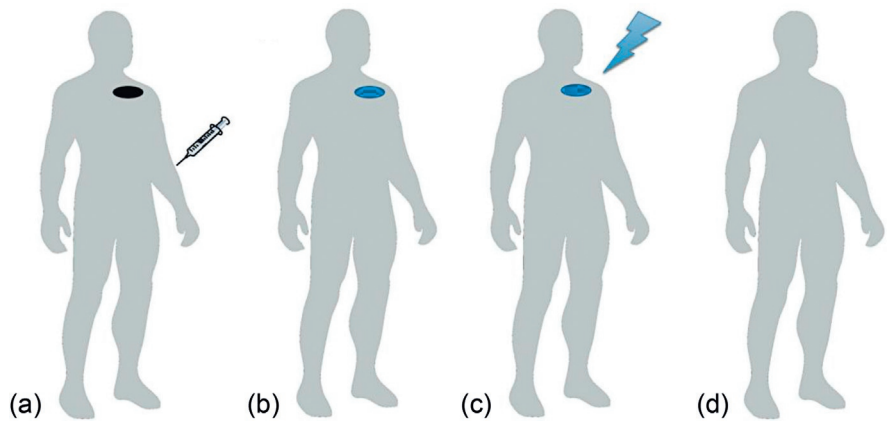


FIGURE 4.53

Schematic representation of the clinical procedure for cancer photodynamic therapy.

Adapted from reference 158.

Photodynamic therapy has been in clinical use for a long time, initially for skin cancers. Subsequently, it has been established as a therapeutic strategy for other types of cancer, such as cervical, esophageal, early stage central type lung,[138] and head and neck cancers,[139] among other applications.[140,141] It has the disadvantage of requiring hospitals to make an expensive capital expenditure on laser machinery.

12.1 PORPHYRINS AS PHOTOSENSITIZERS

Among the many compounds investigated as photosensitizers for PDT,[142] most work has been carried out with porphyrin-based drugs. Porphyrins are aromatic, highly conjugated heterocycles, with a core of four pyrrole rings coupled through four methylene units, that contain 11 conjugated double bonds, leading to light absorption in the red region of the visible spectrum. The excited state thus generated can lead to the formation of singlet oxygen and ROS species (Figure 4.54). Chlorins are analogs of the

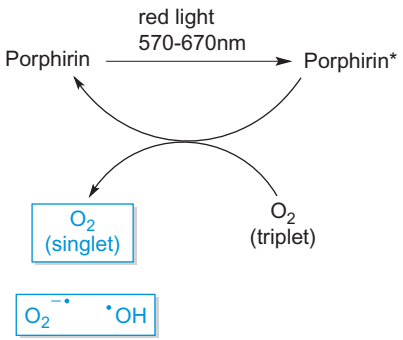


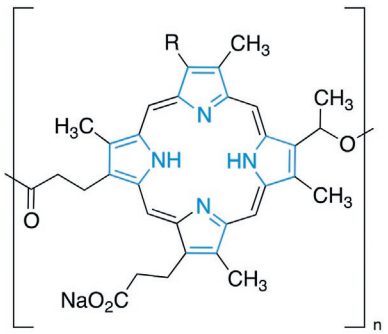
FIGURE 4.54

Photoactivation of porphyrins.

porphyrins, where one of the pyrrole rings has been replaced by a pyrroline, leading to a partial loss of aromaticity of the central core.

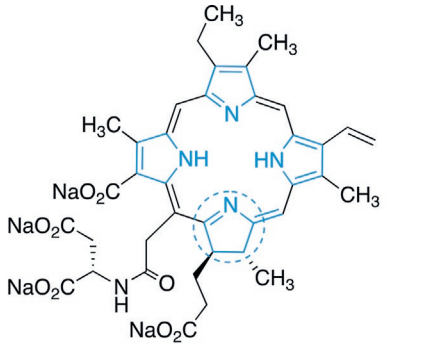
Initial experiments on the use of porphyrins as photosensitizers involved the use of preparations of hematoporphyrin, which were complex mixtures of porphyrin oligomers. They were later replaced by a porfimer sodium oligomer (Photophrin®) with a more regular composition.[143] The semisynthetic chlorin derivative talaporphin (mono-L-aspartylchlorin e_6, Laserphyrin®, Aptocine®) has been approved for early stage lung cancer, and compared to other photosensitizers, it has the advantage of high aqueous solubility and of being associated with minimum cutaneous photosensitivity. It has a long activation wavelength of 664 nm (in the red part of the visible spectrum), allowing relatively deep tissue penetration.[144] In the case of Aptocine®, all components needed for activating the drug are included in the packaging, and treatment can be administered in the outpatient setting.

Another chlorin derivative that has been approved for use as a photosensitizer in PDD is temoporfin (Foscan®), which is activated by red light (652 nm) and employed in Europe for the treatment of squamous cell head and neck carcinoma,[145] although it did not obtain approval from the FDA. Rostaporfin (tin ethyl etiopurpurin, Purlytin®) is a tin complex of another chlorin, with an absorption maximum at 656 nm, that has undergone clinical trials for metastatic breast cancer[146] and also for the treatment of age-related macular degeneration.

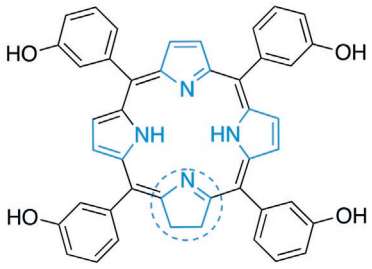


R = -CH=CH_2 and/or -CH(OH)-CH_3
n = 2 to 8

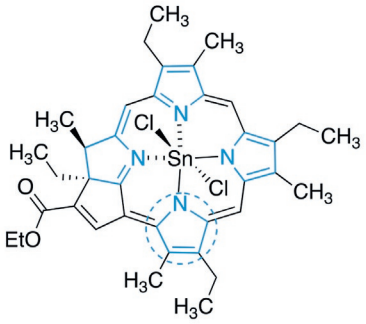
Photophrin (Porfimer sodium oligomer), Photophrin®



Talaporphin sodium, Laserphyrin®, Aptocine®



Temoporphin, Foscan®



Rostaporfin, Purlytin®

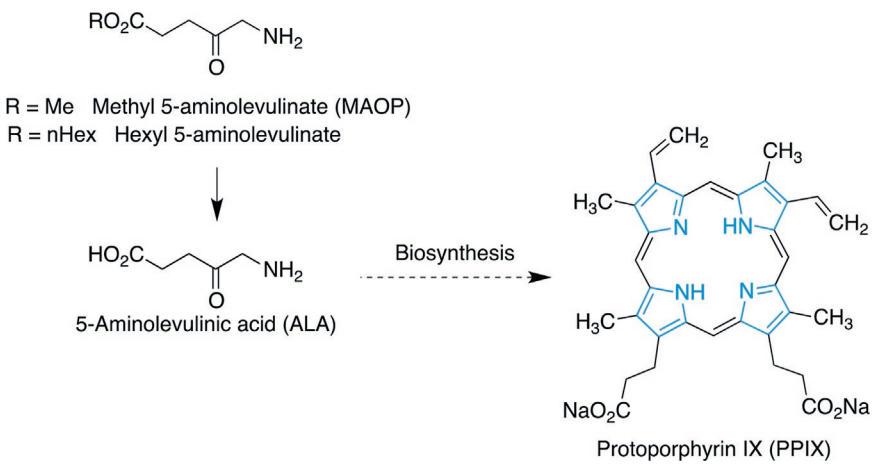


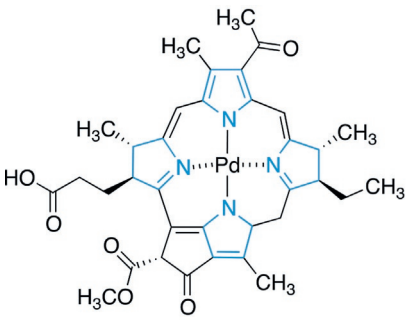
FIGURE 4.55

ALA as a precursor to protoporphyrin IX.

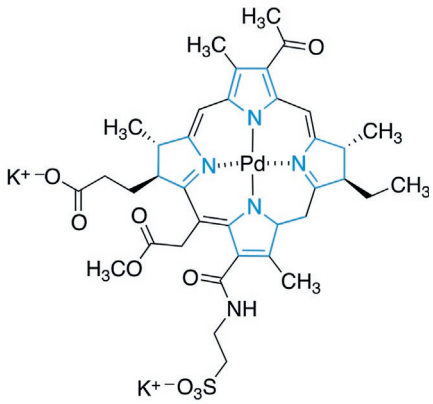
An alternative to porphyrin or chlorin treatment that has also been used in the clinic involves the use of 5-aminolevulinic acid (ALA, Levulan®), a biosynthetic precursor of the natural photosensitizer protoporphyrin IX (Figure 4.55). This compound is normally employed in the form of ester prodrugs, which have an improved absorption when administered as creams.[147] Some of these esters include methyl 5-aminolevulinate (MAOP), Metvix®, and hexyl 5-aminolevulinate (Hexvix®, Cysview®). Protoporphyrin IX thus generated is selectively accumulated in some tumors because of their accelerated metabolism, which includes a faster processing of ALA.

Bacteriochlorophylls are photosynthetic pigments found in some bacteria, and their core (bacteriochlorin) contains two pyrrole and two pyrroline units. Derivatives of bacteriochlorophyll have a unique behavior as photodynamic therapy drugs. Thus, conventional photosensitizers cause tumor damage by accumulating in tissues following extravasation from the intravascular space and activation by light. In contrast, following their intravenous administration, bacteriochlorophylls bind to plasma proteins and therefore show minimal extravasation. When irradiated with NIR light, at an optimal wavelength of 753 nm, they generate singlet oxygen and ROS within the tumor microvasculature network, resulting in its occlusion, followed by vascular dysfunction within the tumor and rapid necrosis of the treated region.[148]

The first bacteriochlorophyll to be studied as a photosensitizer was padoporfin (WST09, T, Tookad®), a bacteriopheophorbide in which the natural Mn ion has been replaced by Pd, thereby achieving a very high singlet oxygen quantum yield and an absorption maximum in the NIR region (763 nm), with the consequent advantages in terms of tissue penetration. Padeliporfin (WST11, TS, Tookad soluble®) is a closely related compound, obtained by retro-Dieckmann opening of the cyclopentenone ring with taurine to improve its aqueous solubility. Padeliporfin has been studied for the treatment of prostate cancer, with 74% of patients showing negative histopathology for prostate cancer after 6 months of the treatment,[149] and it appears to be promising for other types of solid tumors.



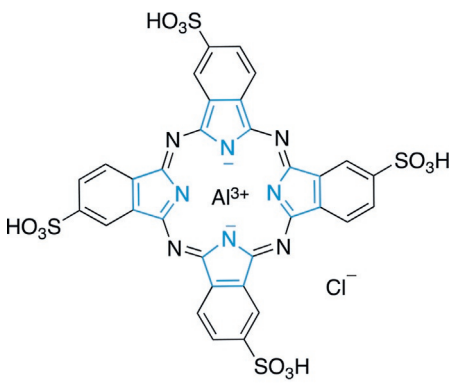
Padoporfin (WST09), Tookad®



Padeliporfin (WST011), Tookad soluble®

12.2 NON-PORPHYRIN PHOTOSENSITIZERS

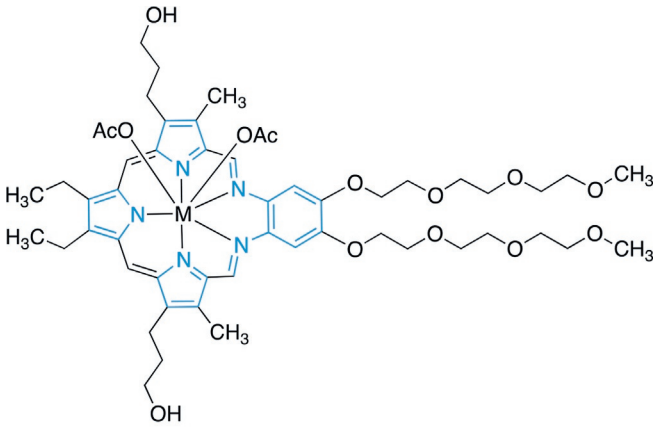
Phthalocyanins are porphyrin-related, second-generation photosensitizers that are used in the form of complexes with diamagnetic metal cations and absorb around 675 nm, in the red region of the electromagnetic spectrum. Chloroaluminum tetrasulfophthalocyanine (ALPcTS) is stable, water-soluble, and has been studied for the PTD treatment of basal cell carcinomas, Kaposi's sarcoma, and lung cancer.[150]



Chloroaluminum tetrasulfophthalocyanine

Texaphyrins are expanded porphyrins having five nitrogen atoms, three of which are pyrrolic and the other two belong to Schiff base functions. Their name derives from the resemblance between this five-nitrogen arrangement and the five-point star in the flag of the state of Texas. This structure leads to an internal core approximately 20% larger than that of the porphyrins, which makes them able to coordinate large metals. Furthermore, they are tumor selective.[151]

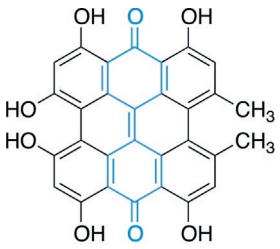
Lutetium texaphyrin (MLu, Lutex®, Lutrin®) absorbs strongly at 730–770 nm, a region with an excellent tissue transparency. This compound has been approved by the FDA for the photodynamic treatment of breast cancer and malignant melanomas. The closely related motexafin gadolinium (MGd, Xcytrin®) has been used in conjunction with whole-brain radiation therapy, leading to improvements in neurocognitive decline and quality of life in non-small cell lung cancer patients with brain metastases.[152] Texaphyrins, especially motexafin gadolinium, have also been developed for use as chemo- and radiosensitisers.[153] Motexafin gadolinium can be viewed as a multitarget anticancer drug because it also behaves as an inhibitor of ribonucleotide reductase.



M = Lu(III) Lutetium texaphyrin (Lutex®, Lutrin®)
M = Gd(III) Motexafin gadolinium (MGd, Xcytrin®)

In addition to their role as photosensitizers, texaphyrins have additional mechanisms of anticancer activity that do not depend on irradiation. They are easier to reduce than porphyrin. Also, in the presence of a variety of reducing metabolites, such as ascorbate, NADPH, thioredoxin, and glutathione, their extended conjugated system can accept one electron, allowing texaphyrins to act as redox mediators and produce ROS in the presence of molecular oxygen, as exemplified in Figure 4.56 for the case of motexafin gadolinium (MGd). Together with the ability to generate oxygen radicals, MGd has been proposed to deactivate the cellular antioxidant system by inhibiting several key enzymes, including the thioredoxin reductase-derived antioxidant system.[138]

Hypericin is a natural extended quinone found in *Hypericum* species (St. John's wort), with a maximum absorbance of 590 nm. It is probably the most potent natural photosensitizer and has been studied for the treatment of a number of tumors.[154]



Hypericin

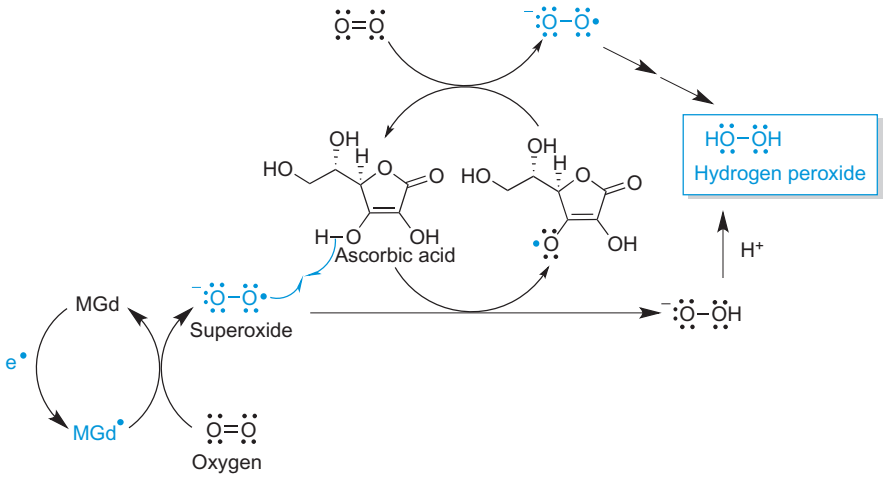


FIGURE 4.56

Generation of oxygen radicals by the texaphyrins.

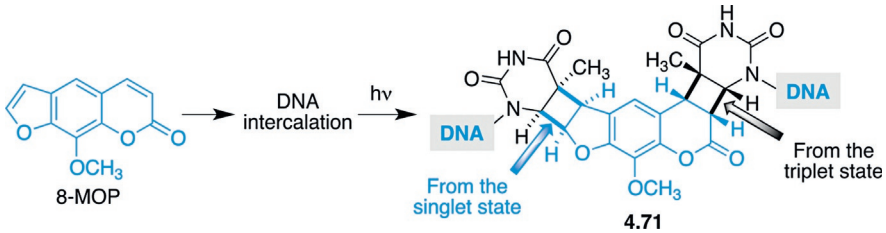


FIGURE 4.57

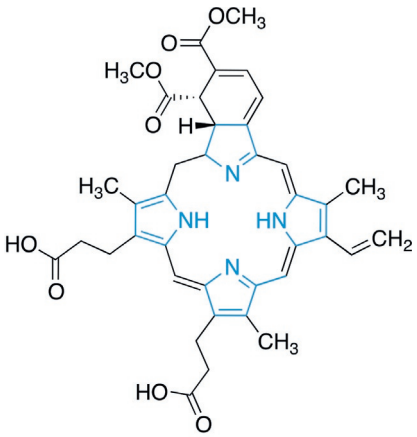
DNA adducts with 8-methoxypsoralen.

Psoralens have been traditionally employed for skin diseases, including psoriasis. The only member of the group approved for cancer treatment is 8-methoxypsoralen (8-MOP, methoxsalen, Uvadex®), which is used in the treatment of cutaneous T cell lymphoma. This compound is administered orally and then some blood is withdrawn, the aberrant white cells are separated and irradiated, and then they are recombined with the other blood constituents and reinjected.[155] In this process, after weakly intercalating into DNA, irradiation of 8-MOP promotes the formation of [2+2] cycloadducts between its 3–4 and 5–6 double bonds and the 5–6 double bonds of adjacent thymidine bases of DNA (Figure 4.57). Both possible monoadducts and bis-adducts **4.71** have been isolated, with the formation of the latter leading to DNA cross-links.[156]

12.3 OTHER APPLICATIONS OF PHOTODYNAMIC THERAPY

Photodynamic strategies have application in the diagnosis of some types of cancer (PDD). Thus, PDD has advantages in terms of sensitivity over conventional white-light cystoscopy in patients with

high-grade, flat, bladder cancer lesions.[157] The dual role of some porphyrinic compounds as imaging and therapeutic tools places them in the increasingly important category of theranostic agents.[158] Furthermore, in addition to cancer treatment, photodynamic therapy is useful for applications such as the treatment of chronic central serous chorioretinopathy.[159] Another important application is the elimination of abnormal blood vessels associated with subretinal choriodal neovascularization in the eye, as exemplified by the treatment of wet macular degeneration using verteporfin (Visudyne®) as a photosensitizer.[160]



Verteporfin (Visudyne®)

REFERENCES

1 Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. 4th ed. Oxford, UK: Oxford University Press; 2007.

2 For a review of drug-induced oxidative stress mechanisms, see Deavall DG, Martin EA, Horner JM, Roberts R. *J Toxicol* 2012;**2012**, Article ID 645460.

3 Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. *Pharmacol Rev* 2004;**56**:185.

4 For a review, see Ray PD, Huang B-W, Tsuji Y. *Cell Signal* 2012;**24**:981.

5 Marnett LJ, Riggins JN, West JD. *J Clin Invest* 2003;**111**:583.

6 Niedernhofer LJ, Daniels JS, Rouzer CA, Greene RE, Marnett LJ. *J Biol Chem* 2003;**278**:31426.

7 Plastaras JP, Dedon PC, Marnett LJ. *Biochemistry* 2002;**41**:5033.

8 Otteneder M, Scott Daniels J, Voehler M, Marnett LJ. *Anal Biochem* 2003;**315**:147.

9 Blair IA. *J Biol Chem* 2008;**283**:15545.

10 For comprehensive treatments of the chemistry of DNA damage, see. (a) Gates KS. In: Platz M, Moss RA, Jones M, editors. *Reviews of reactive intermediate chemistry*. New York: Wiley; 2007; (b) Von Sonntag C. *Free-radical-induced DNA damage and its repair: a chemical perspective*. New York: Springer-Verlag; 2006.

11 McGall GH, Rabow LE, Ashley GW, Wu SH, Kozarich JW, Stubbe J. *J Am Chem Soc* 1992;**114**:4958.

12 Rabow LE, McGall GH, Stubbe J, Kozarich JW. *J Am Chem Soc* 1990;**112**:3203.

13 Dedon PC, Plastaras JP, Rouzer CA, Marnett LJ. *Proc Natl Acad Sci U S A* 1998;**95**:11113.

14 Gajewski E, Rao G, Nackerdien Z, Dizdaroglu M. *Biochemistry* 1990;**29**:7876.
15 Doroshow JH, Synold TW, Somlo G, Akman SA, Gajewski E. *Blood* 2001;**97**:2839.
16 Marnett LJ, Riggins JN, West JB. *J Clin Invest* 2003;**111**:583.
17 Altman SA, Zastawny TH, Randers-Eichhorn L, Cacciuttolo MA, Akman SA, Dizdaroglu M, et al. *Free Rad Biol Med* 1995;**19**:897.
18 For a review, see Nakabeppu Y, Tsuchimoto D, Furuichi M, Sakumi K. *Free Radic Res* 2004;**38**:423–9.
19 For a review see Nakabeppu Y, Tsuchimoto D, Yamaguchi H, Sakumi K. *J Neurosci Res* 2007;**85**:919–34.
20 Gad H, Koolmeister T, Jemth A-S, Eshtad S, Jacques SA, Ström CE, et al. *Nature* 2014;**508**:215.
21 Huber KVM, Salah E, Radic B, Gridling M, Elkins JM, Stukalov A, et al. *Nature* 2014;**508**:222.
22 Laurent G, Jaffrezou JP. *Blood* 2001;**98**:913.
23 Bezombes C, de Thonel A, Apostolou A, Louat T, Jaffrezou JP, Laurent G, et al. *Mol Pharmacol* 2002;**62**:1446.
24 Martin D, Salinas M, Fujita N, Tsuruo T, Cuadrado A. *J Biol Chem* 2002;**277**:42943.
25 Watson J. *Open Biol* 2013;**3**:120144.
26 For a monograph on the anthracyclins, see Krohn K, editor. *Top Curr Chem* 2008;**282/283**.
27 Gewirtz DA. *Biochem Pharmacol* 1999;**57**:727.
28 Siveski-Iliskovic N, Hill M, Chow DA, Singal PK. *Circulation* 1995;**91**:10.
29 Goodman J, Hochstein P. *Biochem Biophys Res Commun* 1977;**77**:797.
30 Vázquez-Vivar J, Martasek P, Hogg N, Masters BS, Pritchard KA, Kalyanaraman B. *Biochemistry* 1997;**36**:11293.
31 Gille L, Nohl H. *Free Radic Biol Med* 1997;**23**:775.
32 Fiallo MML, Drechsel H, Garnier-Suillerot A, Matzanke BF, Kozlowski H. *J Med Chem* 1999;**42**:2844.
33 Tullius TD, Dombrowski BA, Churchill MEA, Kam L. *Methods Enzymol* 1987;**155**:537.
34 Cairo G, Recalcati S, Pietrangelo A, Minotti G. *Free Radic Biol Med* 2002;**32**:1237.
35 Minotti G, Ronchi R, Salvatorelli E, Menna P, Cairo G. *Cancer Res* 2001;**61**:8422.
36 Kwok JC, Richardson DR. *Mol Pharmacol* 2002;**62**:888.
37 Licata S, Saponiero A, Mordente A, Minotti G. *Chem Res Toxicol* 2000;**13**:414.
38 Wiseman LR, Spencer CM. *Drugs* 1998;**56**:385.
39 Schuchter LM, Hensley ML, Meropol NJ, Winer EP. *J Clin Oncol* 2002;**20**:2895.
40 Hasinoff PB, Abram ME, Bernabe M, Khélifa T, Allan WP, Yalowich JC. *Mol Pharmacol* 2001;**59**:453.
41 Skladanowski A, Konopa J. *Biochem Pharmacol* 1994;**47**:2279.
42 Van Rosmalen A, Cullinane C, Cutts SM, Phillips DR. *Nucleic Acids Res* 1995;**23**:42.
43 Wang AHJ, Gao YG, Liaw YC, Li YK. *Biochemistry* 1991;**30**:3812.
44 Taatjes DJ, Gaudiano G, Koch TH. *Chem Res Toxicol* 1997;**10**:953.
45 Cutts SM, Rephaeli A, Nudelman A, Hmelnitzky I, Phillips DR. *Cancer Res* 2001;**61**:8194.
46 Taatjes DJ, Koch TH. *Curr Med Chem* 2001;**8**:15.
47 Koch TH, Barthel BL, Kalet BT, Rudnicki DL, Post GC, Burkhart DJ. *Top Curr Chem* 2008;**283**:141.
48 Wakabayashi I, Groscher K. *Curr Med Chem* 2003;**10**:427.
49 Ziche M, Morbidelli L. *J Neurooncol* 2000;**50**:139.
50 Lown JW, editor. *Anthracycline and anthracenedione-based anticancer agents*. New York: Elsevier; 1988.
51 Monneret C, Eur J. *Med Chem* 2001;**36**:483.
52 Coukell AJ, Faukds D. *Drugs* 1997;**53**:453.
53 Arcamone F, Bernardi L, Giardino P, Patelli B, DiMarco A, Casazza AM. *Cancer Treat Rep* 1976;**60**:829.
54 Umezawa H, Takahashi Y, Kinoschita M, Naganawa H, Matsuda T, Ishizuka M. *J Antibiot* 1979;**32**:1082.
55 Oki T, Matsizawa Y, Yoshimoto A, Numata K, Kitamura I, Hori S. *J Antibiot* 1975;**28**:830.
56 Maral R, Ponsinet G, Jolles G. *C R Acad Sci Ser D* 1972;**275**:301.
57 Israel M, Modest EJ, Frei E. *Cancer Res* 1975;**35**:1365.

58 Melekos MD, Moutzouris GD. *Curr Pharm Design* 2000;**6**:345.
59 Van der Heidjen AG, Witjes JA. *Curr Opin Urol* 2003;**13**:389.
60 Wiley PF, Elrod DW, Houser DJ, Johnson JL, Pschigoda LM, Krueger WC, et al. *J Org Chem* 1979;**44**:4030.
61 Kukuk O, Kilton L, Wade JL, Blough R, Benson AB. *Am J Clin Oncol* 2000;**23**:379.
62 For a review, see Broggini M. *Top Curr Chem* 2008;**283**:191.
63 Sessa C, Zucchett M, Ghielmini M, Bauer J, D'Incalci M, de Jong J, et al. *Cancer Chemother Pharmacol* 1999;**44**:403.
64 Quintieri L, Fantin M, Palatini P, De Martin S, Rosato A, Caruso M, et al. *Biochem Pharmacol* 2008;**76**:784.
65 Marchini S, González O, Ripamonti M, Geroni C, Bargiotti A, Caruso M. *Anticancer Drug Des* 1995;**10**:641.
66 Priebe W, Pérez-Soler R. *Pharmacol Ther* 1993;**60**:215.
67 Arcamone F. *Biochimie* 1998;**80**:201.
68 Cheng CC. *Prog Med Chem* 1983;**20**:83.
69 Adamson RH. *Cancer Chemother Rep* 1974;**58**:293.
70 Estorch M, Carrio I, Martínez-Duncker D, Berna L, Torres G, Alonso C, et al. *J Clin Oncol* 1993;**11**:1264.
71 Thomas X, Le QH, Fiere D. *Ann Hematol* 2002;**81**:504.
72 Gonsette RE. *J Neurol Sci* 2003;**206**:203.
73 Kapuscinski J, Darzynkiewicz Z. *Proc Natl Acad Sci U S A* 1986;**83**:6302.
74 Parker BS, Cutts SM, Cullinane C, Phillips DR. *Nucleic Acids Res* 2000;**28**:982.
75 Edwards SW, Swan TF. *Biochem J* 1986;**237**:601.
76 Kharasch ED, Novak RF. *J Biol Chem* 1985;**260**:500.
77 Parker BS, Cutts SM, Phillips DR. *J Biol Chem* 2001;**276**:15953.
78 Parker BS, Buley T, Evison BJ, Cutts SM, Neumann GM, Iskanders MN, et al. *J Biol Chem* 2004;**279**:18814.
79 Wan X, Zhang W, Li L, Xie Y, Li W, Huang N. *J Med Chem* 2013;**56**:2619.
80 Bachur NR, Gee MV, Gordon SL. *Proc Am Assoc Cancer Res* 1978;**19**:75.
81 Flitter WD, Mason RP. *Arch Biochem Biophys* 1988;**267**:632.
82 Portugal J. *Curr Med Chem Anti-Cancer Agents* 2003;**3**:411.
83 Chen J, Stubbe JA. *Nature Rev Cancer* 2005;**5**:102.
84 Sleijfer S. *Chest* 2001;**120**:617.
85 Takahashi K, Ekimoto H, Minamide S, Nishikawa K, Kuramochi H, Motegi A, et al. *Cancer Treat Rev* 1987;**14**:169.
86 Nicaise C, Hong WK, Dimery W, Usakewicz J, Rozencweig M, Krakoff I. *Invest New Drugs* 1990;**8**:325.
87 Westre TE, Loeb KE, Zaleski JM, Hedman B, Hogdson KO, Solomon EI. *J Am Chem Soc* 1995;**117**:1309.
88 Solomon EI, Brunold TC, Davis MI, Kemsley JN, Lee SK, Lehnert N, et al. *Chem Rev* 2000;**100**:235.
89 Boger DL, Cai H. *Angew Chem Int Ed Engl* 1999;**38**:449.
90 Povirk LF, Hogan M, Dattagupta N. *Biochemistry* 1979;**18**:96.
91 Carter BJ, Murty VS, Reddy KS, Wang SN, Hecht SM. *J Biol Chem* 1990;**265**:4193.
92 Wu W, Vanderwall DE, Stubbe J, Kozarick JW, Turner CJ. *J Am Chem Soc* 1994;**116**:10843.
93 Ohno M. *Pure Appl Chem* 1989;**61**:581.
94 Walker MA, Dalton King H, Dalterio RA, Trail P, Firestone R, Dubowchik GM. *Bioorg Med Chem Lett* 2004;**14**:4323.
95 For a comprehensive online course on the bioorganic chemistry of the enedyines, see Bag SS. http://nptel.ac.in/courses/104103068/37.
96 Abe S, Otsuki M. *Curr Med Chem Anti-Cancer Agents* 2002;**2**:715.
97 Takeshita A, Shinjo K, Naito K, Matsui H, Sahara N, Shigeno K, et al. *Leukemia* 2005;**19**:1306.
98 For a review, see Shao RG, Zhen YS. *Anti-Cancer Agents Med Chem* 2008;**8**:123.
99 Kumar RA, Ikemoto N, Patel DJ. *J Mol Biol* 1997;**265**:187.
100 Bergmann RG. *Acc Chem Res* 1973;**6**:25.

101 Myers AG, Proteau PJ. *J Am Chem Soc* 1989;**111**:1146.
102 Kappen LS, Goldberg IH. *Science* 1993;**261**:1319.
103 Nicolaou KC, Dai WM. *J Am Chem Soc* 1992;**114**:8908.
104 Brown JM. *Cancer Res* 1999;**59**:5863.
105 Brown J, Wang LH. *Anticancer Drug Des* 1998;**13**:529.
106 Lara PN, Frankel P, Mack PC, Gumerlock PH, Galvin I, Martel CL, et al. *Clin Cancer Res* 2003;**9**:4356.
107 Aquino VM, Weitman SD, Winick NJ, Blaney S, Furman WL, Kepner JL, et al. *J Clin Oncol* 2004;**22**:1413.
108 Maxim PG, Carson JJL, Ning S, Knox SJ, Boyer AL, Hsu CP, et al. *Radiat Res* 2004;**162**:185.
109 Kotandeniya D, Ganley B, Gates KS. *Bioorg Med Chem Lett* 2002;**12**:2325.
110 Anderson RF, Shinde SS, Hay MP, Gamage SA, Denny WA. *J Am Chem Soc* 2003;**125**:748.
111 Peters KB, Brown JM. *Cancer Res* 2002;**62**:5248.
112 Liu G, Berlin J, Tutsch KD, Van Ummersen L, Dresen A, Marnocha R, et al. *Clin Cancer Res* 2002;**8**:706.
113 Ringborg U, Bergqvist D, Brorsson B, Cavallin-Ståhl E, Ceberg J, Einhorn N, et al. *Acta Oncol* 2003;**42**:357.
114 Wouters BG, Brown JM. *Radiat Res* 1997;**147**:541–50.
115 For a review of tumor targeting with radiometals, see Ramogida CF, Orvig C. *Chem Commun* 2013;**49**:4720.
116 http://clinicaltrials.gov/show/NCT01618370.
117 Yin L, Hu Q, Hartmann RWH. *Int J Mol Sci* 2013;**14**:13658.
118 Shahri KK, Motavalli RF, Hakimabad HM. *Hell J Nucl Med* 2011;**14**, editorial.
119 For a review of the chemistry of neutron capture therapy, see Soloway AH, Tjarks W, Barnum BA, Rong F-G, Barth RF, Codogni IM, et al. *Chem Rev* 1998;**98**:2389.
120 For a review, see Sivaev IB, Bregadze VI. *Arkivoc* 2008;(4):47.
121 Garabalino MA, Heber EM, Hughes AM, González SJ, Molinari AJ, Pozzi ECC, et al. *Radiat Environ Biophys* 2013;**52**:351.
122 For a review of the use of drugs to improve radiotherapy, see Begg AC, Stewart FA, Vens C. *Nature Rev Cancer* 2011;**11**:239.
123 For reviews, see. (a) Weinmann M, Welz S, Bamberg M. *Curr Med Chem Anti-Cancer Agents* 2003;**3**:364; (b) Wardman P. *Clin Oncol* 2007;**19**:397.
124 Eschwege F, Sancho-Garnier H, Chassagne D, Brisgand D, Guerra M, Malaise EP, et al. *Int J Radiat Oncol Biol Phys* 1997;**39**:275.
125 Bennewith KL, Raleigh JA, Durand RE. *Cancer Res* 2002;**62**:6827.
126 Overgaard J, Sand Hansen A, Overgaard M, Bastholtd L, Berthelsen A, Specht L, et al. *Radiother Oncol* 1998;**46**:135.
127 Cowen RL, Williams KJ, Chinje EC, Jaffar M, Sheppard FCD, Telfer BA, et al. *Cancer Res* 2004;**64**:1396.
128 Laino C. *Oncol Times* 2012;**9**:6.
129 http://www.cancer.gov/clinicaltrials/search/view?cdrid=373922&version=HealthProfessional&protocolsearchid=12356206.
130 Laidig KE, Gainer JL, Daggett V. *J Am Chem Soc* 1998;**120**:9394.
131 Sheehan J, Ionescu A, Pouratian N, Hamilton DK, Schlesinger D, Oskouian Jr RJ, et al. *J Neurosurg* 2008;**108**:972.
132 For representative reviews, see. (a) Nair CCK, Parida DK, Nomura T. *J Radiat Res* 2001;**42**:21; (b) Citrin D, Cotrim AP, Hyodo F, Baum BJ, Krishna MC, Mitchell JB. *Oncologist* 2010;**15**:360.
133 For a review of amifostine, see Kouvaris JR, Kouloulias VE, Vlahos LJ. *Oncologist* 2007;**12**:738.
134 Castano A, Demidova TN, Hamblin MR. *Photodiagn Photodyn Ther* 2004;**1**:279.
135 Chen Q, Hetzel FW. *J Clin Laser Med Surg* 1998;**16**:9.
136 Tacquet JP, Frochot C, Manneville V, Barberi-Heyob M. *Curr Med Chem* 2007;**14**:1673.
137 Dolmans DE, Fukumura D, Jain RK. *Nat Rev Cancer* 2003;**3**:380.
138 Kato H. *J. Photochem Photobiol* 1998;**42**:96.

139 Allison RR, Cuenca RE, Downie GH, Camnitz P, Brodish B, Sibata CH. *Photodiagn Photodyn Ther* 2005;**2**:205.
140 Allison MR, Mota HC, Sibata CH. *Photodiagn Photodyn Ther* 2004;**1**:263.
141 For a review of preclinical and clinical advances in photodynamic therapy, see O'Connor AE, Gallagher WM, Byrne AT. *Photochem Photobiol* 2009;**85**:1053.
142 For reviews, see. (a) Wainwright M. *Rev Progr Color* 2004;**34**:95; (b) Wilson BB, Patterson MS. *Phys Med Biol* 2008;**53**:R61.
143 Marcus SL, McIntyre WR. *Emerging Drugs* 2002;**7**:321.
144 Kikuchi T, Asakura T, Aihara H, Shiraki M, Takagi S, Kinouchi Y, et al. *Anticancer Res* 2003;**23**:4897.
145 Lorenz KJ, Maier H. *HNO* 2008;**56**:402.
146 Mang TS, Allison R, Hewson G, Snider W, Moskowoitz R. *Cancer J Sci Am* 1998;**4**:378.
147 Brunner H, Hausmann F, Krieg RC, Endlicher E, Scholmerich J, Knuechel R, et al. *Photochem Photobiol* 2001;**74**:721.
148 Coleman J, Scherz A. *Eur Urolog Rev* 2012;**107**.
149 Azzouzi AR, Barret E, Moore CM, Villers A, Allen C, Scherz A, et al. *BJU Int* 2013;**112**:766.
150 Kalka K, Merk H, Mukhtar H. *J Am Acad Dermatol* 2000;**42**:389.
151 For reviews, see. (a) Arambula JF, Preihs C, Borthwick D, Magda D, Sessler JL. *Anticancer Agents Med Chem* 2011;**11**:222; (b) Preihs C, Arambula JF, Magda D, Jeong H, Yoo D, Cheon J, et al. *Inorg Chem* 2013;**52**:12184.
152 Richards GM, Mehta MP. *Expert Opin Pharmacother* 2007;**8**:351.
153 Magda D, Richards GM. *Semin Cancer Biol* 2006;**16**:466.
154 For a review, see Agostinis P, Vantieghem A, Merlevede W, Witteb PAM. *Int J Biochem Cell Biol* 2002;**34**:221.
155 Cimino GD, Gamper HB, Isaacs ST, Hearst JE. *Ann Photochem Photobiol* 1997;**66**:141.
156 Kanne D, Straub K, Hearst JE, Rapopport H. *J Am Chem Soc* 1982;**104**:6754.
157 Zaak D, Wieland WF, Stief CG, Burger M. *Eur Urol Suppl* 2008;**7**:536.
158 For a review of this aspect of photodynamic therapy, see Josefsen LB, Boyle RW. *Theranostics* 2012;**2**:916.
159 Karim SP, Adelman RA. *Clin Ophthamol* 2013;**7**:1867.
160 Reeves BC, Harding SP, Langham J, Grieve R, Tomlin K, Walker J, et al. *Health Technol Assess* 2012;**16**:1.

CHAPTER

DNA ALKYLATING AGENTS

5

CONTENTS

1 INTRODUCTION

Anticancer drugs that target DNA have been used in the clinic for more than 60 years.[1,2] Despite the recent major advances in cancer research, the mechanism by which most clinically relevant anticancer drugs kill cells consists of interference with replication, which can be achieved most simply by DNA alkylation. Other agents that also interfere with replication, such as compounds interacting with the DNA minor groove (sometimes via alkylation) and DNA intercalators, are discussed in the following chapters.

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00005-3

Alkylating agents can be defined as compounds capable of covalently attaching an alkyl group to a biomolecule under physiological conditions (aqueous solution, 37°C, pH 7.4). DNA alkylating agents interact with resting and proliferating cells in any phase of the cell cycle, but they are more cytotoxic during the late G_1 and S phases because not enough time is available to repair the damage before DNA synthesis takes place.

In principle, covalent bonds can arise from attack of either nucleophilic or electrophilic species to DNA, and indeed some nucleophiles (e.g., hydrazine, hydroxylamine, and bisulfite) are known to attack DNA bases under physiological conditions. However, because all nitrogen and oxygen atoms of these bases are nucleophiles, with the exception of the nitrogen atoms involved in the nucleoside bond (N^9 or N^1 in purines or pyrimidines), therapeutically useful drugs always behave as carbon electrophiles.[3] Two related but independent interactions govern attraction between nucleophiles and electrophiles: electrostatic attraction between positive and negative charges (electrostatic control) and orbital overlap between the HOMO of the nucleophile and the LUMO of the electrophile (orbital control). These two types of reactivity have been termed "hard" and "soft," respectively. Thus, the highly electronegative oxygen atoms tend to react under electrostatic control and are considered as "hard" nucleophiles, and accordingly they react with "hard" electrophiles—that is, those with a more pronounced cationic character. Due to the fact that nitrogen atoms of DNA bases are softer nucleophiles than oxygen atoms and that many therapeutically useful alkylating agents are relatively "soft" electrophiles, DNA alkylating compounds react mainly at nitrogen sites in the following order: N^7 of guanine > N^1 of adenine > N^3 of cytosine > N^3 of thymine. On the other hand, diazonium salts, generated from nitrosoureas and other antitumor agents, are examples of therapeutically relevant "hard" electrophiles, which tend to preferentially alkylate oxygen atoms at phosphate residues and carbonyl oxygen atoms in DNA bases, especially O^6 of guanine. DNA alkylation is also governed to a great extent by steric effects, and nucleophilic sites placed inside the double helix are less exposed to alkylation, whereas those in the major and minor grooves are more easily attacked.[4]

The structure and dynamics of DNA are greatly affected by alkylation of its bases, which leads to several types of effects. First, alkylation prevents DNA replication and RNA transcription from the affected DNA molecule. It also leads to the fragmentation of DNA by hydrolytic reactions and by the action of repair enzymes when attempting to remove the alkylated bases. Alkylation also induces the mispairing of the nucleotides by alteration of the normal hydrogen bonding between bases. Finally, compounds capable of bis-alkylation can form bridges within a single DNA strand (intrastrand cross-linkage, also known as limpet attachment) or between two complementary DNA strands (interstrand cross-linkage), preventing their separation during DNA replication or transcription. They can also lead to cross-linking between DNA and associated proteins (Figure 5.1). It has been proven that bifunctional alkylating compounds are considerably more cytotoxic than their monofunctional counterparts, and also that there is a direct correlation between the degree of interstrand cross-linking and cytotoxicity.

2 NITROGEN MUSTARDS

2.1 INTRODUCTION

Sulfur mustard (mustard gas, yperite) was used in World War I for chemical warfare because it is an extremely irritant vesicant agent. After the war, it was realized that it also caused systemic effects such as leukopenia, aplasia of the bone marrow, dissolution of lymphoid tissue, and ulceration of the

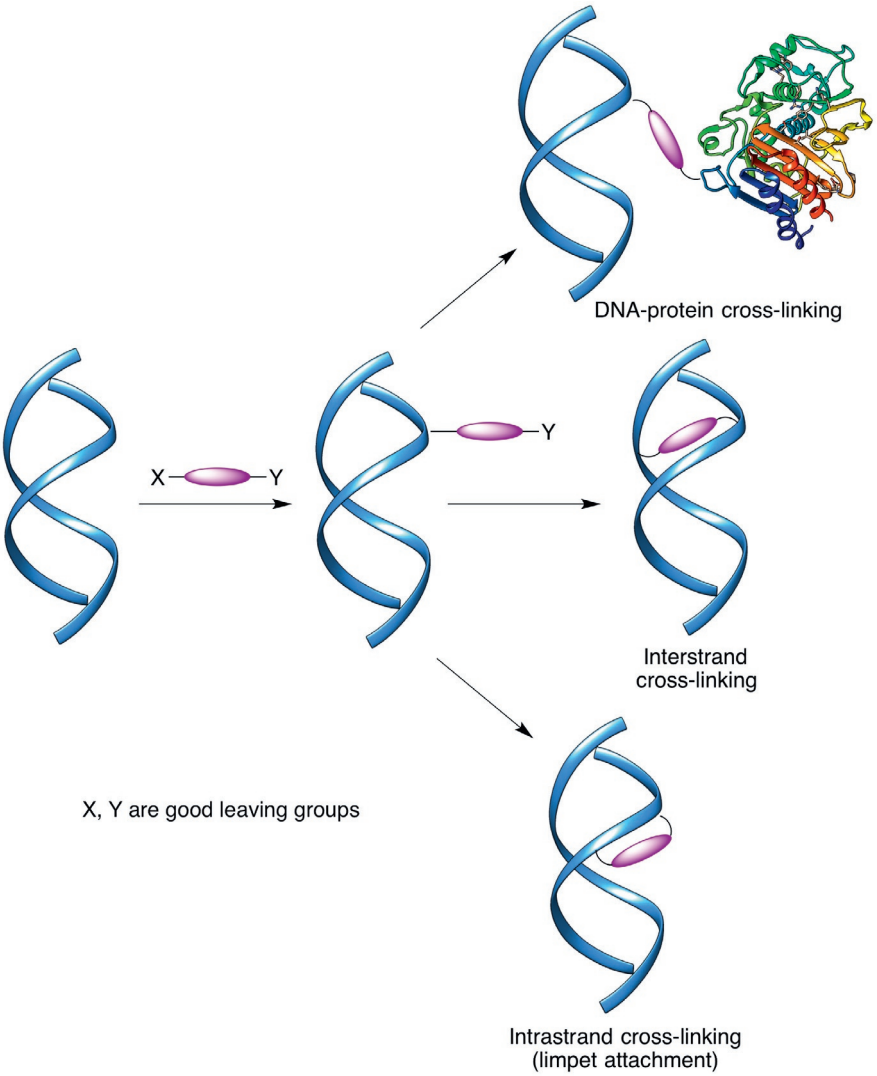
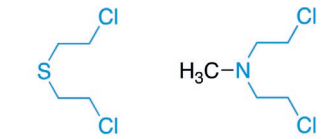


FIGURE 5.1

Different types of links produced on DNA by bis-alkylating agents.

gastrointestinal tract. These effects suggested a possible role for this compound in cancer treatment, but after an exploratory study it was considered too toxic for systemic use.[5] In late 1943, a ship (SS *John Harvey*) carrying a secret cargo of this compound was sunk in Bari, Italy, by German aircraft, leading to the release of sulfur mustard vapor in the harbor and over the city and causing at least 83 deaths. In the course of the investigation of this disaster, it was observed that mustard gas led to profound lymphoid and myeloid suppression, which suggested that it might also be able to kill rapidly dividing cancer cells.

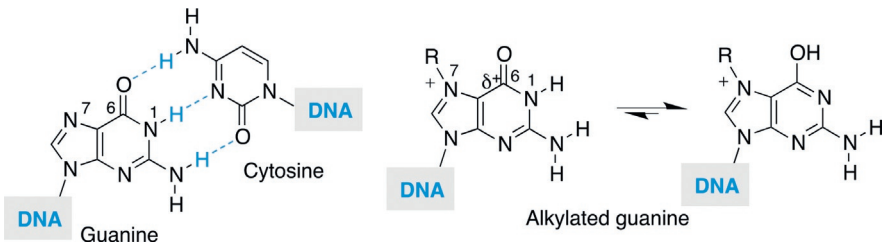
Prompted by this report and by their own previous work on the effects of mustard gas on animals, the Yale pharmacologists Louis Goodman and Alfred Gilman tested mechlorethamine (chlormethine, mustine), a nitrogen analog of mustard gas, on animals and then humans and found it to be effective as a treatment for lymphoma, including Hodgkin's lymphoma and acute lymphoblastic leukemia. This study was classified at that time and was not published until 1946, when it started the modern era of cancer chemotherapy.[6] Even at this early stage, it was soon apparent that the therapeutic effect of nitrogen mustards was limited by marrow toxicity and the development of resistance, which are still a source of problems in cancer chemotherapy today. These problems notwithstanding, mechloretamine (Mustargen®) was approved in 1949, and it is still used as part of some antitumor regimes for the chemotherapy of Hodgkin's lymphoma.[7] In 2013, a mechloretamine gel (Valchlor®) was approved for the topical treatment of stage IA/IB mycosis fungoides-type cutaneous T-cell lymphoma.



2.2 DNA ALKYLATION BY NITROGEN MUSTARDS AND CYTOTOXICITY MECHANISMS

Due to the relative unreactivity of alkyl chlorides as electrophiles, direct attack of DNA nucleophilic centers by nitrogen mustards under physiological conditions is too slow to be of therapeutic relevance. The reason why nitrogen mustards have a high reactivity as alkylating agents under mild conditions is the anchimeric assistance from the nitrogen atom—that is, the formation through an intramolecular nucleophilic substitution of the aziridinium cation **5.1**, which is highly reactive because of the positive charge at the leaving group and the high strain of the three-membered ring, which is relieved in the alkylation process. Because the most nucleophilic atom in DNA is the N-7 nitrogen of guanine, the most common species arising from alkylation is **5.2** (Figure 5.2).

As mentioned previously, one consequence of alkylation is the alteration of the normal pairing of DNA bases between adenine–thymine and guanine–cytosine (Watson–Crick base pairs). For instance, the three hydrogen bonds normally linking guanine and cytosine require the existence of a carbonyl group at the purine C-6 position. Because alkylation at N-7 creates a positive charge on this center, which is adjacent to the partial positive charge at C-6 due to the electron deficiency of the carbonyl group, the tautomeric equilibrium is displaced to the more stable 6-hydroxy form.[8] This change in the normal tautomeric form converts hydrogen bond acceptor groups into donors and vice versa.



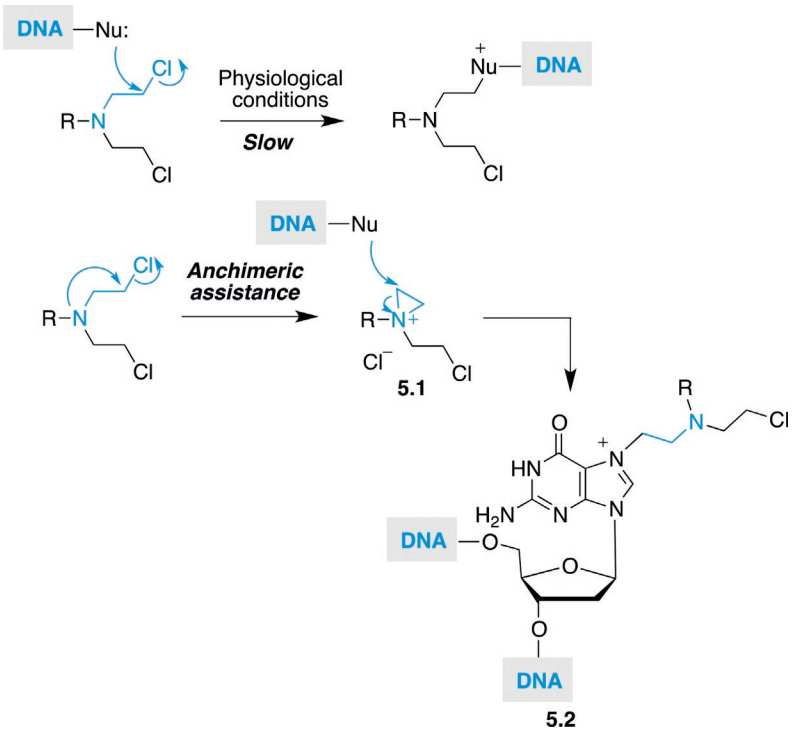
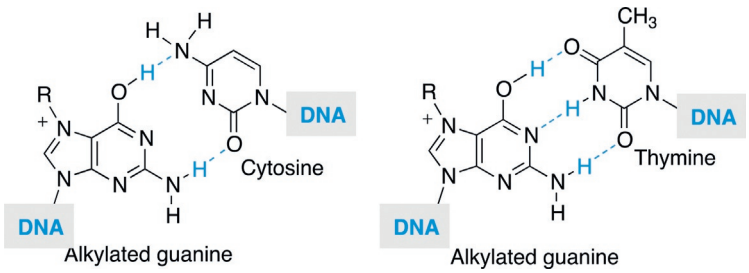


FIGURE 5.2

Mechanism of DNA alkylation by nitrogen mustards.

As a consequence, hydrogen bonding with cytosine is weakened because only two bonds can be established at best, whereas pairing of the 6-hydroxy species with thymine leads to a more stable complex (three hydrogen bonds). The normal pairing is altered to guanine–thymine, leading to mutations.



Another consequence of guanine alkylation is an increase in the electrophilicity of positions adjacent or conjugated to the positive charge at N-7, which leads to several hydrolytic reactions that alter the DNA structure. Thus, cleavage of the heteroside bond in structure **5.2**, although slow,[9] induces DNA depurination to give **5.3**. This structure is in equilibrium with the open form **5.4**, which has a good leaving

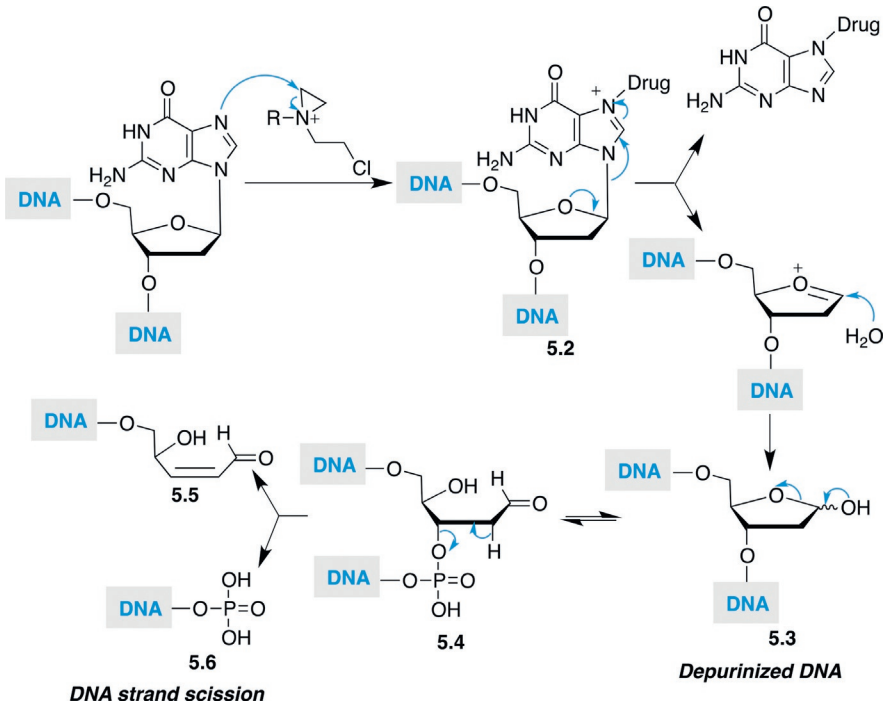


FIGURE 5.3

DNA fragmentation triggered by guanine alkylation.

group (phosphate oxygen) at the β position with respect to the carbonyl group, leading to an easy elimination process whereby DNA is fragmented to **5.5** and **5.6** (Figure 5.3).

Another position of the purine ring that acquires increased electrophilicity upon alkylation is C-8, which is adjacent to the positive charge generated in the alkylation reaction. Water addition to C-8 leads to intermediate **5.7**, which then evolves to **5.8**. This compound has an imine structure that upon hydrolysis gives **5.4** with subsequent DNA strand scission (Figure 5.4).

Because nitrogen mustards are bifunctional alkylating agents, one of their cytotoxicity mechanisms is related to their ability of DNA-monoalkylated species **5.9** to give covalent DNA interstrand and intrastrand cross-links (**5.10**) or DNA–protein complexes (**5.11**), leading to disruption of replication or transcription (Figures 5.1 and 5.5).

2.3 STRUCTURE–ACTIVITY RELATIONSHIPS IN NITROGEN MUSTARDS

Although mechloretamine was an improvement over sulfur mustard, it was still highly vesicant and chemically labile because of its very rapid reaction with biological material and water, respectively. Replacement of its methyl group by an aromatic ring lowers its reactivity because the electron-withdrawing effect of this type of substituent hampers anchimeric assistance to alkylation by the

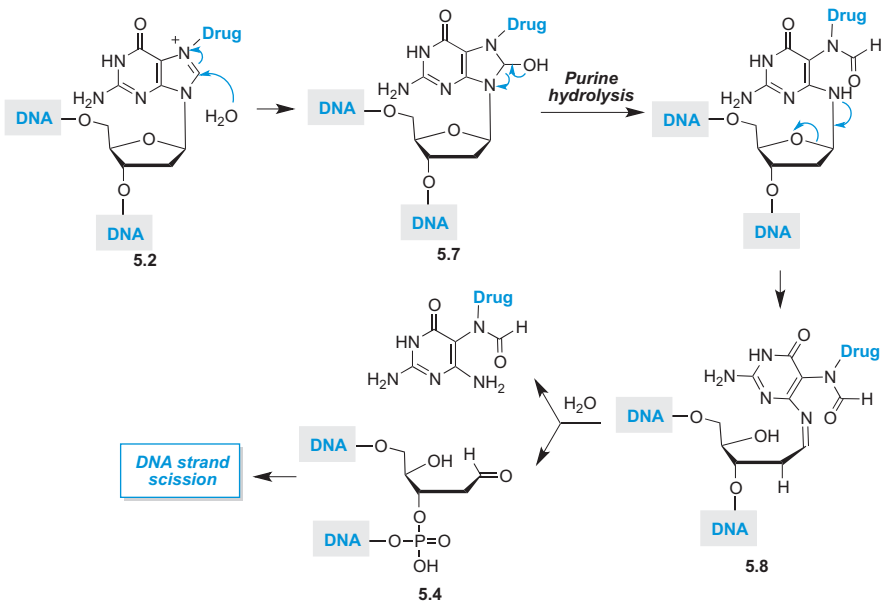
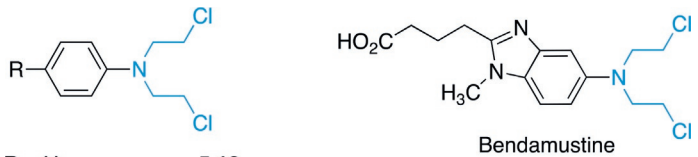


FIGURE 5.4

Other DNA fragmentation processes that take place following guanine alkylation.

nitrogen atom. The increased stability gives enough time for absorption and distribution before alkylation takes place and therefore allows oral administration. The simplest aromatic nitrogen mustard is compound **5.12**, which has the disadvantage of not being water-soluble. Addition of a carboxyl group led to the soluble analog **5.13**, which was inactive. However, simple separation of the carboxylic group from the aromatic ring by a spacer yielded active compounds such as chlorambucil (Leukeran®). This compound, approved in 1957, is an orally administered alkylating agent mainly used to treat chronic lymphocytic leukemia and lymphomas. Bendamustine (Ribomustin®, Treandra®, Levact®) is a related nitrogen mustard in which the benzene ring has been replaced by a benzimidazole. It is sometimes used to treat chronic lymphocytic leukemia, B-cell non-Hodgkin's lymphoma, and myeloma, and its use has undergone a recent renaissance.[10]



The butyric acid moiety of chlorambucil and bendamustine modulates not only their aqueous solubility and reactivity as alkylating agents but also their metabolism. Thus, a significant fraction of

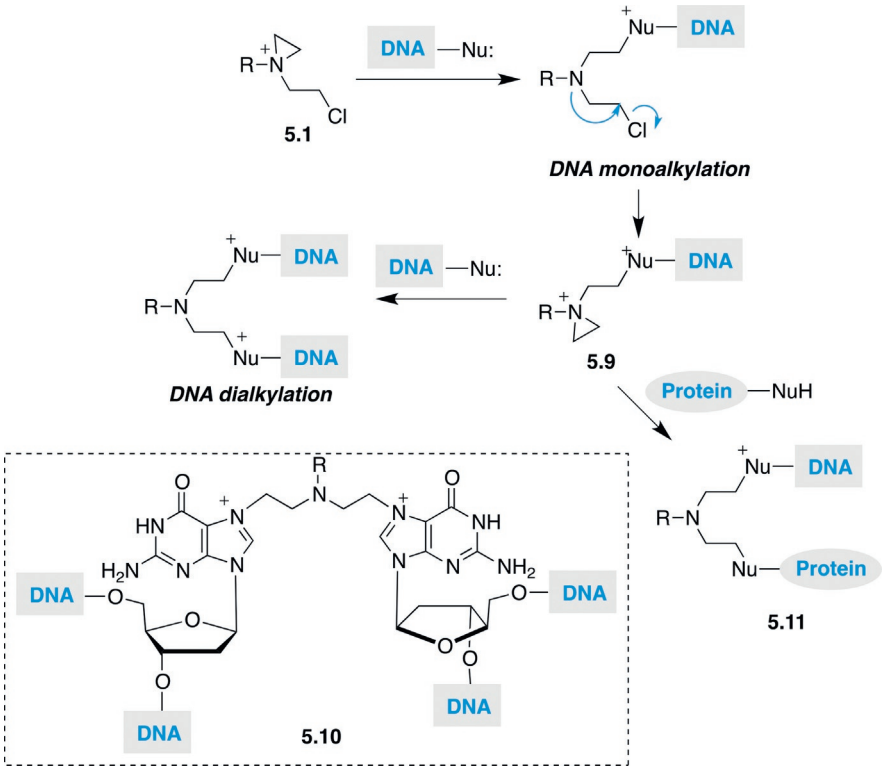
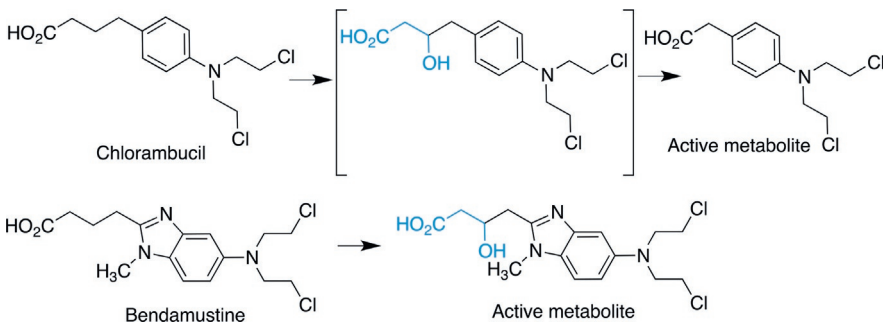


FIGURE 5.5

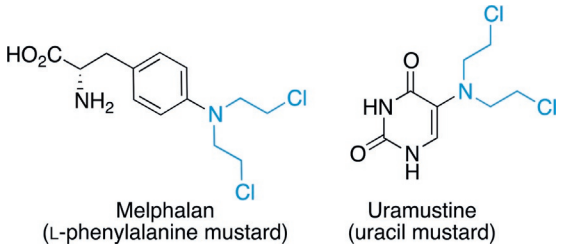
DNA cross-linking by nitrogen mustards.

chlorambucil is metabolically degraded to an active phenylacetic acid-derived mustard via β-hydroxylation, following the biochemical pathway employed for fatty acid degradation. In the case of bendamustine, the hydroxy metabolite, which is also active, is more stable and is apparently not transformed into the acetic acid analog.[11]



2.4 SITE-DIRECTED NITROGEN MUSTARDS

Due to their high toxicity, considerable effort has been devoted to the development of site-directed mustards. Initial strategies were based on the incorporation of moieties that were expected to accumulate preferentially in tumor cells. Thus, melphalan (Alkeran®), which contains an L-phenylalanine unit, was postulated to concentrate in melanomas because melanine is a product of phenylalanine metabolism. Although this original rationale was not correct, melphalan is used in certain types of bone marrow tumors such as multiple myeloma and ovarian or breast cancers. In addition, because of its myeloablative properties and broad antitumor effects as a DNA alkylating agent, melphalan remains the most widely used agent in preparative regimes for hematopoietic stem cell transplant.[12] The main role of its side chain is to facilitate drug uptake by employing two amino acid transport systems to enter tumor cells: the sodium-independent L-amino acid system and the sodium-dependent transporter for alanine, serine, and cysteine (ASC carrier). Other compounds designed on similar principles are uramustine (uracil mustard) and estramustine (see later).



In contrast to chlorambucil, the bioavailability of orally administered melphalan is highly variable, which can be attributed to its very rapid chemical degradation to the mono- and dihydroxy derivatives **5.14** and **5.15**.[13] *In vitro* studies have shown that this hydrolysis is pH dependent and takes place preferentially under neutral or basic conditions,[14] suggesting that the electron-withdrawing effect of the protonated amino group hampers aziridinium ion generation and its subsequent hydrolysis (Figure 5.6).

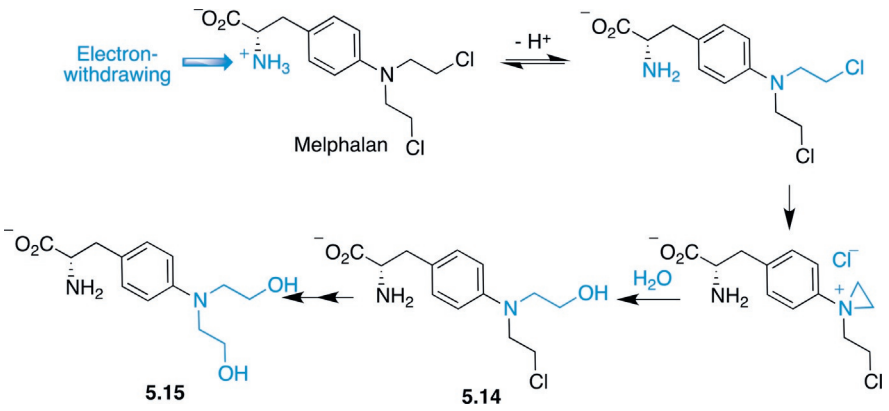
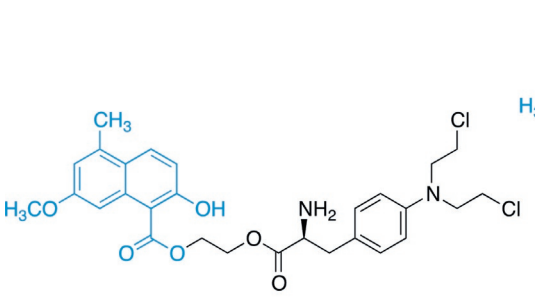


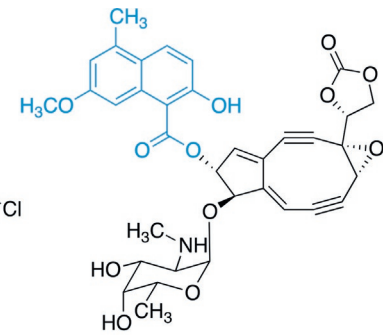
FIGURE 5.6

Chemical degradation of melphalan.

The hydrolytic stability of melphalan has been improved by the preparation of the analog **5.16**. This compound contains a naphthoate portion that can be found in the antitumor enediyne antibiotic neocarzinostatin (see Chapter 4, Section 8), and its design was based on the knowledge of the role of this structural fragment in the complexation of neocarzinostatin to a protein called apo-neocarzinostatin, which greatly increases its stability.[15]



5.16



Neocarzinostatin

In another approach to stabilizing melphalan toward hydrolysis, a compound known initially as J-1 and later as melphalan–flufenamide was designed, which showed promising preclinical activity in several types of solid tumors[16] that prompted further clinical investigation in adult patients with advanced solid tumors, advanced ovarian cancer, or non-small cell lung cancer.[17] The intact peptide is less effective in the alkylation of nucleophilic sites at DNA, probably due to steric hindrance. Nevertheless, melphalan–flufenamide exhibits significantly higher *in vitro* and *in vivo* cytotoxicity than melphalan. *In vitro* studies show that following rapid incorporation into the cytoplasm of tumor cell lines, intracellular hydrolysis results in the release of melphalan. Melphalan–flufenamide may thus be considered as a prodrug of melphalan, and its enzymatic activation (Figure 5.7) is performed by aminopeptidases such

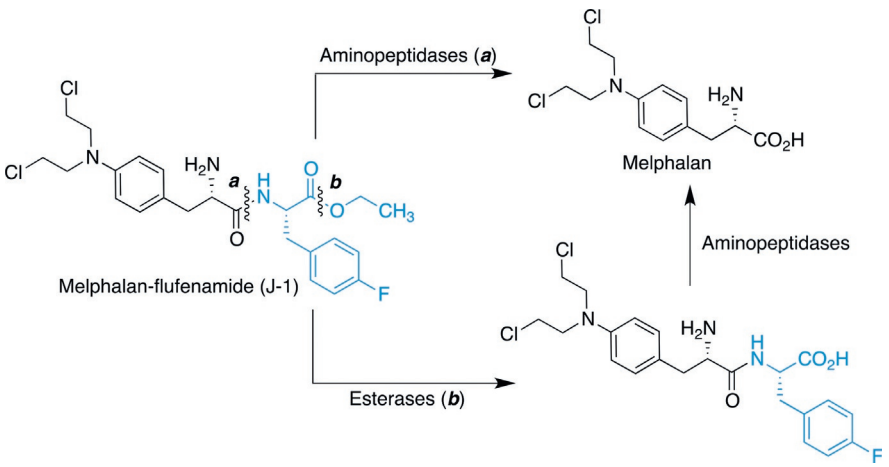


FIGURE 5.7

Bioactivation of melphalan–flufenamide.

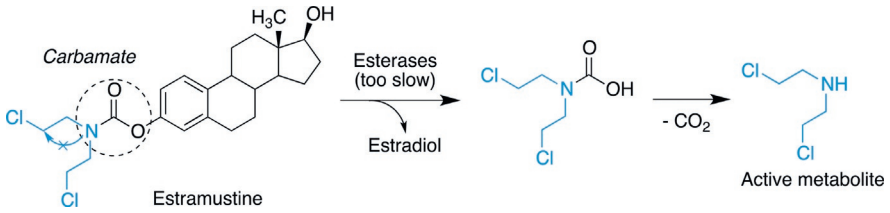


FIGURE 5.8

Metabolism of estramustine.

as aminopeptidase N (APN).[18] Interestingly, this enzyme is overexpressed in certain malignancies, which provides an opportunity to achieve some tumor selectivity.[19]

Estramustine (Estracyt®, Emcyt®), which consists of a β-estradiol unit linked to a nitrogen mustard portion via a carbamate bridge, was designed as a prodrug because the electron-withdrawing effect of the carbonyl group makes the electron density of its nitrogen atom insufficient to trigger aziridinium formation. Thus, estramustine was expected to target estrogenic hormone receptors before release of the active nitrogen mustard group following cleavage of the carbamate ester link. However, this function turned out to be too stable for enzymatic cleavage, and therefore estramustine does not possess significant alkylating activity (Figure 5.8).[20] On the other hand, although estramustine is metabolized to estrogens both *in vitro* and *in vivo*, it is also active in tissues and cell lines that lack estrogen receptors, and therefore its antitumor activity is not hormone related; in fact, it is due to microtubule disassembly, as will be discussed in Section 4 of Chapter 9.

Another strategy for the development of site-directed nitrogen mustards can be selective bioactivation of prodrug forms if biochemical differences can be found between a tumor and normal tissues. Thus, cyclophosphamide (Cytoxan®, Endoxana®), first reported in 1958, which is inactive because the electron-withdrawing effect of the P=O bond prevents the generation of aziridinium cations (Figure 5.9), was expected to be activated by phosphoramide enzymatic hydrolysis on the basis of a report stating that some tumors contain high levels of phosphoramidases. This compound has become the main antitumor drug of the alkylating class, being used to treat Hodgkin's disease and other lymphomas, leukemias, and lung, breast, and ovarian cancers, often in combination with other drugs. Its combination with bortezomib and dexamethasone is highly effective in multiple myeloma.[21] Other uses include the treatment of Wegener's granulomatosis, severe rheumatoid arthritis, and lupus

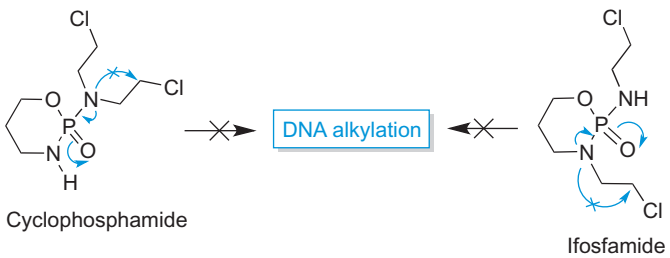


FIGURE 5.9

Cyclophosphamide and ifosfamide are inactive as such.

erythematosus; the drug also has immunosuppressant action in smaller doses. Ifosfamide (Mitoxana®), a more recent related compound, is usually used to treat sarcoma, testicular cancer, and some types of lymphomas.

The original assumption about cyclophosphamide hydrolytic bioactivation soon proved to be wrong, and several studies showed that cyclophosphamide is not metabolized by hydrolysis but, rather, by hepatic P450 oxidation to give 4-hydroxycyclophosphamide, which is in equilibrium with its acyclic aldophosphamide form. Hepatic alcohol dehydrogenase transforms these compounds into the inactive metabolites 4-ketocyclophosphamide and carboxyphosphamide, respectively, which explains the low hepatic toxicity of this drug. Some of the hydroxycyclophosphamide is carried throughout the body by the bloodstream and is further activated by a spontaneous elimination reaction that yields acrolein and phosphoramide mustard, the main cytotoxic species. The negative charge on the phosphoramidate oxygen atom balances the electron-withdrawing effect of the P=O group and allows its activation to an aziridinium cation. Phosphoramide mustard can be hydrolyzed to nornitrogen mustard, which is also active (Figure 5.10).

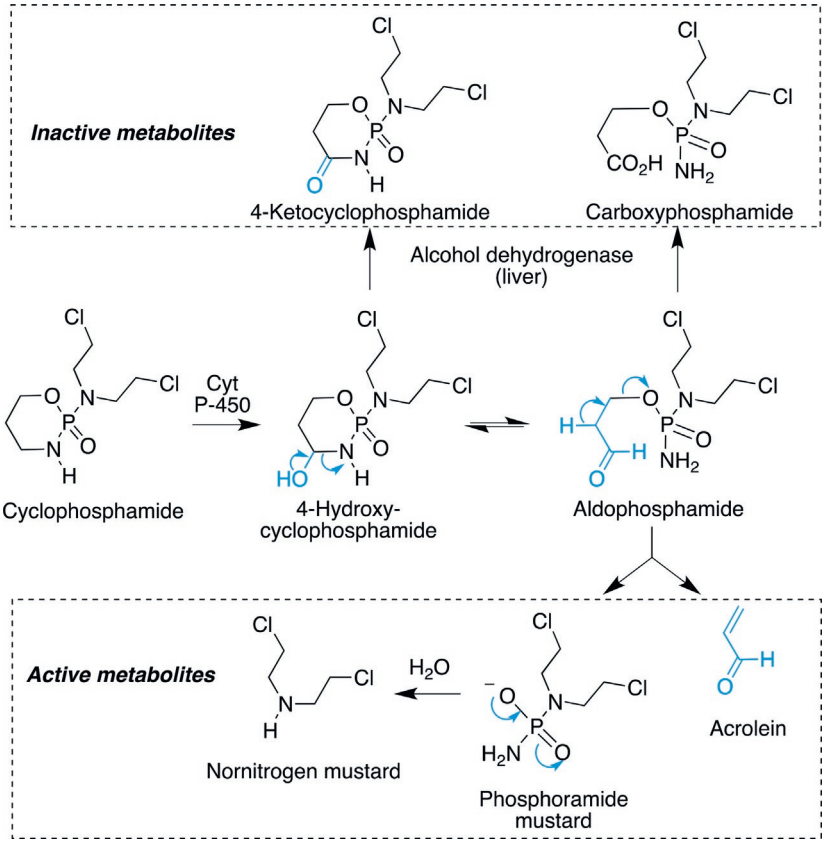


FIGURE 5.10

Bioactivation of cyclophosphamide.

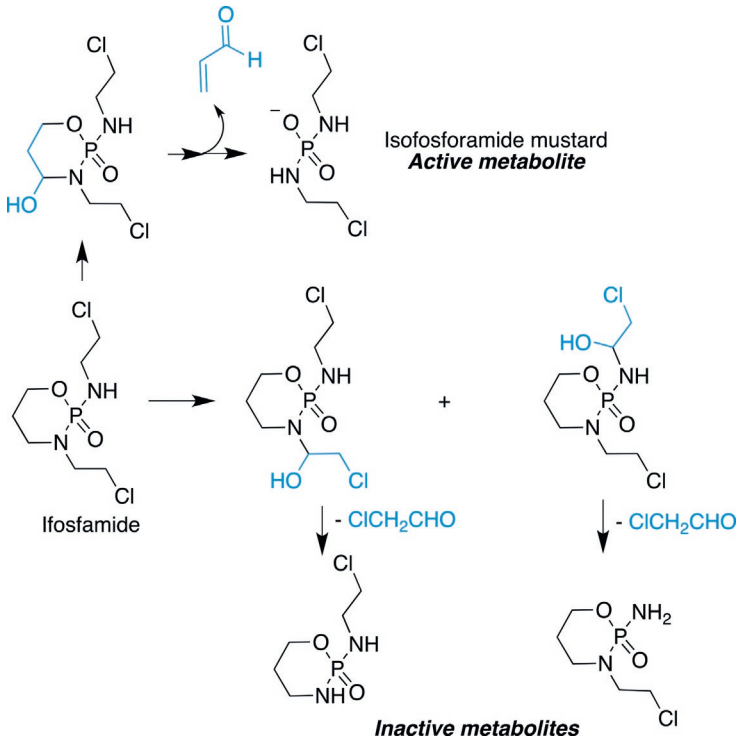


FIGURE 5.11

Bioactivation of ifosfamide.

Despite their structural similarity, ifosfamide metabolism is different from that of cyclophosphamide. Thus, although the active metabolite, isofosforamide mustard, is generated from the 4-hydroxy derivative by the same mechanism described for cyclophosphamide, this hydroxylation is slower than that of the chloroethyl side chains attached to the exocyclic nitrogen, probably because of steric hindrance on the C-4 position, which allows formation of inactive metabolites by competing *N*-dealkylation (Figure 5.11). These differences explain the need for higher doses to achieve the same effect, when compared to cyclophosphamide.

Acrolein, the second product from the elimination reaction, is less active as an antitumor agent despite its high electrophilicity. On the other hand, it appears to be responsible for a major side effect of cyclophosphamide and iphosphamide, namely the development of hemorrhagic cystitis.[22] This problem can be reduced by coadministration of both drugs with a thiol acrolein scavenger such as *N*-acetylcysteine or mesna (sodium 2-mercaptoethanesulfonate). Because these thiol compounds are found as disulfides in plasma, they do not react with the alkylating species responsible for the cytotoxic activity. In the kidney, they are reduced by glutathione transferase, liberating the thiol, which inactivates acrolein through a conjugate addition, giving compounds such as **5.17** in the case of mesna (Figure 5.12).

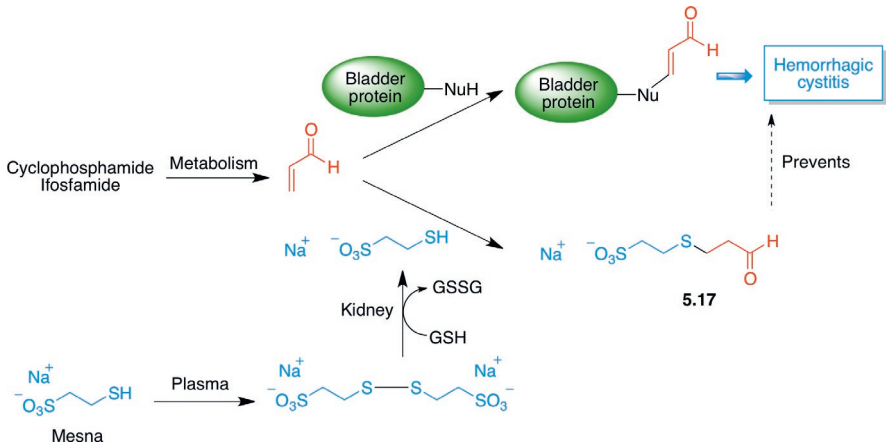


FIGURE 5.12

Detoxification of acrolein by mesna.

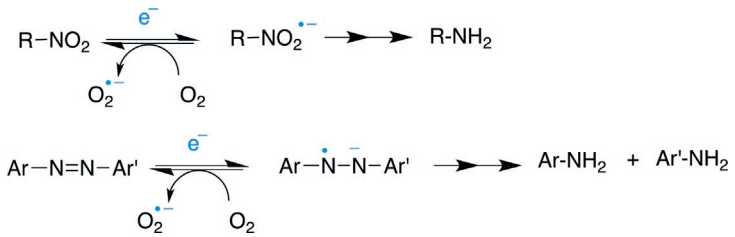


FIGURE 5.13

Reduction of nitro and azo compounds.

An important biochemical difference between some tumors and normal tissues is their different reducing capacities. The inner regions of solid tumors have little vascularization, and therefore their oxygen content is low (for a more detailed treatment of hypoxia-based strategies for tumor-specific prodrug activation, see Chapter 13, Section 2.2). Reductive metabolism is a multistep process that includes an initial equilibrium that is reverted by molecular oxygen. For this reason, reductive metabolism of some functional groups such as nitro and azo is enhanced in hypoxic tissues (Figure 5.13).

On this basis, some aromatic nitrogen mustard prodrugs bearing nitro or azo groups have been designed for activation in these hypoxic environments. In the simplest of them, the presence of these electron-withdrawing groups in the *p*-position with respect to the nitrogen atom prevents cyclization to an aziridinium cation, but after metabolic reduction they are transformed into an electron-releasing group (Figure 5.14).[23]

Similarly, some nitrogen mustard cobalt(III) complexes, such as SN-24771, are activated by reduction in hypoxic tumor microenvironments because one-electron reduction of Co(III) to Co(II) greatly

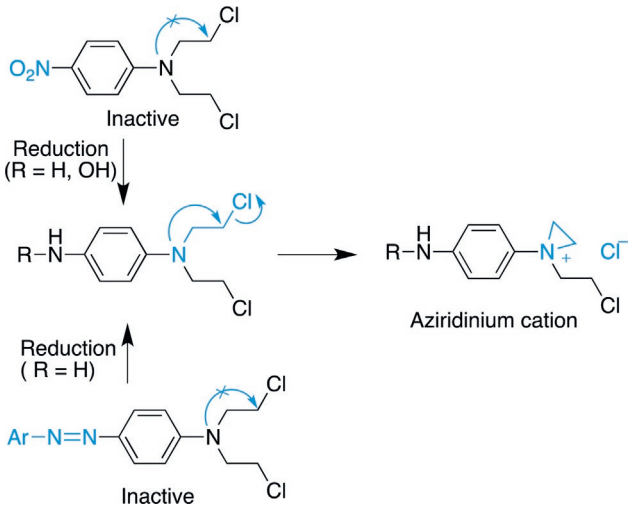


FIGURE 5.14

Aromatic nitrogen mustard prodrugs for selective activation in hypoxic tissues.

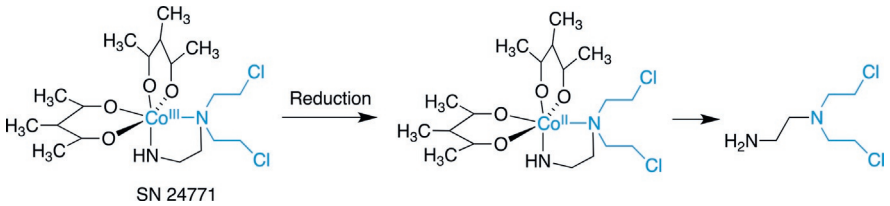


FIGURE 5.15

Nitrogen mustard Co(III) complexes and their activation in hypoxic tissues.

labilizes the Co–N bonds, causing the release of the active nitrogen mustard (Figure 5.15).[24] See Section 2.2.4 in Chapter 13 for further details on the use of Co(III) complexes in hypoxia-based strategies for tumor-specific prodrug activation.[25]

More complex approaches are based on the distal activation concept, in which the reductive process uncovers a nucleophilic or basic center that then triggers the liberation of the alkylating agent by reaction with a distant part of the molecule. For instance, the phosphoramide mustard prodrug **5.18** is activated by reduction of its nitro group to amino, which increases the basicity of the quinoline nitrogen sufficiently to allow the elimination reaction depicted in Figure 5.16.

A related example is based on the reduction of a quinone system, which uncovers two nucleophilic hydroxyl groups, as shown in Figure 5.17 for the case of the melphalan double prodrug **5.19**.[26] The conformation needed for the reaction that liberates the active drug is favored by the presence of the methyl groups because it is less sterically compressed than alternative conformations (Thorpe–Ingold effect).

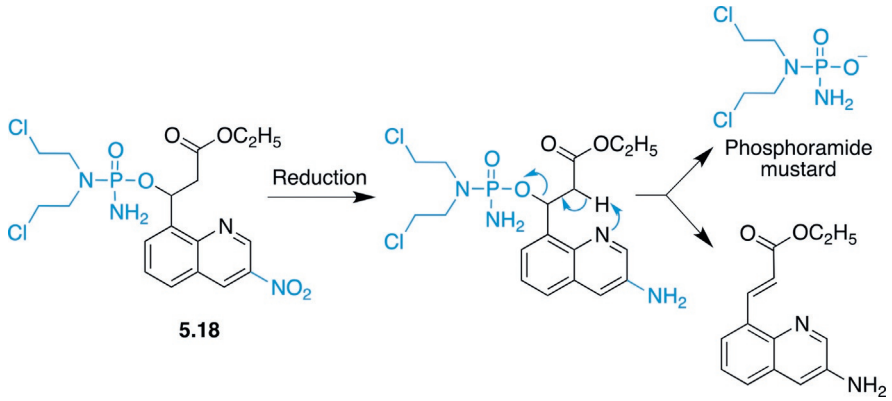


FIGURE 5.16

Distal activation of a nitrogen mustard prodrug mediated by reductive processes.

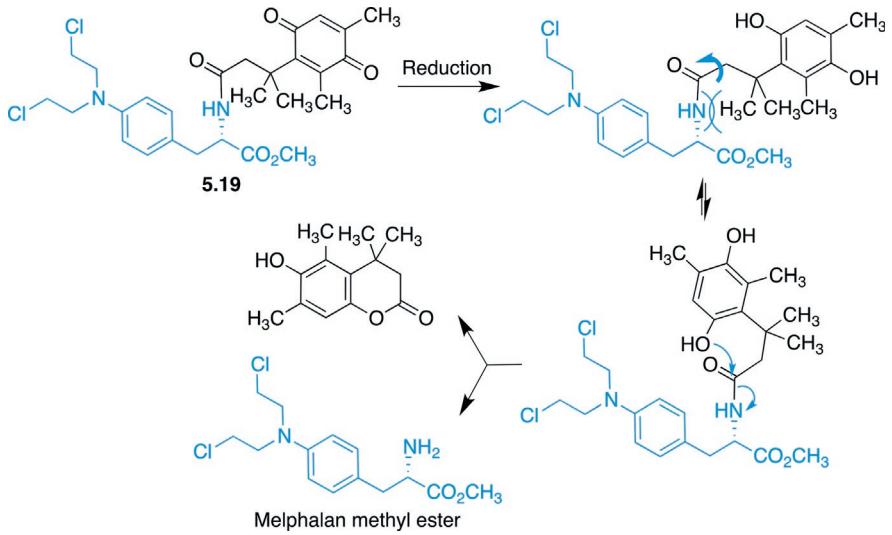


FIGURE 5.17

Bioreductive activation of a melphalan prodrug.

3 AZIRIDINES (ETHYLENEIMINES)

Because the active species involved in DNA alkylation by nitrogen mustards is an aziridinium cation, several aziridine derivatives have also been tested as antitumor agents.

Electron-releasing substituents raise the aziridine nitrogen pK_a and lead to a high concentration of aziridinium cations **5.20**, which renders these compounds too reactive to be of therapeutic value. For this reason, the aziridine units are attached to electron-withdrawing groups, which reduces their

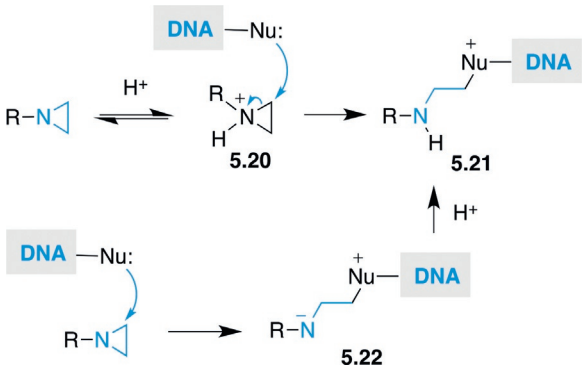
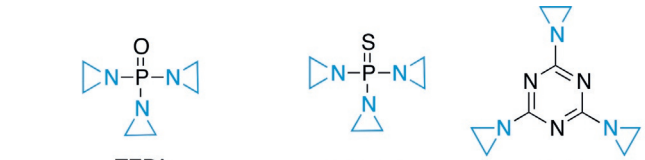


FIGURE 5.18

DNA alkylation by aziridines.

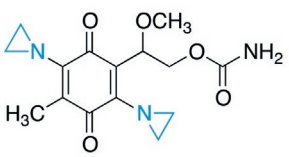
reactivity as bases but still allows formation of DNA-alkylation products such as **5.22**, which then are protonated to **5.21**. The driving forces of this reaction are the stabilization of the nitrogen negative charge by the electron-withdrawing group and the liberation of ring strain upon opening of the aziridium (Figure 5.18).

Early studies showed that at least two aziridine units were necessary for good activity, but no improvements were observed by addition of a third or fourth aziridine, suggesting that cytotoxicity is mainly due to a cross-linking mechanism, as in the case of nitrogen mustards. The first compounds of this family to be introduced in therapeutics were triethylenemelamine (Tetramine®)[27] and thiotepa (Thioplex®), so called because it is a sulfur analog of triethylenephosphoramide (TEPA). Thiotepa is still used in bladder carcinoma and requires intracavitary administration because of its low stability in the acid conditions prevalent in the stomach. Because it produces myelosuppression as a main toxicity effect, it was designated as "orphan drug" by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2007 as a conditioning treatment prior to hematopoietic stem cell transplantation. It is also employed as conditioning treatment prior to allogeneic or autologous hematopoietic progenitor cell transplantation (HPCT) in hematological diseases, sometimes combined with total body irradiation.

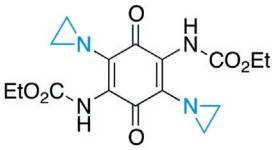


Other antitumor compounds contain two or three aziridine rings linked to a benzoquinone system and can act as DNA bis-alkylators and cross-linking agents. They were designed to cross the blood–brain barrier because of their high lipophillicity and low ionization. Carboquone (carbazilquinone) and diaziquone (AZQ) have been extensively evaluated in the treatment of several cancers, but they have found little use due to their toxicity and low effectiveness. AZQ, one of the most active compounds,[28]

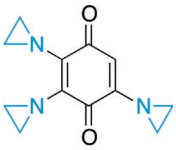
was the first compound to receive orphan drug status from the FDA in the early 1980s, but it showed no clear advantage over preexisting drugs. Triaziquone (Oncovedex®) was used clinically in the 1960s for the treatment of a number of cancers, but because of its toxicity to bone marrow and blood vessel walls, it has been replaced by more effective agents.[29] Apaziquone (EO9), which contains only one azidirine moiety, is a bioreductive drug that showed no activity in phase II clinical trials when administered intravenously because of rapid pharmacokinetic elimination and poor penetration through vascular tissue. However, later studies showed that it is active and well tolerated in patients with superficial transitional cell carcinoma of the bladder after intravesical administration, and it has been granted "fast track" status by the FDA for this indication.[30] The related aziridinylquinone BZQ has also been the subject of clinical investigation in humans.[31]



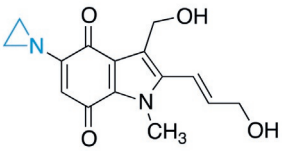
Carboquone



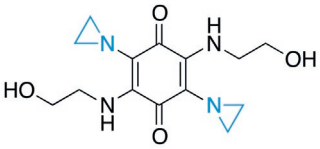
Diaziquone (AZQ)



Triaziquone



Apaziquone (EO-9)



BZQ

In general, the mechanism of DNA alkylation by aziridinilbenzoquinones[32,33] involves bioreductive processes[34] by the action of one-electron or two-electron reductases. The oxidation state of the quinone function modulates the alkylating activity, and it is also involved in the generation of cytotoxic reactive oxygen species. Indeed, the quinone group is a structural motif commonly found in reductively activated antitumor agents due to the fact that quinones exhibit reduction potentials similar to substrates of endogenous reductases.[35] Reduction of the quinone to a hydroquinone increases the pK_a of the aziridine nitrogen because of the replacement of the electron-withdrawing carbonyls by two electron-releasing hydroxy groups and therefore allows its protonation to a more reactive aziridinium cation. Furthermore, intramolecular hydrogen bonding of these groups with the aziridine nitrogen may assist this protonation (Figure 5.19). In some cases, alkylation is possible in the absence of reduction,[36] and it has been proven that simple aziridinilbenzoquinones can directly cross-link DNA in a pH-dependent process presumably related to the protonation mechanism shown in Figure 5.19.[37]

DT-diaphorase (DTD, NQO1), is an obligate two-electron reductase that is particularly interesting as a target for antitumor compounds[38] because it is present in the cell nucleus and its levels are increased in several tumors, such as non-small cell lung cancer.[39] Since the importance of DTD in the activation of aziridinylbenzoquinones was recognized,[40] there has been much effort to produce novel agents that efficiently target this enzyme.[41] SAR studies have shown that electron-donating groups on the benzoquinone ring increase DNA damage, whereas electron-withdrawing and sterically bulky groups at the C-6 position lead to less active or inactive compounds.[42] In some cases, such as in Me-DZQ, the usefulness of the compound is limited by poor solubility, a problem that has been

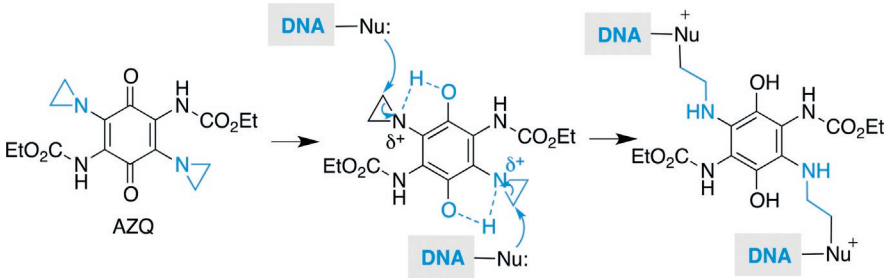
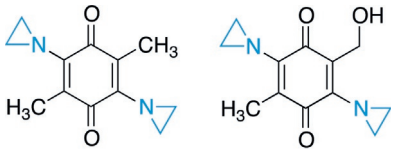


FIGURE 5.19

Bioreductive activation of aziridinylbenzoquinones.

partially overcome by introduction of hydrophilic hydroxyl groups in the side chains. For instance, RH1[43] is an excellent substrate for DTD, which has potent DNA cross-linking activity and high antitumor potency *in vitro* and *in vivo*[44] with reduced toxicity in normal tissues. It underwent phase I clinical studies in patients with solid tumors under the auspices of Cancer Research UK,[45,46] although because of its limited aqueous stability and solubility, it will require a suitable formulation for more advanced clinical trials.



In addition to alkylation, other reactions are possible on the hydroquinone form of aziridinylquinones such as **5.23**. One of them, which leads to its inactivation, is a 1,5-sigmatropic shift of hydrogen to give **5.24**,[47] which is then transformed into ethylaminoquinone **5.25** by tautomerism or into aminoquinone **5.26** through a second 1,5-sigmatropic shift followed by hydrolysis (Figure 5.20).

An additional transformation that inactivates **5.23** takes place by loss of the aziridine ring on its tautomer **5.27**, leading to quinone **5.28** (Figure 5.21).

One-electron metabolic reduction of aziridinylquinones is also possible, leading to semiquinones. Their protonated derivatives **5.29** also undergo a 1,5-sigmatropic shift, leading to inactive compounds **5.25** and **5.26**, the same as in the two-electron reduction process (Figure 5.22). As expected, semiquinone intermediates can also generate oxygen radical species upon reaction with O_2.[48]

These degradation pathways have therapeutic implications because the lower pharmacokinetic stability of indoloquinone aziridines, such as EO-9, with regard to their benzoquinone analogs is due to higher concentrations of the corresponding protonated semiquinone **5.29** due to the fact that the electron-releasing effect of the indole nitrogen leads to a low acidity for **5.29**. The pK_a of the semiquinone derived from EO-9 is 9.3, whereas the corresponding pK_a values of benzosemiquinones are below neutrality. For this reason, benzosemiquinones are mostly deprotonated, and the hydrogen sigmatropic shift mentioned previously cannot occur.[49]

Several natural products, including the mitomycins, FR-900482, and FR-69979, contain one fused aziridine ring,[50] but because of their specificity toward the minor groove, they are discussed in Chapter 6.

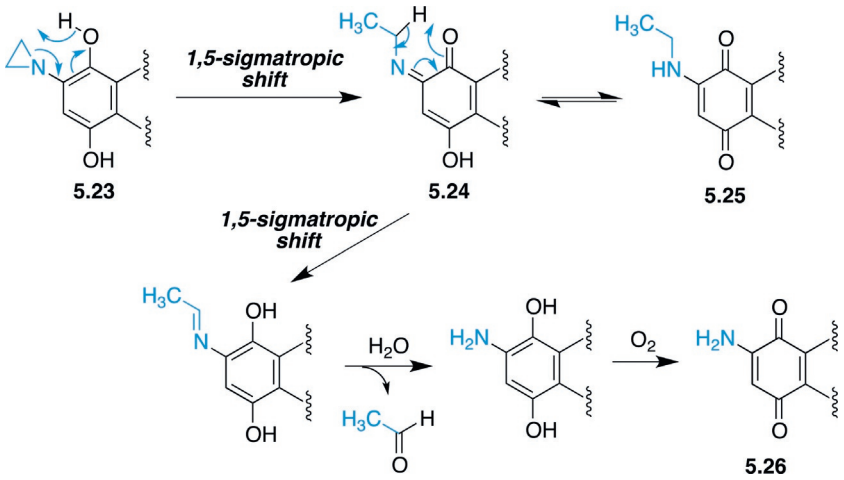


FIGURE 5.20

Inactivation of aziridinylbenzoquinones through a 1,5-sigmatropic shift.

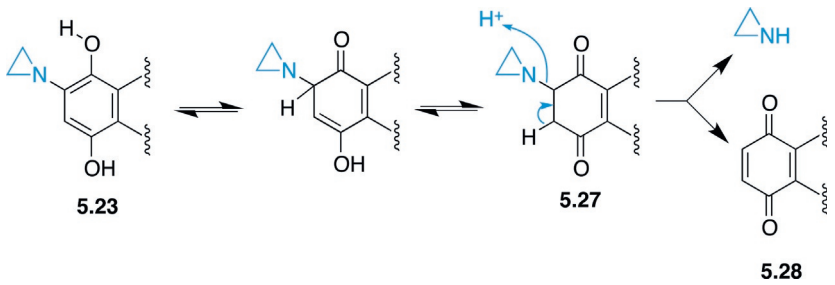


FIGURE 5.21

Inactivation of the hydroquinone forms of aziridinylbenzoquinones by loss of the aziridine ring.

4 EPOXIDES

The high reactivity of the epoxide ring toward nucleophilic groups in biomolecules is the basis of the use of ethylene oxide to sterilize substances that would be damaged by heat, including medical supplies such as bandages, sutures, and surgical implements. It has also become quite common as a substructure in side chains of compounds aimed at alkylating DNA[51] or other macromolecular targets.[52]

Diepoxybutane **5.30** is the simplest epoxide that is able to cross-link DNA. This compound is not employed as such but is instead nonenzymatically generated from treosulfan (Ovastat®), a member of the methanesulfonate family (see Section 5) that can therefore be regarded as its prodrug (Figure 5.23).[53] Similarly to nitrogen mustards, treosulfan alkylates DNA at guanine bases. It is employed mainly for the treatment of ovarian cancer. In addition, it has demonstrated preclinical and clinical activity against some other solid tumors and hematological malignancies, and it is used for bone marrow ablation before stem cell transplantation[54] and to treat malignant melanoma and breast

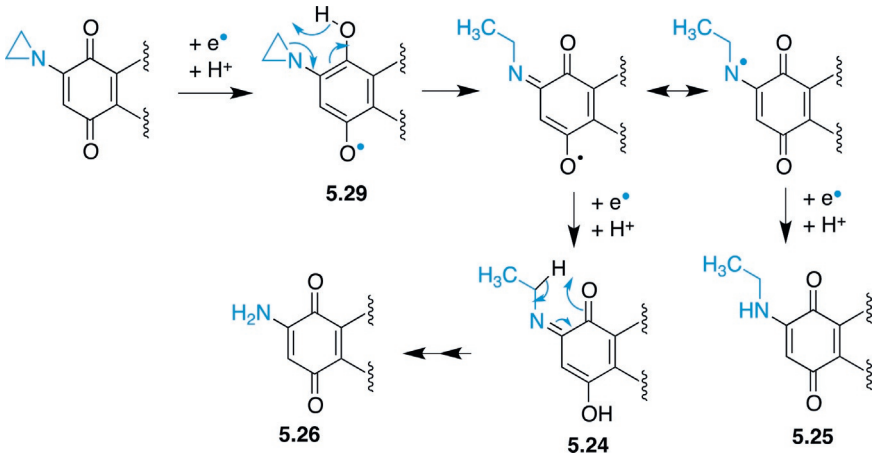


FIGURE 5.22

Inactivation of the hydroquinone forms of aziridinylbenzoquinones by one-electron reduction.

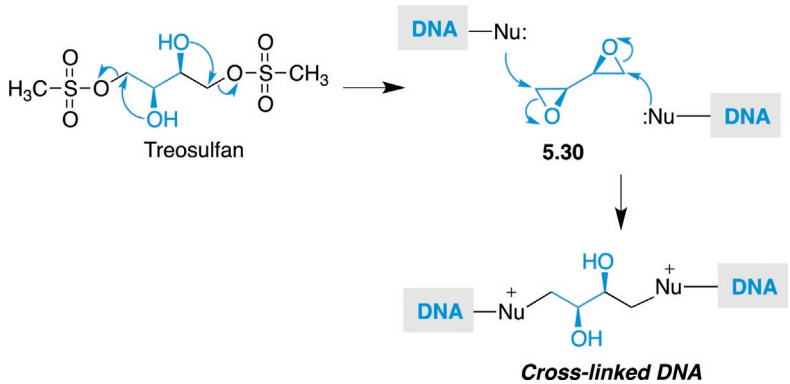


FIGURE 5.23

DNA alkylation by treosulfan.

cancer. In combination with other drugs, treosulfan is being studied in patients with nonmalignant inherited disorders, such as thalassemia major.[55]

Mitobronitol (Myelobromol®), the 1,6-dibromo analog of mannitol, is a bromhydrine prodrug that, similarly to treosulfan, undergoes a double intramolecular nucleophilic displacement to give diepoxide **5.31**, another DNA cross-linking reagent (Figure 5.24). It is used for myelosuppression prior to allogenic bone marrow transplantation in accelerated chronic granulocytic leukemia, showing lower toxicity than other alkylating agents such as busulfan.[56]

VAL-083 (dianhydrodulcitol, dianhydrogalactitol) is a diastereomer of the bis-epoxide **5.31** that acts as an intermediate in the previous mechanism. This compound was approved in China for the treatment of chronic myelogenous leukemia (CML) and lung cancer, and has undergone extensive clinical testing for other indications including glioblastoma multiforme (GBM), the most common and aggressive form of brain cancer.

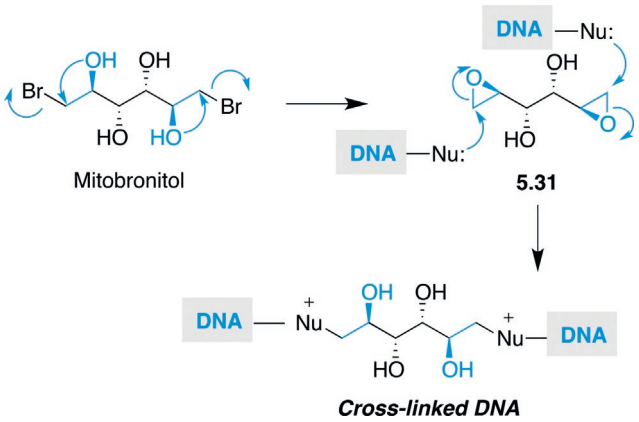
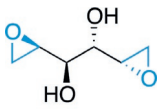


FIGURE 5.24

DNA alkylation by mitobronitol.



VAL-083

Mixed epoxide-aziridine antitumor compounds are also known. For instance, azinomycin B (carzinophilin) is a complex natural product isolated from *Streptomyces sahachiroi* that contains densely assembled functionalities and has potent antitumor activity *due to* DNA intercalation and interstrand cross-linking (Figure 5.25).[57,58] It was used with some success in a clinical study in humans with several types of cancer.

5 METHANESULFONATES

Methanesulfonate is a good leaving group because of the efficient delocalization of negative charge between three oxygen atoms. For this reason, several compounds containing two methanesulfonate groups separated by a polymethylene chain were tested as antitumor agents, finding that the optimal activity corresponded to the compound with 4 carbon atoms busulfan. Other members of this family are piposulfan, improsulfan, hepsulfam and the previously mentioned treosulfan, a diepoxide prodrug.

Busulfan (Myleran®) was the mainstay of the chemotherapeutic treatment of CML until it was displaced by imatinib, although it is still in use to a degree as a result of the drug's relatively low cost. It is also used in the treatment of chronic granulocytic leukemia and, in high-dose combination with cyclophosphamide, to condition patients for allogeneic bone marrow transplantation, although it is particularly toxic for pulmonary tissue and this toxicity may be dose limiting. Hepsulfam underwent some early clinical studies.[59]

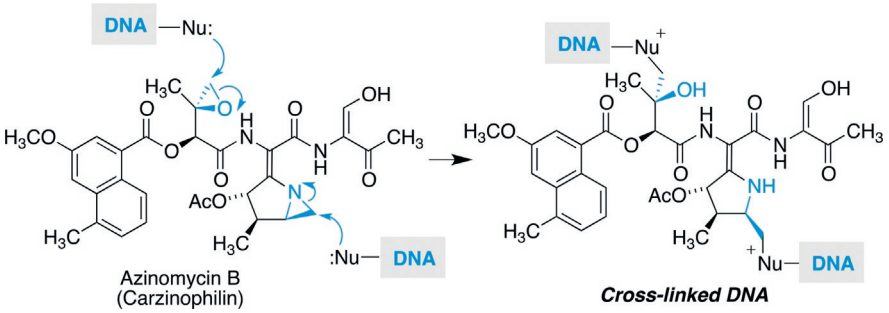
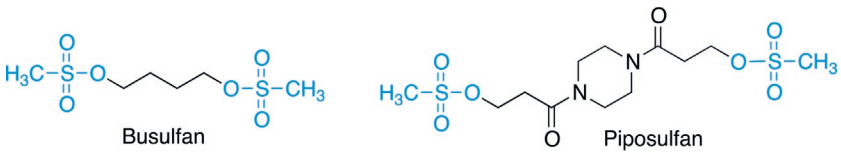
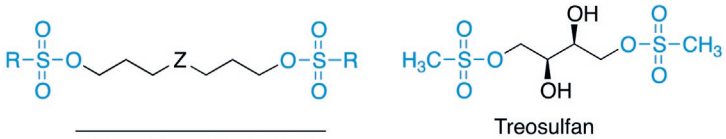


FIGURE 5.25

DNA cross-linking by azinomycin B.





R	Z	Compound
CH_3	NH	Improsulfan
NH_2	CH_2	Hepsulfam

The formation of interstrand DNA cross-links has been demonstrated for busulfan[60] and treosulfan.[61] Busulfan also produces DNA intrastrand cross-links, mainly at the 5′-GA-3′ sequence.[62] In contrast with nitrogen mustards, in which the rate-limiting step is the unimolecular formation of the aziridinium ion, busulfan reacts with guanine N-7 by a S_N2 mechanism (Figure 5.26), in which the rate-limiting step depends on the concentration of both reaction partners.[63]

The study of metabolites of busulfan suggests that it is also able to alkylate cysteine residues. The urinary excretion of compound **5.32** can be explained by the mechanism summarized in Figure 5.27, which involves a double nucleophilic attack by this amino acid.

6 NITROSOUREAS

In a random screening carried out by the National Cancer Institute in 1959, 1-methyl-3-nitro-1-nitrosoguanidine showed a weak antileukemic activity. Assays with analogs of this compound led to the discovery of the antitumor activity of 1-methyl-1-nitrosourea, the lead compound of the nitrosourea group. It was soon discovered that introduction of a 2-chloroethyl chain on the nitrogen atom

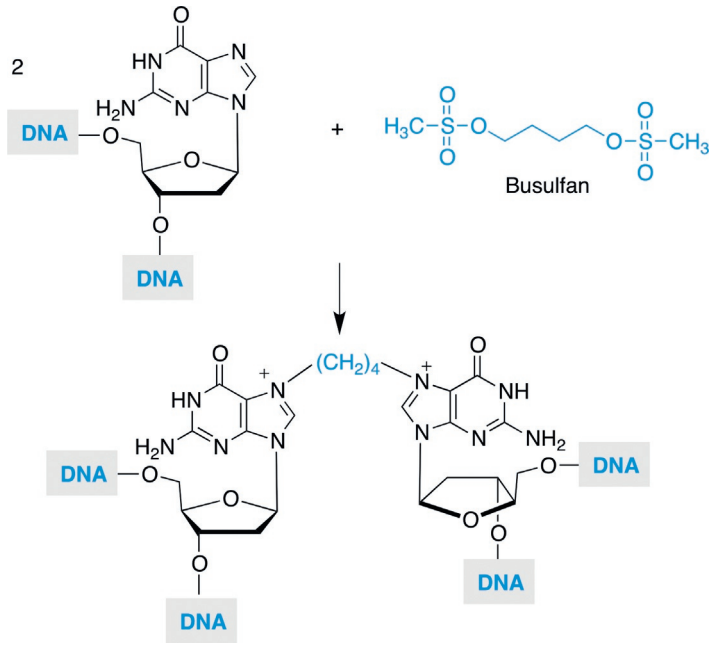


FIGURE 5.26

DNA alkylation by busulfan.

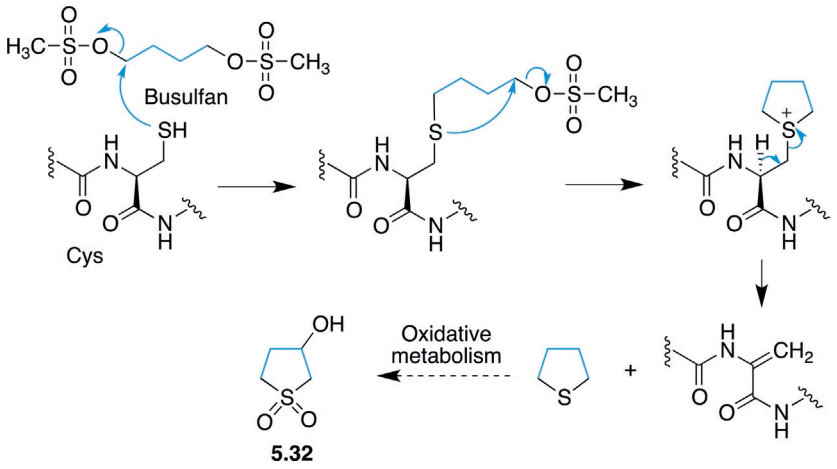


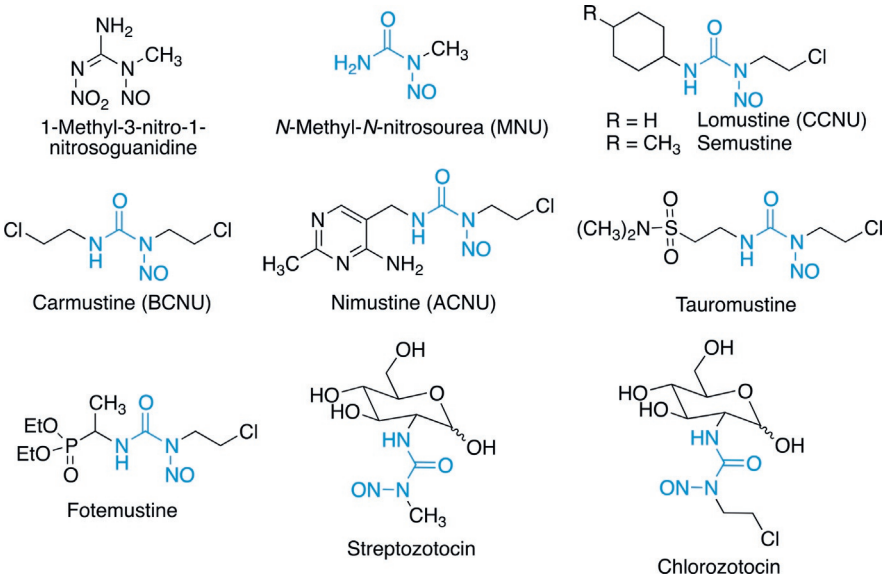
FIGURE 5.27

Alkylation of cysteine residues by busulfan.

bearing the nitroso group (CNUs) led to much increased activity. These chloroethyl derivatives were lipophilic enough to cross the blood–brain barrier and therefore were useful in the treatment of brain tumors. This property led to the synthesis of a large number of nitrosoureas, including lomustine (CCNU) and its methyl derivative semustine, carmustine (BCNU), nimustine (ACNU), and the water-soluble tauromustine and fotemustine, but toxicity problems have prevented their widespread use. In 1967, streptozotocin (Zanosar®), a hydrophilic natural nitrosourea, was isolated from a strain of *Streptomyces achromogens*. This compound was chosen as a lead because initial SAR studies suggested that hydrophilic nitrosoureas were more potent and less toxic, and a number of analogs, such as chlorozotocin, were prepared.

Currently, the most clinically relevant nitrosoureas are lomustine, BCNU, ACNU, and streptozotocin. Lomustine (CCNU, CeeNU®) is used in brain tumors; breast, pancreatic, and lung cancers; Hodgkin's lymphoma; melanoma; multiple myeloma; and ovarian cancer. Carmustine (BiCNU®) is used in several types of brain cancer (including glioma, glioblastoma multiforme, medulloblastoma, and astrocytoma), multiple myeloma, and lymphoma (Hodgkin's and non-Hodgkin's lymphoma). A new formulation of carmustine with reduced systemic toxicity has been developed for the local treatment of brain tumors. Formulated into a slow-release "wafer" dosage form (Gliadel Wafer®; polifeprosan 20 with carmustine), it is implanted into the resection cavity left after surgical removal of the tumor.[64] It was approved by the FDA in 1997 for use as an adjunct to surgery to prolong survival in patients with recurrent GBM for whom surgical resection is indicated. Nimustine is used in combination with teniposide as a second- or third-line chemotherapy for recurrent glioblastoma.[65]

After the discovery in the mid-1960s that streptozotocin was selectively toxic to the β cell of the pancreatic islets, it was assumed that this drug might be used in pancreatic cancers. Indeed, it was approved by the FDA as a treatment for pancreatic islet cell cancer in 1982 and marketed as Zanosar®, although its use is generally limited to patients whose cancer cannot be removed by surgery. Regarding chlorozotocin, a phase II study showed that it is active against metastatic melanoma to the same degree as other chloroethylnitrosoureas in clinical use,[66] but without causing bone marrow toxicity.



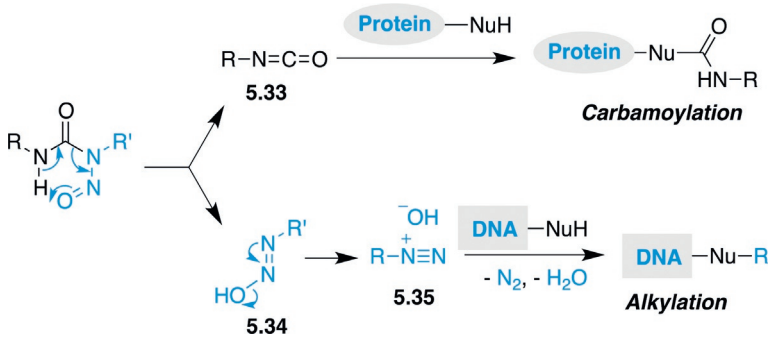


FIGURE 5.28

Products that arise following the thermal decomposition of nitrosoureas.

Nitrosoureas have been widely studied from a mechanistic standpoint. The presence of the nitroso group labilizes the nitrogen–carbon bond, leading to spontaneous decomposition into two electrophiles: an isocyanate **5.33** and a diazene hydroxide **5.34** that has been detected in some cases by electrospray ionization mass spectroscopy.[67] This intermediate generates a diazonium salt **5.35** (Figure 5.28).[68] Alkylation seems to be the main reaction responsible for antitumor activity, whereas carbamoylation takes place primarily on amino groups in proteins, leading to inhibition of several DNA repair mechanisms. *N*-nitrosoamides and *N*-nitrosocarbamates, which can act as alkylating (but not carbamoylating) agents, also have antitumor activity,[69] which supports the assumption that alkylation is the key mechanism.

The previously discussed fragmentation pathway was proposed mainly on the basis of studies of the thermal decomposition of nitrosoureas under anhydrous conditions,[47] but in water solution the reaction is much more complex and has been explained by the mechanism shown in Figure 5.29. Addition of a molecule of water to the nitrosourea, in its tautomeric form,[70] gives the tetrahedral intermediate **5.36**, which is decomposed into a primary amine, carbon dioxide, and **5.34**. This elimination requires an anti-periplanar conformation for **5.37**. Addition of a nucleophile other than water to the nitrosourea tautomer explains the isolation of carbamoylated products, formed by elimination of **5.34**.

Most nitrosoureas (CNUs) contain one chloroethyl chain on the nitrosated nitrogen, which allows them to act as DNA cross-linking agents. Reaction of electrophilic diazonium species **5.37** with guanine is assumed to take place on O-6 to give **5.38**. This mono-alkylated product reacts subsequently with the N-3 atom of the cytosine unit in the complementary DNA strand by anchimeric assistance of the guanine N-1 atom through intermediate **5.39**, giving the cross-linked product **5.40** (Figure 5.30). In fact, addition of O^6-alkylguanine DNA alkyltransferase, an enzyme that breaks O-6 guanine adducts, prevents cross-linking.

Alternatively, intact nitrosourea molecules rather than diazonium species can directly alkylate DNA. Thus, the nucleophilic attack of guanine O-6 to the nitrosourea tautomer **5.41** gives intermediate **5.42**. Although alternative mechanisms have been proposed, according to labeling experiments, it is probable that **5.42** cyclizes to the nitrosoisoxazolidine **5.43**, which is attacked by another O-6 atom of a neighboring guanine unit to give **5.44**. In this adduct, the O-6 of the first guanine is carbamoylated

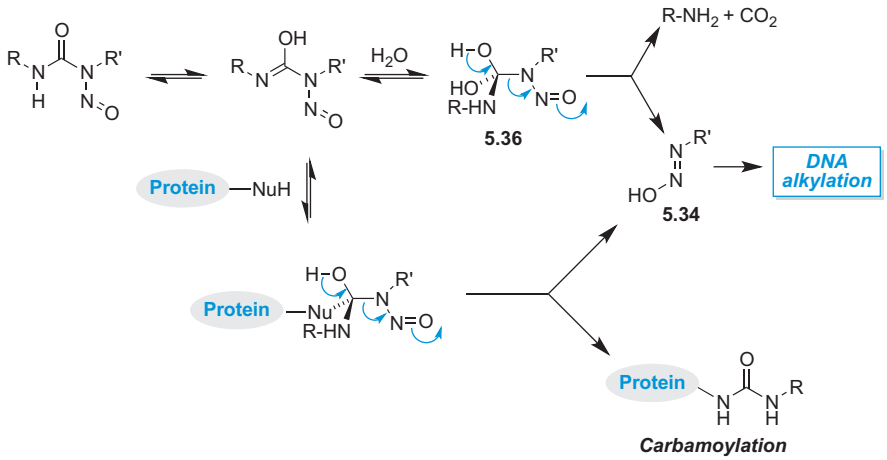


FIGURE 5.29

Decomposition of nitrosoureas in aqueous solution.

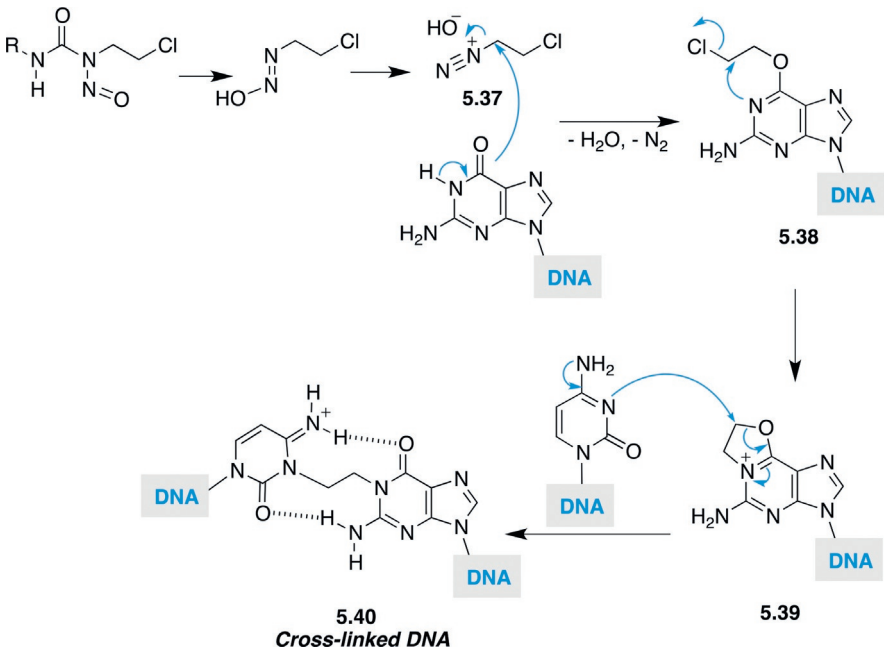


FIGURE 5.30

DNA cross-linking by nitrosoureas.

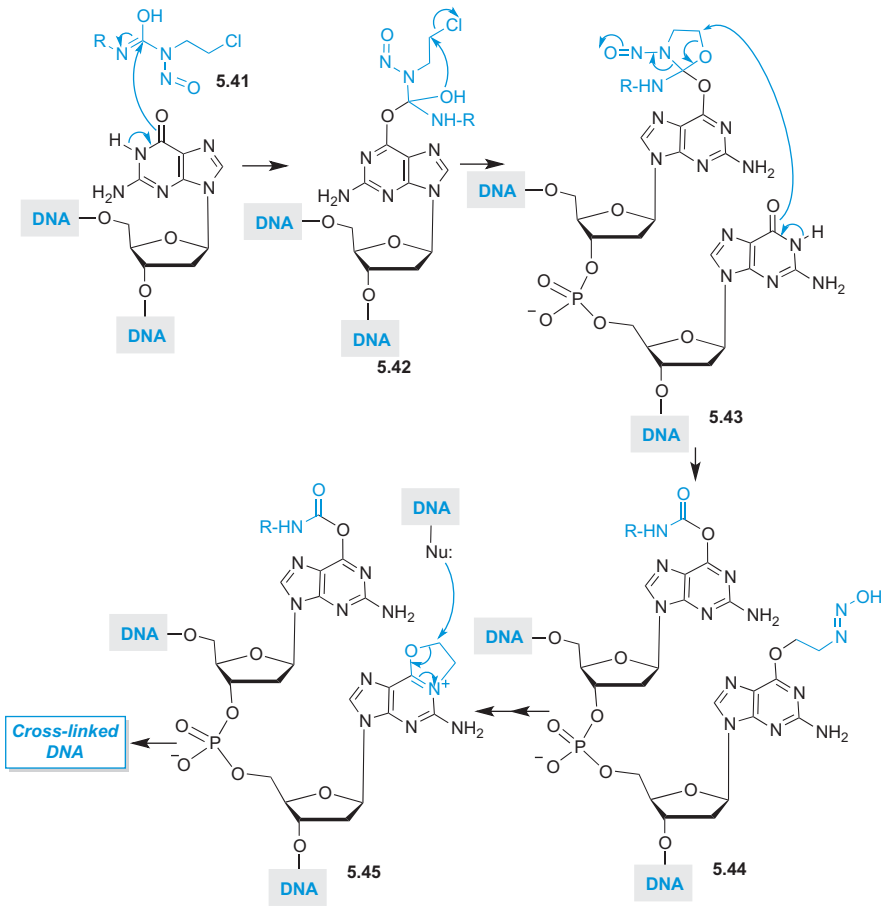


FIGURE 5.31

Alternative mechanisms for DNA cross-linking by nitrosoureas.

and the O-6 of the second guanine is alkylated with a 2-hydoxydiazoethyl group (Figure 5.31). Diazonium generation and attack of N-3 from a cytosine of the opposite DNA strand, with anchimeric assistance from guanine N-1, finally gives the carbamoylated cross-linked product **5.45**.

Streptozotocin differs from other nitrosoureas in that it does not cross the blood–brain barrier because of its high hydrophilicity, and it also shows a relatively low myelosuppression because of decreased entry into bone marrow cells. The selective cytotoxicity of streptozotocin against the pancreas β cells is due to its resemblance to glucose, which facilitates drug uptake to the islets making use of the glucose transport protein GLUT2. Therefore, the main applications of streptozotocin are the induction of diabetes mellitus in experimental animals and the treatment of islet cell pancreatic tumors, normally in association with nicotinamide for reasons that are explained later. As expected from its nitrosourea structure, streptozotocin methylates DNA, especially at the guanine N-7 and O-6

positions,[71] but there is also much evidence that shows that free radicals play an essential role in its cytotoxicity.[72] It has been shown that streptozotocin induces the generation of nitric oxide,[73] superoxide and hydroxyl radicals, and also that association with oxygen radical scavengers, such as nicotinamide, prevents streptozotocin-induced cleavage of islet DNA.[74]

7 TRIAZENES

Dacarbazine (DTIC-Dome®) is employed in combination therapy for the treatment of metastatic malignant melanoma, Hodgkin's lymphoma, sarcoma,[75] and islet cell carcinoma of the pancreas. This compound was initially designed as an antimetabolite because it is an analog of 5-aminoimidazole-4-carboxamide, an intermediate in purine biosynthesis (see Figure 2.38). However, it is a prodrug, and its cytotoxic activity is due to the generation during its metabolism of methyldiazonium, which methylates DNA.[76] Methyldiazonium has a very short half-life of approximately 0.4 sec in aqueous solution, which is nevertheless sufficient to allow it to reach its target. A mechanism for this process is summarized in Figure 5.32, in which activation of dacarbazine by metabolic oxidative demethylation to MTIC (5-methyltriazenoimidazole-4-carboxamide) was proven by the isolation of labeled formaldehyde and 5-aminoimidazole-4-carboxamide (AIC) when dacarbazine was labeled with ^{14}C at one of the methyl groups. This intermediate is then transformed by tautomerism into **5.46**, a diazonium precursor. The major methylation reaction takes place at the guanine N-7 atom and is relatively nontoxic. Methylation at guanine O-6 also occurs, and it is thought to be the main cytotoxic mechanism.[77] It is interesting to note that compounds that act as precursors to the ethyldiazonium cation lack any DNA alkylating properties, which has been explained by the lower stability in aqueous solution of

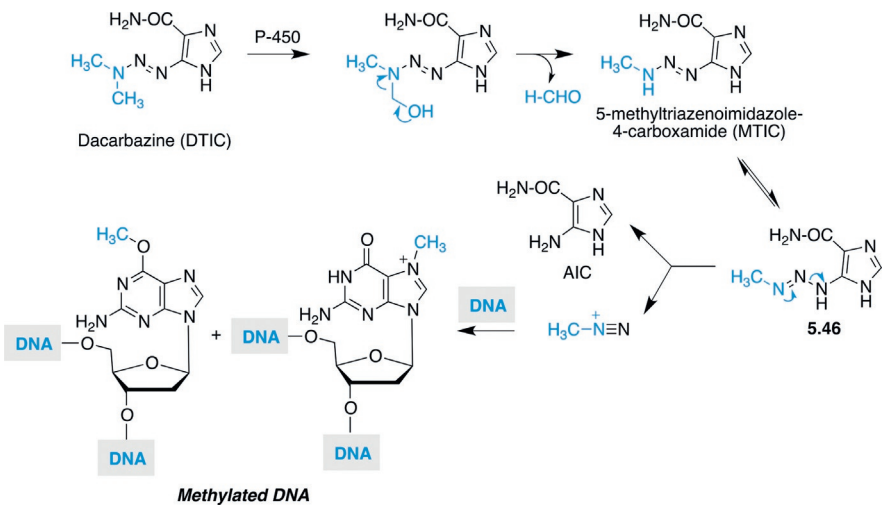


FIGURE 5.32

Generation of methyldiazonium from dacarbazine and subsequent methylation of DNA.

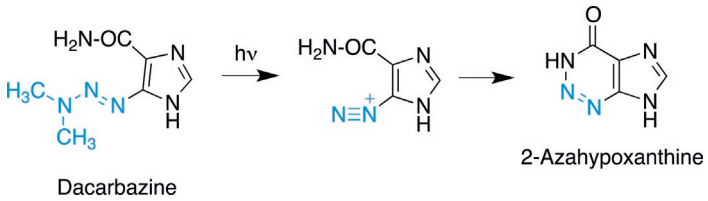


FIGURE 5.33

Photodegradation of dacarbazine.

ethyldiazonium with regard to methyldiazonium,[78] leading to its evolution to ethylene by elimination or to ethanol by reaction with a molecule of water before reaching DNA.

In addition to its toxicity, dacarbazine has several drawbacks due to its excessive hydrophilicity, which leads to slow and incomplete oral absorption and therefore intravenous administration becomes necessary. Another disadvantage is its high photosensitivity, with a very short half-life (~30 min), decomposing to 2-azahypoxanthine via an intermediate diazonium species (Figure 5.33). For this reason, intravenous infusion bags of dacarbazine must be protected from light.

These problems have stimulated the synthesis of dacarbazine analogs, the most important of which is temozolomide (Temodal®). Its development is associated with research teams at the Universities of Nottingham, Aston, and Strathclyde, and started as a purely synthetic project related to the chemistry of bicyclic systems with bridgehead nitrogen atoms. In this project, the imidazotetrazine **5.47** was obtained, with the aim of taking advantage of the easy hydrolysis of the tetrazinone fragment to liberate a DNA alkylating moiety having a 2-chloroethyl substituent attached to a nucleophilic nitrogen. However, **5.47** lacked significant antitumor activity. At a later stage, a related compound, which was first named azolastone and then mitozolomide, showed a remarkable preclinical antitumor activity, but early phase I studies showed that it induced irreversible thrombocytopenia. Its analog, temozolomide, was eventually selected for clinical trials (Figure 5.34).[79]

Temozolomide is one of the few drugs specifically approved for a brain tumor, namely anaplastic astrocytoma, and the first that can be administered orally. It has also been shown to improve survival in patients with other malignant gliomas, including glioblastoma.[80] Similar to dacarbazine, it is a prodrug that is converted into the same intermediate (MTIC), but in the case of temozolomide the bioactivation

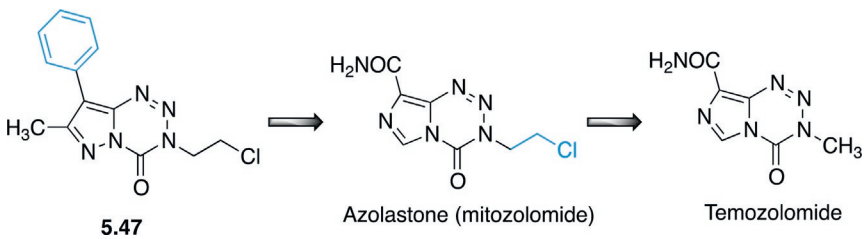


FIGURE 5.34

Milestones in the design of temozolomide.

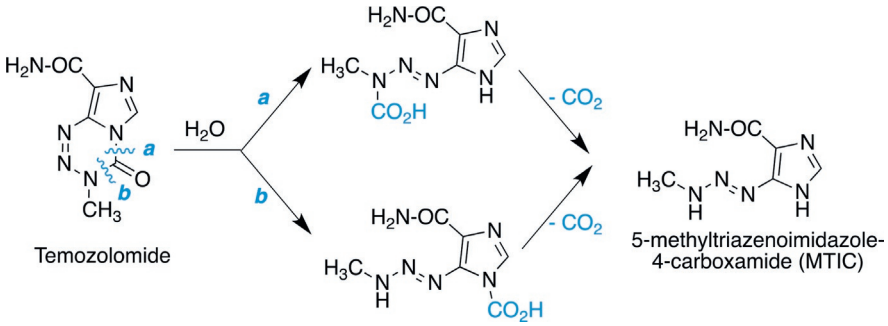


FIGURE 5.35

Temozolomide hydrolysis.

process involves a nonenzymatic hydrolysis reaction followed by spontaneous decarboxylation (Figure 5.35). The absence of hepatic activation is an advantage because metabolic individual variation in patient microsomal activity needs not be taken into account. The main problem associated with temozolomide administration is its bone marrow toxicity.

8 METHYLHYDRAZINES

When a series of *N*,*N*′-dialkylhydrazine derivatives that had been prepared as monoaminooxidase (MAO) inhibitors were routinely submitted to cytotoxicity tests, it was shown that compounds with an *N*-methyl substituent had anticancer potential. This discovery ultimately led to the development of procarbazine (Matulane®), which was approved for use in combination therapy for advanced Hodgkin's disease. Mechanistically, procarbazine is a unique agent with multiple mechanisms of action that is not cross-resistant with other alkylating agents. It inhibits the incorporation of small DNA precursors, as well as RNA and protein synthesis. Procarbazine can also directly damage DNA through a methylation reaction, whose precise mechanism is unclear. The major species found in plasma after its administration is azoprocarbazine **5.48**, formed by oxidation by P450 or MAO. This reaction also generates hydrogen peroxide, which was initially believed to be responsible for the antitumor activity, although much evidence has accumulated against this hypothesis, including the fact that procarbazine did not produce DNA breaks *in vivo*, probably because the required amount of drug was above its LD_{50}.[81] Tautomerism can transform **5.48** into hydrazone **5.49**, which gives by hydrolysis aldehyde **5.57**, a precursor of the primary excreted metabolite *N*-isopropylterephthalamic acid **5.58**, and methylhydrazine **5.50**. Although this route can potentially lead to methylating species such as **5.52**, it appears to lack physiological significance, but it explains the low stability of procarbazine in aqueous solution. Azoprocarbazine (**5.48**) may also be further metabolized by cytochrome P450 to azoxy derivatives **5.53** and **5.54**. The first of these intermediates is responsible for anticancer activity, with DNA methylation being explained by generation of methyldiazonium **5.55**. Liberation of alcohol **5.56** and subsequent oxidative metabolism of this compound explains the excretion of acid **5.58**. Alternatively, a side chain rearrangement in **5.54** to give a diazo compound followed by its fragmentation can also explain the generation of methyldiazonium **5.55** and alcohol **5.56** (Figure 5.36).

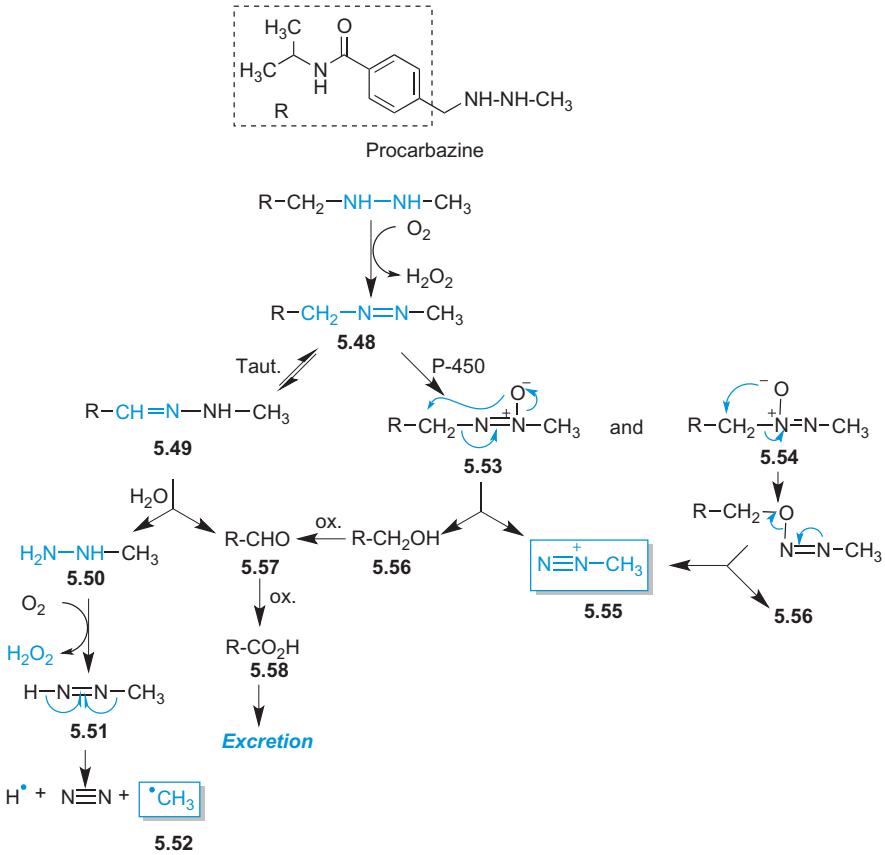


FIGURE 5.36

Generation of methyldiazonium during procarbazine metabolism.

9 1,3,5-TRIAZINES: HEXAMETHYLMELAMINE AND TRIMELAMOL

Altretamine (hexamethylmelamine, Hexalen®) was originally prepared as a resin precursor, but it was studied as an antitumor compound because of its structural analogy with the previously mentioned aziridine derivative triethylenemelamine (TEM). Although it is active in several types of tumors, its main therapeutic role is in the treatment of recurrent ovarian cancer, following first-line treatment with cisplatin.[82] The precise mechanism of altetramine cytotoxicity is unknown, although several proposals have been made. The main metabolic pathway is oxidative cytochrome P450-catalyzed *N*-demethylation, with carbinolamine **5.59** as an intermediate, which yields the pentamethyl derivative **5.62**, formaldehyde, and smaller amounts of inactive compounds arising from further demethylation. Alternatively, elimination of the hydroxy group from **5.59** gives the iminium species **5.60** that appears to be the alkylating species[83] rather than the formaldehyde generated in the demethylation process, which then reacts with DNA to give **5.61** (Figure 5.37).

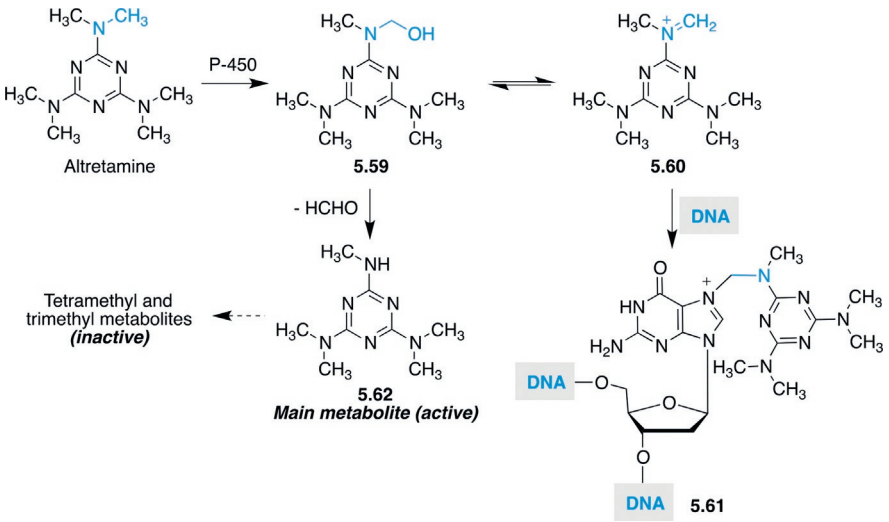


FIGURE 5.37

DNA alkylation by altretamine metabolites.

Trimelamol is a tris(hydroxymethyl) analog of altretamine that is water soluble and has the advantage of not requiring metabolic activation, although it is obviously less stable due to the presence of its carbinolamine moieties.[84] Involvement of formaldehyde in the cytotoxicity of trimelamol has been established,[85] as evidenced by the isolation of adduct **5.64** from reaction of formaldehyde with two adenine amino groups, although participation of iminium species **5.63** cannot be discarded. Reaction with the more nucleophilic guanine N-7 should be easily reversed due to the positive charge at nitrogen in the adduct (Figure 5.38). Trimelamol was studied in refractory ovarian cancers,[86] but it had to be withdrawn from further clinical studies due to formulation difficulties related to its low stability.

10 TRANSITION METAL SPECIES

10.1 PLATINUM COMPLEXES

Cisplatin (CDDP, cisdiamminedichloroplatinum II, cisplatinum) provides an excellent example of serendipity in the discovery of antitumor drugs. In 1965, in the course of studying the effects of electric currents on cells, it was discovered that *Escherichia coli* cells formed long filaments, but they did not divide. Further research showed that inhibition of bacterial cell division was due to cisplatin, generated from the platinum electrodes and the ammonium chloride present in the media.

Since its approval in 1978, cisplatin (Platinol®) has become an important component in chemotherapy regimens for the treatment of ovarian, testicular, lung, and bladder cancers, as well as lymphomas, myelomas, and melanoma. Unfortunately, its continued use is greatly limited by severe dose-limiting side effects and intrinsic or acquired drug resistance. These side effects include nephrotoxicity, neurotoxicity, ototoxicity, and myelosuppression. The nephrotoxicity, which can be attributed to

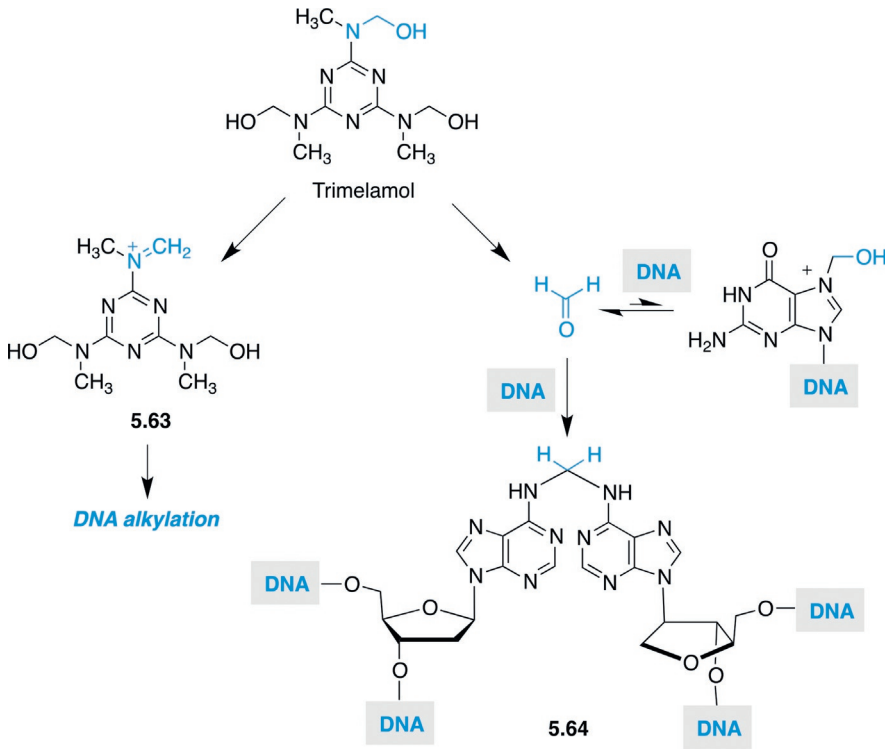


FIGURE 5.38

DNA alkylation by trimelamol.

interactions with renal components leading to tubular necrosis of both proximal and distal renal tubules, can be reduced to some degree through the use of saline hyperhydration before and after treatment. Other side effects limit the dose delivered to patients, which can be sublethal to tumors (particularly ovarian cancers), and this may stimulate the development of resistance to further drug treatment. Mechanisms of drug resistance include reduced drug uptake and/or increased drug efflux, degradation and deactivation by intracellular thiols such as glutathione, and improved repair or tolerance of DNA–cisplatin adducts.[87]

Cisplatin is a square–planar complex, containing two labile chlorines and two relatively inert ammonia molecules coordinated to the central Pt(II) atom in a *cis* configuration. When this compound enters the cell, it reacts with water to give the positively charged active species **5.65** and especially **5.66**, a process that is favored by the relatively low intracellular chloride concentration. These species enter the nucleus and are responsible for the formation of DNA Pt complexes that account for the antitumor activity (see below). Cytoplasmic deactivation of cisplatin is also possible and is mainly due to its reaction with mercapto groups in glutathione because the "soft" nature of both Pt and S favors their mutual binding. In plasma, the high chloride concentration somewhat hampers this reaction, but nevertheless the extracellular hydrolysis of cisplatin followed by the formation of Pt adducts with mercapto

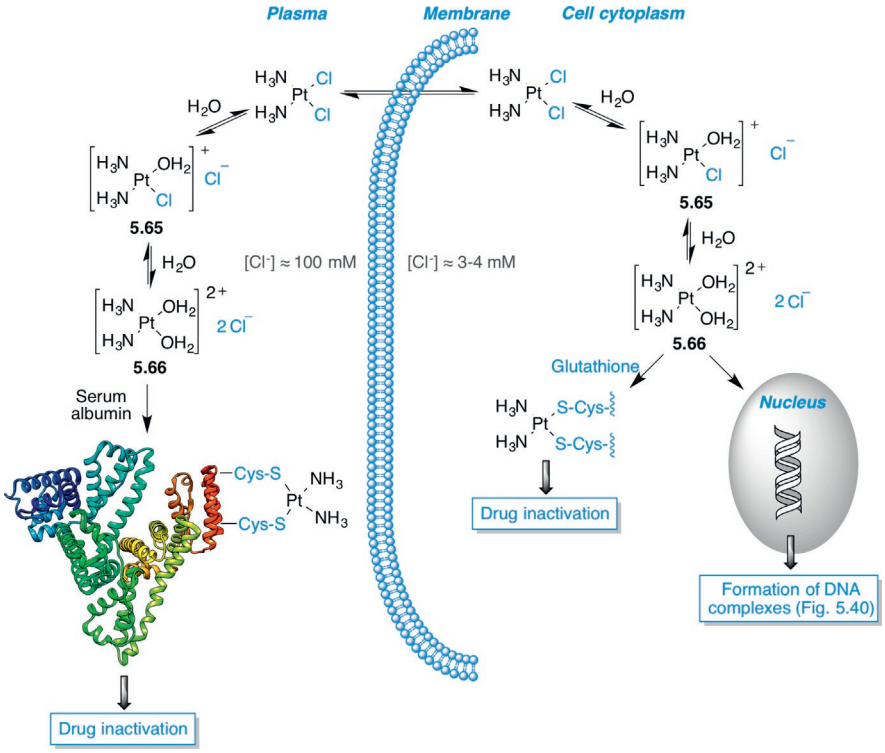


FIGURE 5.39

Intracellular bioactivation of cisplatin.

groups of cysteine residues present in serum albumin and other proteins is estimated to deactivate up to 98% of the drug after its intravenous administration (Figure 5.39).[88]

The active complexes **5.65** and **5.66** enter the nucleus and become attracted by the negatively charged DNA. This electrostatic interaction is followed by complexation with nitrogen atoms of purine bases, normally the N-7 atoms of two vicinal guanine units,[89] that displace the two water molecules leading to intrastrand cross-linking. This reaction deforms the DNA tertiary conformation, as shown by X-ray crystallography, and causes its unwinding at the complexation site (Figure 5.40).[90] As a consequence, high-mobility-group proteins become attached to DNA by intercalation of a phenylalanine unit at the unwound DNA damage site along the widened minor groove, preventing DNA replication.[91] Although, strictly speaking, Pt coordination with DNA bases cannot be considered an alkylation reaction, cisplatin and its analogs are normally studied among the alkylating agents because of the electrophilicity of the active species.

The cisplatin-induced cross-linking can also take place between two opposing DNA strands. In this case, the portion of the DNA double helix close to the coordinating deoxyguanosines is unwound and bent toward the minor groove, together with the *cis*-diammineplatinum (II) fragment, and the complementary deoxycytidines are displaced to an extrahelical arrangement (Figure 5.41).[92]

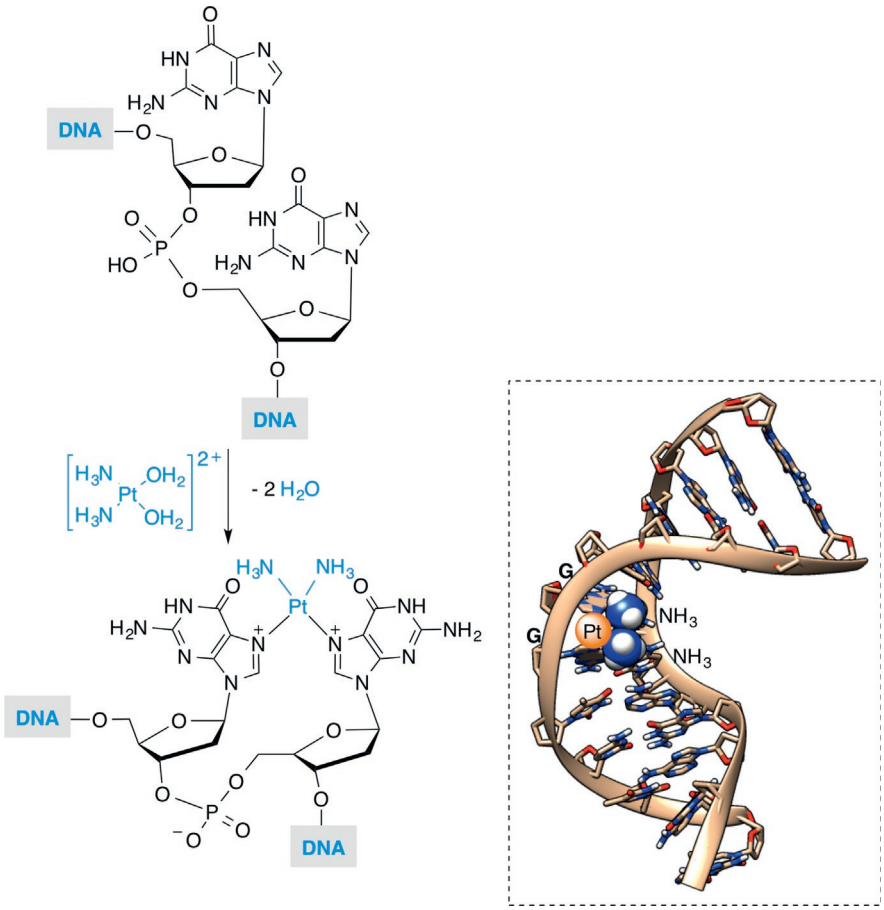


FIGURE 5.40

Reaction between DNA and cisplatin, leading to intrastrand cross-linking. The three-dimensional complex was generated from Protein Data Bank reference 1A84 and displayed with Chimera 1.8.1.

An additional mechanism for prevention of DNA transcription is replacement of Zn by Pt in the zinc-finger protein transcription factor. The existence of the zinc cation is essential to coordinate amino acids of the protein, usually cysteine and histidine, packing together the DNA binding domains into a dense structure. Replacing the zinc ion with platinum disrupts this conformation and binds the zinc finger permanently to DNA–polymerase-α, which is a transcription enzyme vital for cell replication (Figure 5.42). Platinum–DNA adducts also activate other cellular processes that mediate the cytotoxicity of these anticancer drugs.[93] Additional cytotoxicity mechanisms that have been proposed include the interactions of Pt complexes with the cell membrane[94] or with regulatory proteins.[95]

Due to the very high toxicity of cisplatin and the existence of intrinsic or acquired drug-resistance problems, thousands of analogs have been prepared in an effort to improve its selectivity and

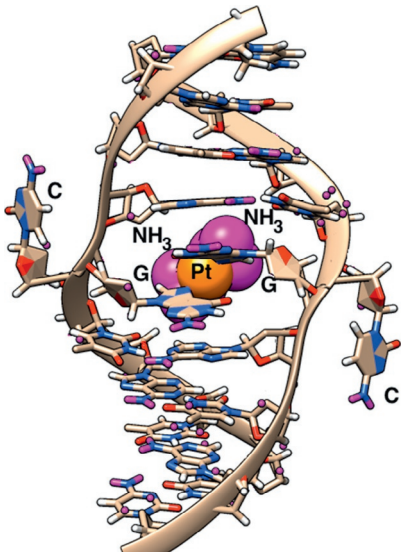


FIGURE 5.41

Structure of a cisplatin-induced DNA interstrand cross-link. Generated from Protein Data Bank reference 1DDP and displayed with Chimera 1.8.1.

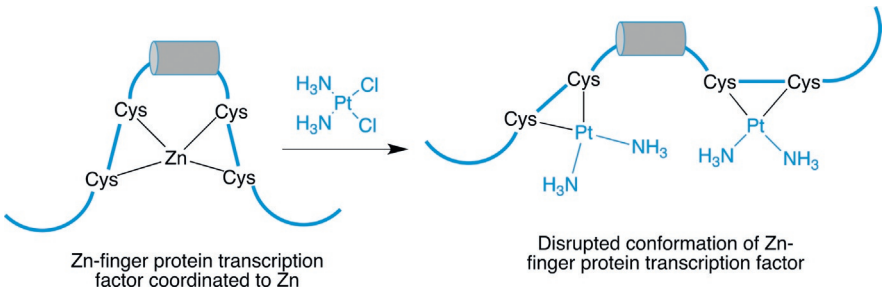


FIGURE 5.42

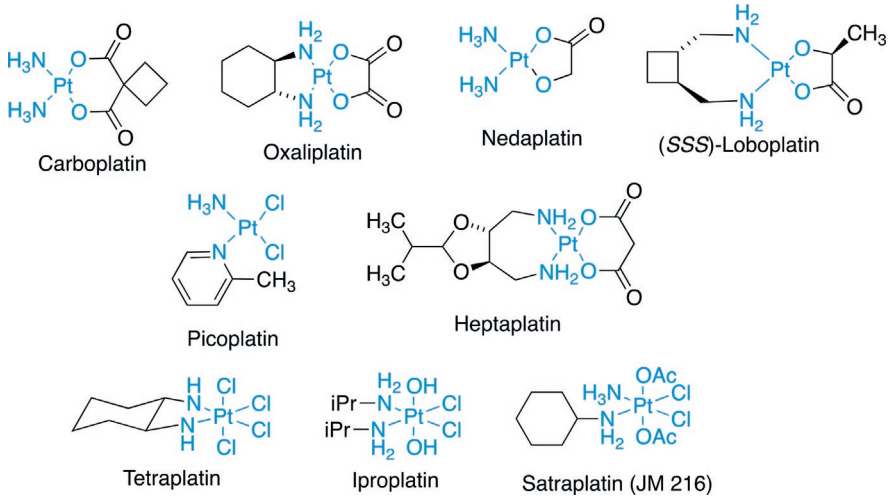
Replacement of Zn by Pt in Zn-finger transcription factors.

therapeutic index.[96] Many platinum-based drugs have entered clinical trials, with more failures than successes.[97] Among these drugs, only two (carboplatin and oxaliplatin) have gained international marketing approval, three more (nedaplatin, lobaplatin, and heptaplatin) have gained approval in individual nations, and currently there are a few additional drugs in various phases of clinical trials (satraplatin and picoplatin). The focus in this area has shifted toward drug delivery, as in the case of lipoplatin, a liposomal derivative of cisplatin (see Chapter 13, Section 7.1).[98]

Cisplatin analogs include tetragonal Pt(II) complexes, such as carboplatin, nedaplatin, oxaliplatin, ZD-0473, and SKI-2053R. Octahedral Pt(IV) complexes are also known, including tetraplatin,

iproplatin, and satraplatin (JM 216).[99] The development of these cisplatin analogs has revealed common requirements that are necessary for their use as an anticancer drug:

1. Electroneutrality to allow for the drug to pass through cell membranes, although the active form is charged after ligand exchange.
2. The presence of at least two good leaving groups, preferentially *cis* to one another, although *trans* complexes also show activity in some cases (discussed later).
3. The presence of "inert" carrier ligands, usually nontertiary amine groups that increase adduct stabilization through hydrogen bonding with nearby bases.



Carboplatin (Paraplatin®) was approved in 1989 for the initial treatment of advanced ovarian cancer in combination with other chemotherapeutic agents. It has a mechanism of action identical to that of cisplatin, forming cross-links with guanine in DNA. At effective doses, it produces substantially reduced nephrotoxicity because the dicarboxylate ligands facilitate its excretion. Oxaliplatin (Eloxatin®), which was licensed in Europe in 1999 but gained FDA approval only in 2002,[100] showed *in vitro* and *in vivo* efficacy against many tumor cell lines, including some that are resistant to cisplatin and carboplatin. The presence of the bulky diaminocyclohexane ring is thought to result in the formation of platinum–DNA adducts more effective at blocking DNA replication than in the case of cisplatin. Oxaliplatin has a spectrum of activity different from that of either cisplatin or carboplatin and lacks cross-resistance with them, suggesting that it has different molecular targets and/or mechanisms of resistance.[101] It was the first platin-based drug to be active against metastatic colorectal cancer in combination with fluorouracil and folinic acid.[102]

Three other platin-based drugs have been approved: nedaplatin (Aqupla®) in Japan, lobaplatin in China, and heptaplatin in South Korea. Nedaplatin is less toxic than cisplatin, but it is only moderately successful in overcoming cisplatin resistance. Lobaplatin, a nearly 1/1 mixture of the (*SSS*)- and (*RRS*)-diastereoisomers of 1,2-diammino-methyl-cyclobutaneplatinum(II) lactate, was approved in China for the treatment of CML, metastatic breast cancer, and small cell lung cancer.[103] SKI-2053R (Heptaplatin), which has also considerably less toxicity than the parent molecule,[104] was approved for the treatment of gastric cancers.[105]

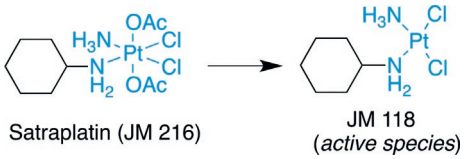


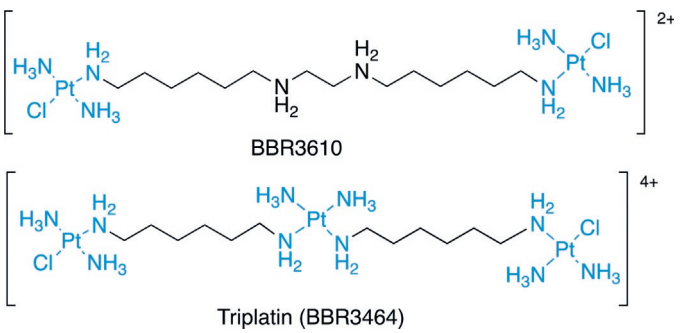
FIGURE 5.43

Bioactivation of a Pt(IV) complex.

Aiming at overcoming resistance due to interaction with thiol-containing molecules, ZD-0473 (picoplatin) was designed, in which one of the amines linked to Pt was replaced by a bulky methyl-substituted pyridine, allowing a longer half-life.[106,107] On the other hand, efforts to find new orally administered analogs led to the octahedral Pt(IV) complex satraplatin (JM 216). The last two drugs are still under clinical trials.

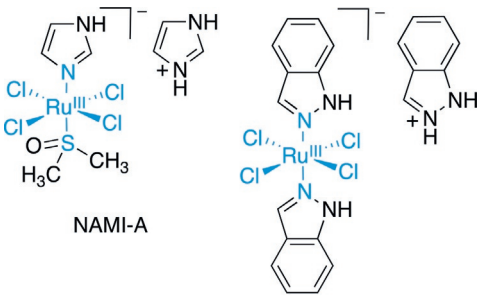
Pt(IV) complexes appear to act by a different mechanism, and evidence suggests that reduction to the corresponding Pt(II) derivatives is necessary for activity. For instance, the active species for satraplatin is believed to be compound JM 118 (Figure 5.43).[108] Unlike Pt(II) complexes, Pt(IV) compounds are highly stable in plasma and therefore can reach the tumor site unchanged and then be transformed into the biologically active Pt(II) species by gradual reaction with biological reducing agents such as ascorbate, glutathione, or NAD(P)H). The hypoxic environment that is usually present inside solid tumors helps this bioactivation to take place in a selective manner.

During the 1990s, it was reported that some *trans*-platinum complexes had activity against tumors resistant to cisplatin, implying differences in the DNA binding of both types of complexes. The *trans* isomer of cisplatin, called TDDP (*trans*-diaminedichloroplatinum(II)), is unable to form 1,2-intrastrand adducts due to its stereochemistry, but it forms interstrand cross-links between complementary guanine and cytosine and 1,3-intrastrand adducts, causing a different type of conformational distortion of the double helix.[109] Other types of *trans*-platinum antitumor compounds are dinuclear and trinuclear platinum (II) complexes (containing two or three reactive platinum centers), designed to form long-range interstrand and intrastrand DNA cross-links. Two examples are the diplatinum and the triplatinum complexes BBR3610 and triplatin (BBR3464). The latter compound forms DNA interstrand cross-links as well as 1,4 and 1,5 intrastrand cross-links, showing preference for the guanine–guanine sequence.[110] It has a broad spectrum of antitumor activity and is undergoing clinical trials.[111]



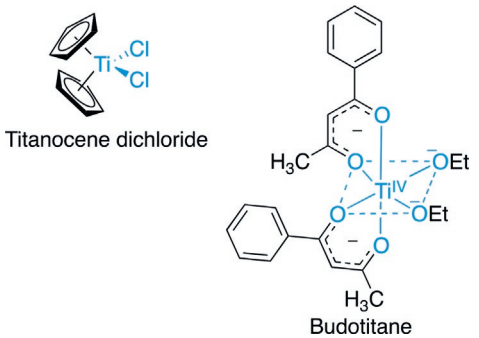
10.2 RUTHENIUM COMPLEXES

In view of the high systemic toxicity of platinum complexes and their propensity to develop drug resistance, complexes of other transition metals, particularly ruthenium, are being investigated.[112] The most advanced compounds in this area are NAMI-A and KP-1019, which have entered clinical trials. Similarly to Pt(IV) complexes, these Ru(III) derivatives need reductive activation to Ru(II) cytotoxic species. Thus, the relatively inert Ru(III) species are transported due to their affinity for transferrin and delivered into the tumors, where they are reduced to the Ru(II) state with preference over healthy tissues because of the combined effects of hypoxia and an acidic environment.[113]



10.3 TITANOCENES

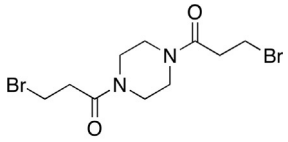
Titanocene dichloride was the first organometallic compound to be studied as an anticancer agent. It progressed to phase II clinical trials, but it was found to have insufficient activity. Similarly, budotitane showed some activity *in vitro* but did not give encouraging results in clinical trials. In both cases, the low *in vivo* stability of the titanocenes toward hydrolysis was probably the cause of their disappointing performance. A number of modified titanocenes have been designed to address this problem, but they have not yet reached the clinical stage.[114]



Although the mechanism of the anticancer action of titanocenes is complex and is not known in detail, it has been proposed that, similarly to the platin complexes, their activity is primarily due to direct DNA damage and alteration of Zn^{2+} homeostasis.[115]

11 MISCELLANEOUS ALKYLATING AND ACYLATING ANTITUMOR AGENTS

Pipobroman (Vercyte®) is used for treatment of polycythemias, such as polycythemia vera (Vaquez's disease), a relatively rare chronic disease of the blood in which the red cells are increased in number,[116] and essential thrombocythemia.[117] Pipobroman has a chemical structure similar to that of alkylating agents, although its exact mechanism of action has not been demonstrated.



Pipobroman

Among the many DNA-damaging natural cytotoxins, leinamycin is of particular interest because of its ability to simultaneously generate both DNA-damaging radicals and electrophiles by completely novel chemical pathways. Although it has not been clinically tested, it represents a new structural type of DNA-damaging agent.[118] Leinamycin was isolated from a strain of *Streptomyces* found in soil samples collected in Japan.[119] Early *in vitro* experiments revealed that the DNA damage is thiol triggered and is due to its unique 1,2-dithiolan-3-one-1-oxide moiety. Although leinamycin is relatively stable in water, upon entering the thiol-rich environment of the cell, a cascade of chemical reactions is initiated that leads to oxidative DNA damage (and perhaps general oxidative stress),[120] as well as DNA alkylation,[121] which is sequence specific.[122] As shown in Figure 5.44, the initial reaction with thiols gives

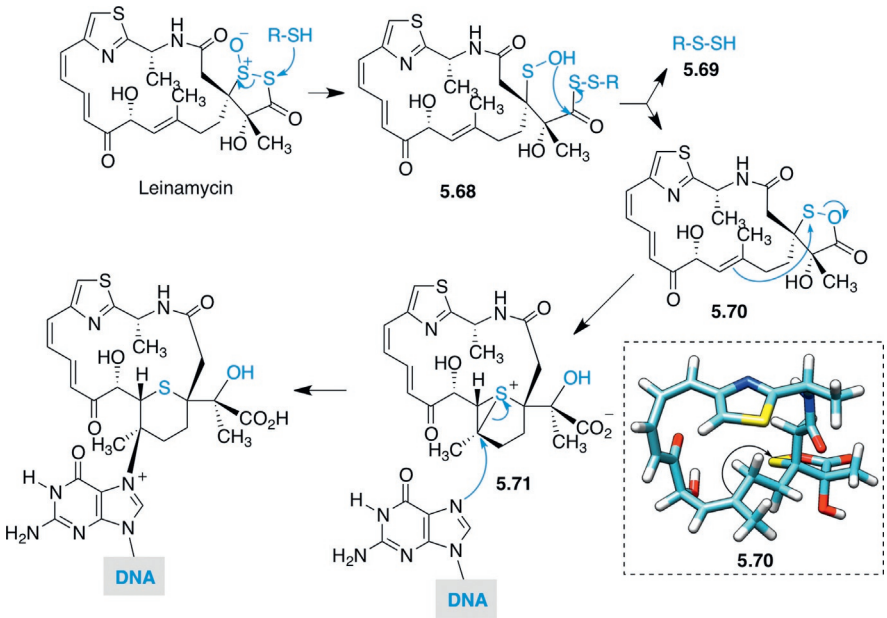


FIGURE 5.44

DNA alkylation by leinamycin.

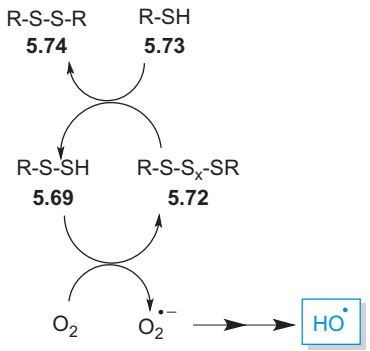


FIGURE 5.45

Generation of hydroxyl radicals from a leinamycin-derived hydrodisulfide.

intermediates **5.68** that cyclize to **5.70** with release of hydrodisufides **5.69**, which cause oxidative DNA damage (see later). The spatial arrangement of **5.70** allows nucleophilic attack of the alkene to the electrophilic sulfur to give the highly electrophilic episulfonium ion **5.71**, which alkylates DNA at the N-7 position of guanine residues.

Hydrodisulfides are more easily oxidized than sulfides because of their higher acidity, which leads to complete ionization under physiological pH. Therefore, it was proposed that compounds **5.69** liberated from leinomycin can transfer one electron to molecular oxygen, leading to the generation of oxygen radicals. Catalytic amounts of **5.69** are sufficient to cause oxidative DNA damage and subsequent strand breaking by this mechanism because polysulfides **5.72** are transformed back into **5.69** by reaction with thiols **5.73**, which are thus depleted from the cell (Figure 5.45). However, recent data indicate that reactive oxygen species are not crucial for leinamycin-induced DNA damage.[123]

REFERENCES

1 Hurley LH. *Nat Rev Cancer* 2002;**2**:188.
2 For a review of DNA binders in clinical trials and chemotherapy, seeAli A, Bhattacharya S. *Bioorg Med Chem* 2014;**22**:4506.
3 Nelson SM, Ferguson LR, Denny WA. *Cell Chromosome* 2004;**3**:2.
4 Blackburn GM, Gait MJ, Loakes D, Williams DM. *Nucleic acids in chemistry and biology*. 3rd ed. Washington, DC: RSC Publishing; 2006 [chapter 8].
5 Adair FE, Bagg HJ. *Ann Surg* 1931;**93**:190.
6 Gilman A, Phillips FS. *Science* 1946;**103**:409.
7 Busia A, Laffranchi A, Viviani S, Bonfante V, Villani F. *Anticancer Res* 2010;**30**:4381.
8 Persmark M, Guengerich FP. *Biochemistry* 1994;**33**:8662.
9 Greenberg MM, Hantosi Z, Wiederholt CJ, Rithner CD. *Biochemistry* 2001;**40**:15856.
10 For reviews, see. (a) Cheson BD, Rummel MJ. *J Clin Oncol* 2009;**27**:1492; (b) Sheridan M. *Drugs* 2012;**72**:1929.
11 Werner W, Letsch G, Ihn W, Sohr R, Preiss R. *Pharmazie* 1991;**46**:113.
12 Bayraktar UD, Bashir Q, Qazilbash M, Champlin RE, Clurea SO. *Biol Blood Marrow Transplant* 2012;**18**:372.

13 Wu ZY, Thompson MJ, Roberts MS, Addison MS, Cannell GR, Grabs AJ, et al. *J Chromatogr* 1995;**673**:267.
14 Brightman K, Finlay G, Jarvis I, Knowlton T, Manktelowa CT. *J Pharm Biomed Anal* 1999;**20**:439.
15 Urbaniak MD, Bingham JP, Hartley JA, Woolfson DN, Caddick S. *J Med Chem* 2004;**47**:4710.
16 Wickström M, Johnsen JI, Ponthan F, Segeström L, Sveinbjörnsson B, Lövborg H, et al. *Mol Cancer Ther* 2007;**6**:2409.
17 Wickström M, Haglund C, Lindman H, Nygren P, Larsson R, Gullbo J. *Invest New Drugs* 2008;**26**:195.
18 Gullbo J, Wickström M, Tullberg M, Ehrsson H, Lewensohn R, Nygren P, et al. *J Drug Target* 2003;**11**:355.
19 (a) Wickström M, Viktorsson K, Lundholm L, Aesoy R, Nygren H, Sooman L, et al. *Biochem Pharmacol* 2010;**79**:1281; (b) Chauhan D, Ray A, Viktorsson K, Spira J, Paba-Prada C, Munshi N, et al. *Clin Cancer Res* 2013;**19**:3019.
20 Tew KD. *Semin Oncol* 1983;**10**:21.
21 Mikhael JR, Schuster SR, Jiménez-Zepeda VH, Bello N, Spong J, Reeder CB, et al. *Blood* 2012;**119**:4391.
22 Cox PJ. *Biochem Pharmacol* 1979;**28**:2045.
23 Palmer BD, Wilson WR, Cliffe S, Denny WA. *J Med Chem* 1992;**35**:3214.
24 Ware DC, Palmer BD, Wilson WR, Denny WA. *J Med Chem* 1993;**36**:1839.
25 Ahn GO, Brown M. *Front Biosci* 2007;**12**:3483.
26 Killian D, Gharat L, Chikhale P. *Drug Deliv* 2000;**7**:21.
27 Wilson HM. *Ann Intern Med* 1954;**41**:118.
28 Eagan RT, Dinapoli RT, Cascino TL, Scheithauer B, O'Neill BP, O'Fallon JR. *J Neurooncol* 1987;**5**:3009.
29 Obe G, Beek B. *Mutat Res* 1979;**65**:21.
30 Phillips RM, Hendriks HR, Peters GJ. *Br J Pharmacol* 2012;**10**:1476.
31 Begleiter A. *Front Biosci* 2000;**5**:153.
32 Hargreaves RHJ, Hartley JA, Butler J. *Front Biosci* 2000;**5**:172.
33 Pierce SE, Guziec LJ, Guziec FS, Brodbelt JS. *Chem Res Toxicol* 2010;**23**:1097.
34 Naylor MA, Thomson P. *Mini Rev Med Chem* 2001;**1**:17.
35 Moore HW. *Science* 1977;**197**:527.
36 Butler J, Dzielendziak A, Lea JS, Ward TH, Hoey BM. *Free Radic Res Commun* 1990;**8**:231.
37 Akhtar HM, Begleiter A, Johnson D, Lown JW, McLaughlin L, Sim SK. *Can J Chem* 1975;**53**:2891.
38 Beall HD, Winski SL. *Front Biosci* 2000;**5**:639.
39 Danson S, Ward TH, Butler J, Ranson M. *Cancer Treatment Rev* 2004;**30**:437.
40 Gibson NW, Hartley JA, Butler J, Siegel D, Ross D. *Mol Pharmacol* 1992;**42**:531.
41 Begleiter A. *Front Biosci* 2000;**5**:E153.
42 Fourie J, Guziec F, Guziec L, Monterrosa C, Fiterman DJ, Begleiter A. *Cancer Chemother Pharmacol* 2004;**53**:191.
43 Winski SL, Hargreaves RHJ, Butler J, Ross D. *Clin Cancer Res* 1998;**4**:3083.
44 Ward TH, Danson S, McGown AT, Ranson M, Coe NA, Jayson GC, et al. *Clin Cancer Res* 2005;**11**:2695.
45 Danson S, Ranson M, Denneny O, Cummings J, Ward TH. *Cancer Chemother Pharmacol* 2007;**60**:851.
46 Danson SJ, Johnson P, Ward TH, Dawson M, Denneny O, Dickinson G, et al. *Ann Oncol* 2011;**22**:1653.
47 Zhou R, Skibo EB. *J Med Chem* 1996;**39**:4321.
48 Li BB, Gutiérrez PL, Amstad P, Blough NV. *Chem Res Toxicol* 1999;**12**:1042.
49 Xing C, Skibo EB. *Biochemistry* 2000;**39**:10770.
50 Coleman RS. *Curr Opin Drug Discov Dev* 2001;**4**:435.
51 Eilon GF, Gu J, Slater LM, Hara K, Jacobs JW. *Cancer Chemother Pharmacol* 2000;**45**:183.
52 Xie X, Lemcke T, Gussio R, Zaharevitz DW, Leost M, Meijer L, et al. *Eur J Med Chem* 2005;**40**:655.
53 Hartley JA, O'Hare CC, Baumgart J. *Br J Cancer* 1999;**79**:264.
54 Danylesko I, Shimoni A, Nagler A. *Bone Marrow Transpl* 2012;**47**:5.
55 Bernardo ME, Piras E, Vacca A, Giorgiani G, Zecca M, Bertaina A, et al. *Blood* 2012;**120**:473.

56 Szebeni J, Barna K, Uher F, Milosevits J, Paloczi K, Gaal D, et al. *Leukemia* 1997;**11**:1769.
57 Coleman RS, Pérez RJ, Burk CH, Navarro A. *J Am Chem Soc* 2002;**124**:13008.
58 Foulke-Abel J, Agbo H, Zhang H, Mori S, Watanabe CMH. *Nat Prod Rep* 2011;**28**:693.
59 Ravdin PM, Havlin KA, Marshall MV, Brown TD, Koeller JM, Kuhn JG, et al. *Cancer Res* 1991;**51**:6268.
60 Bedford P, Fox BW. *Biochem Pharmacol* 1983;**32**:2297.
61 Hartley JA, O'Hare CC, Baumgart J. *Br J Cancer* 1999;**79**:264.
62 Iwamoto T, Hiraku Y, Oikawa S, Mizutanei H, Kojima M, Kawanishi S. *Cancer Res* 2004;**95**:454.
63 Farmer PB. *Pharmacol Ther* 1987;**35**:301.
64 Puppa AD, Rossetto M, Ciccarino P, Denaro L, Rotilio A, d'Avella D, et al. *World Neurosurg* 2011;**76**:156.
65 Glas M, Hundsberger T, Stuplich M, Wiewrodt D, Kurzwelly D, Nguyen-Huu B, et al. *Oncology* 2009;**76**:174.
66 Samson MK, Baker LH, Cummings G, Talley RW, McDonald B, Bhathena DB. *Cancer Treat Rep* 1982;**66**:371.
67 Hayes MT, Bartley J, Parsons PG, Eaglesham GK, Prakask AS. *Biochemistry* 1997;**36**:10646.
68 Montgomery JA, James R, McCaleb GS, Johnston TP. *J Med Chem* 1967;**10**:668.
69 Johnston TP, Montgomery JA. *Cancer Treat Rep* 1986;**70**:13.
70 Buckley N. *J Org Chem* 1987;**52**:484.
71 Murata M, Takahashi A, Saito I, Kawanishi S. *Biochem Pharmacol* 1999;**57**:881.
72 Bolzán AD, Bianchi MS. *Mutat Res* 2002;**512**:121.
73 Kroncke KD, Fehsel K, Sommer A, Rodriguez ML, Kolb-Bachofen V. *Biol Chem Hoppe Seyler* 1995;**376**:179.
74 Bedoya FJ, Solano F, Lucas M. *Experientia* 1996;**52**:344.
75 García del Muro X, López-Pousa A, Maurel J, Martín J, Martínez-Trufero J, Casado A, et al. *J Clin Oncol* 2011;**29**:2528.
76 Meer L, Janzer RC, Kleihues P, Kolar GF. *Biochem Pharmacol* 1986;**35**:3243.
77 Kyrtopoulos SA, Souliotis VL, Valavanis C, Boussiotis VA, Pangalis GA. *Environ Health Perspect* 1993;**99**:143.
78 Glaser R, Sik-Cheung Choy G, Kirk Hall M. *J Am Chem Soc* 1991;**113**:1109.
79 For a review of temozolomide development, see Stevens MFG. In: Neidle S, editor. *Cancer drug design and discovery*. 2nd ed. New York: Elsevier; 2014 [chapter 5].
80 Fukushima T, Takeshima H, Kataoka H. *Anticancer Res* 2009;**29**:4845.
81 Renschler MF, Eur J. *Cancer* 2004;**40**:1934.
82 Chan JK, Loizzi V, Manetta A, Berman ML. *Gynecol Oncol* 2004;**92**:368.
83 Ames MM. *Cancer Treat Rev* 1991;**18**(Suppl. A):3.
84 Jackso C, Crabb TA, Gibson M, Godfrey R, Saunders R, Thurston DE. *J Pharm Sci* 1991;**80**:245.
85 Coley HM, Brooks N, Phillips DH, Hewer A, Jenkins TC, Jarman M, et al. *Biochem Pharmacol* 1995;**11**:1203.
86 Judson IR, Calvert AH, Gore ME, Balmanno K, Gumbrell LA, Perren T, et al. *Br J Cancer* 1991;**63**:311.
87 Kehe K, Szinicz L. *Toxicology* 2005;**214**:198.
88 Reedijk J, Eur J. *Inorg Chem* 2009;**2009**:130.
89 Lippard SJ. *Pure Appl Chem* 1987;**59**:731.
90 Takahara PM, Rosenzweig AC, Frederick CA, Lippard SJ. *Nature* 1995;**377**:649.
91 Ohndorf U, Rould MA, He Q, Pabo CO, Lippard SJ. *Nature* 1999;**399**:708.
92 Huang H, Zhu L, Reid BR, Drobny GP, Hopkins PB. *Science* 1995;**270**:1842.
93 Dong W, Lippard SJ. *Nature Rev Drug Discov* 2005;**4**:307.
94 Rebillard A, Lagadic-Gossmann D, Dimanche-Boitrel M-T. *Curr Med Chem* 2008;**15**:2656.
95 Sheikh-Hamad D, Am J. *Physiol* 2008;**295**:F42.
96 Monneret C. *Ann Pharm Fr* 2011;**69**:286.
97 For a review of the status of platinum anticancer drugs in the clinic and in clinical trials, see Wheate NJ, Walker S, Craig GE, Oun R. *Dalton Trans* 2010;**39**:8113.

98 Boulikas T, Pantos A, Bellis E, Christofis P. *Cancer Ther* 2007;**5**:537.
99 Kelland L. *Nat Rev Cancer* 2007;**7**:573.
100 Ibrahim A, Hirschfeld S, Cohen MH, Griebel DJ, Williams GA, Pazdur R. *Oncologist* 2004;**9**:8.
101 Raymond E, Faivre S, Woynarowski JM, Chaney SG. *Semin Oncol* 1998;**25**:4.
102 Graham J, Mushim M, Kirkpatrick P. *Nature Rev Drug Discov* 2004;**3**:11.
103 Wu Q, Qin Sh-K, Chen Ch-J, Wang R. *J Hematol Oncol* 2010;**3**:43.
104 Kim NK, Kim TY, Shin SG. *Cancer* 2001;**91**:1549.
105 Lee JW, Park JK, Lee SH, Kim SY, Cho YB, Kui HJ. *Anticancer Drugs* 2006;**17**:377.
106 Hoctin-Boes G, Cosaert J, Koehler M. *Proc Am Soc Clin Oncol* 2001;**20**:344a.
107 Gelmon KA, Vandernberg TA, Panasci L, Norris B, Crump M, Douglas L, et al. *Ann Oncol* 2003;**14**:543.
108 Kelland LR. *Expert Opin Invest Drugs* 2000;**9**:1373.
109 Radulovic S, Tesic Z, Manic S. *Curr Med Chem* 2002;**9**:1611.
110 Perego P, Caserini C, Gatti L, Carenini N, Romanelli S, Supino R, et al. *Mol Pharmacol* 1999;**55**:528.
111 Jodrell DI, Evans TRJ, Steward W, Cameron D, Prendiville J, Aschele C, et al. *Eur J Cancer* 2004;**40**:1872.
112 For selected reviews, see. (a) Reisner E, Arion VB, Keppler BK, Pombeiro AJL. *Inorg Chim Acta* 2008;**361**:1569; (b) Levina A, Mitra A, Lay PA. *Metallomics* 2009;**1**:458.
113 Schluga P, Hartinger CG, Egger A, Reisner E, Galanski M, Jakupec MA, et al. *Dalton Trans* 2006;**14**:1796.
114 Pizarro AM, Habtemariam A, Sadler PJ. *Top Organomet Chem* 2010;**32**:21.
115 Olszewski U, Hamilton G. *Anticancer Agents Med Chem* 2010;**10**:302.
116 Kiladjian JJ, Gardin C, Renoux M, Bruno F, Bernard JF. *Hematol J* 2003;**4**:198.
117 Passamonti F, Lazzarino M. *Leuk Lymphoma* 2003;**44**:1483.
118 Gates KS. *Chem Res Toxicol* 2000;**13**:953.
119 Hara M, Takahashi I, Yoshida M, Kawamoto I, Morimoto M, Nakano H. *J Antibiot* 1989;**42**:333.
120 Mitra K, Kim W, Daniels JS, Gates KS. *J Am Chem Soc* 1997;**119**:11691.
121 Asai A, Hara M, Kakita S, Kanda Y, Yoshida M, Saito H, et al. *J Am Chem Soc* 1996;**118**:6802.
122 Zang H, Gates KS. *Chem Res Toxicol* 2003;**16**:1539.
123 Sinha P, Shin YJ, Hays AM, Gates K, Sun D. *J Cancer Sci Ther* 2012;**S8**:3.

CHAPTER

ANTICANCER DRUGS THAT INTERACT WITH THE DNA MINOR GROOVE

6

CONTENTS

1 INTRODUCTION

Besides nonspecific electrostatic interaction with phosphate groups, there are two main ways in which a small molecule can bind to DNA in a reversible way:

1. Groove-binding interactions, which do not require conformational changes in DNA and usually show high sequence specificity.
2. Intercalation of planar or quasi-planar aromatic ring systems between adjacent base pairs, which requires separation of the latter and normally takes place with low sequence specificity (Figure 6.1). Intercalation is discussed in detail in Chapter 7.

2 NETROPSIN, DISTAMYCIN, AND RELATED COMPOUNDS

Minor groove interaction was first discovered in the natural products netropsin and distamycin A. Although these compounds do not have relevant antitumor activity, they are the prototype minor groove binders (MGBs), and for this reason they are briefly discussed here. They bind noncovalently to the minor groove of DNA, thereby preventing DNA and RNA synthesis by inhibition of the corresponding polymerase reaction, and display a pronounced sequence specificity, which has led to much interest in them.[1]

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00006-5

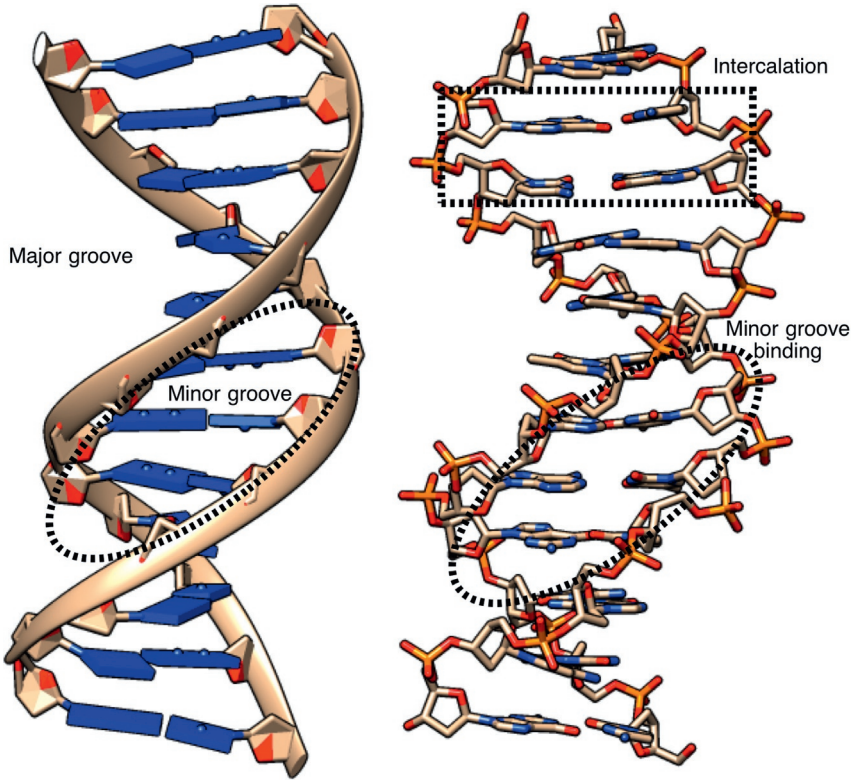
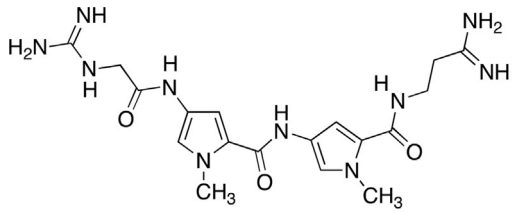
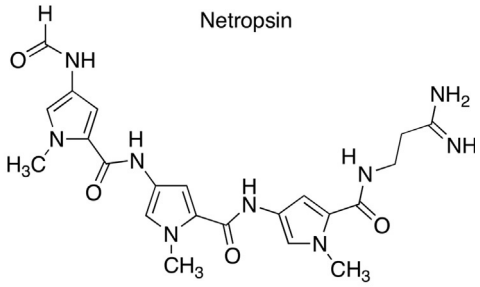


FIGURE 6.1

Main types of reversible interactions with DNA.



Netropsin



Distamycin A

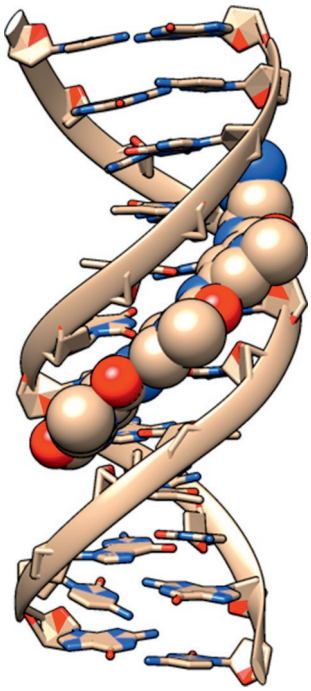


FIGURE 6.2

Binding of distamycin A to DNA. Generated from Protein Data Bank entry 2DND and displayed with Chimera 1.8.1.

Because of the differences in electrostatic potential, hydration, hydrogen bonding ability, and steric hindrance, the major and minor grooves differ in their molecular recognition properties. Thus, the major groove normally binds to large molecules, such as proteins and oligonucleotides, and the minor groove has a tendency to bind to small molecules. Due to the curved shape of the minor groove, molecules with torsional freedom interact with it more easily (Figure 6.2); for this reason, many of the compounds discussed in this chapter contain several simple aromatic or heteromatic rings linked by torsionally free bonds. The interaction with the minor groove of some antitumor agents was mentioned in Chapter 4 (anthracyclines in Section 3, bleomycins in Section 7, and enediynes in Section 8).

Studies on drug specificity toward the minor groove have been carried out mainly on distamycin and related compounds, which have shown a pronounced specificity for AT sequences.[2] Ligand recognition by the minor groove is governed, in the first place, by hydrogen bonding interactions involving hydrogen acceptor groups in DNA bases, particularly N_3 and $C_2=O$ of the adenine–timine or guanine–citosine pairs. As shown in Figure 6.3, these interactions are hampered in the latter pair, mainly for steric reasons. In addition, the minor groove is strongly solvated, and liberation of water molecules into the bulk solvent upon complex formation leads to a favorable binding entropy (hydrophobic effect) because AT-rich regions are more hydrated than GC-rich regions and hence they provide a larger entropic contribution. Finally, the negative electrostatic potential is greater in AT-rich than in GC-rich regions, thus favoring an initial electrostatic interaction with positively charged groups in the ligand.

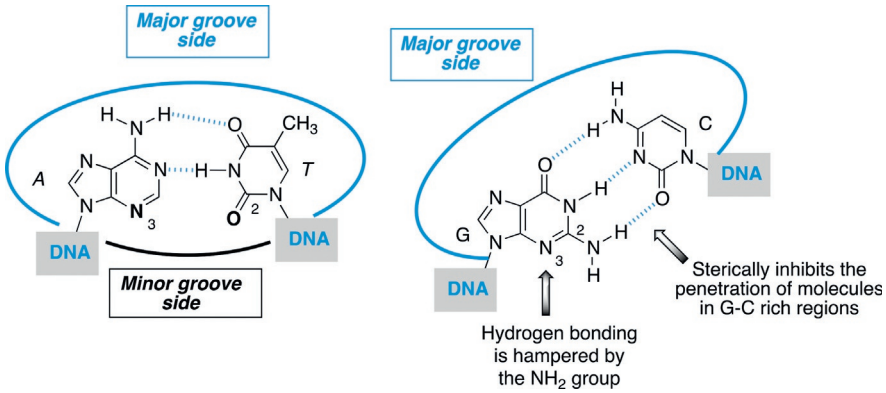


FIGURE 6.3

Adenine–thymine and guanine–cytosine pairs.

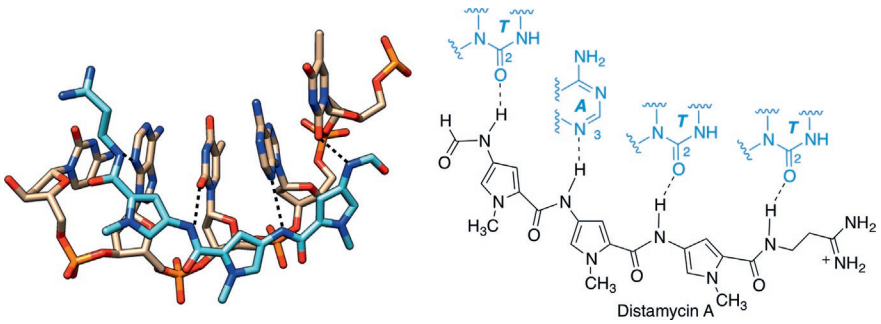


FIGURE 6.4

Hydrogen bonds between distamycin A and the DNA minor groove.

Hydration of the ligand molecules is also an important factor in the understanding of differences in binding affinity.[3]

Hydrogen bonds involve the amido or amidino groups of the drugs as hydrogen donors and the N_3 of adenine and $C_2=O$ groups of thymine as hydrogen acceptors, as shown in Figure 6.4 for the case of distamycin A.

Theoretical and X-ray diffraction studies suggest the formation of bifurcated (three-centered) hydrogen bonds,[4] where each carboxamide is bound to two acceptor groups belonging to bases in complementary DNA strands (Figure 6.5). Contrary to initial expectations, the protonated guanidine or amidine groups do not bind directly to DNA phosphate groups but, rather, line the floor of the minor groove.

The synthesis of analogs of distamycin A by increasing the number of *N*-methylpyrrole-2-carboxamide units or replacement of some pyrrole nuclei by imidazole, and also by preparation of hybrid structures with intercalating or alkylating portions, has led in some instances to much enhanced cytotoxicity.[5] The most promising compounds in this area are tallimustine, brostallicin (PNU-166196), and PNU- 145156E (FCE 26644).

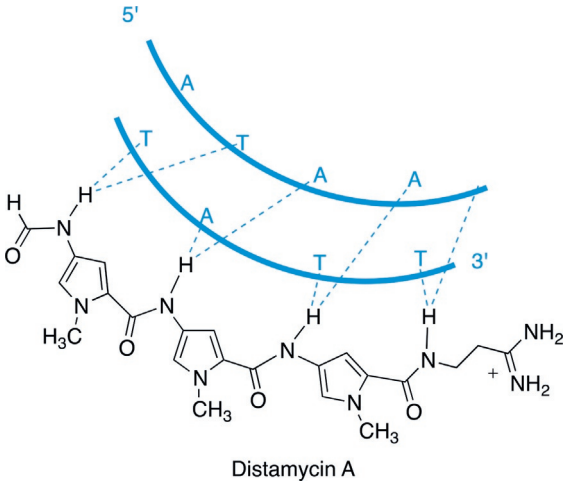
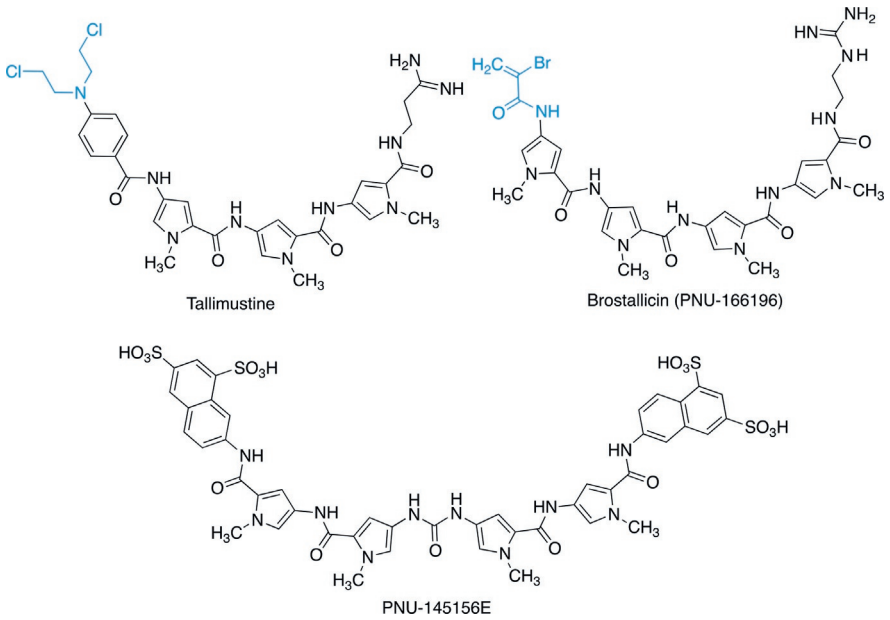


FIGURE 6.5

Three-centered hydrogen bonds in the distamycin A–DNA interaction.



Tallimustine (FCE 24517) contains a benzoyl nitrogen mustard unit, which acts as an alkylating moiety, attached to the distamycin A framework. This compound exhibits a most striking DNA sequence specificity of alkylation, which has been studied using the combinatorial selection method restriction endonuclease protection, selection, and amplification (REPSA).[6] The highest affinity tallimustine

binding sites contain one of two sequences, either the expected distamycin hexamer binding sites followed by a CG base pair (e.g., 5′-TTTTTTC-3′ and 5′-AAATTTC-3′) or the unexpected sequence 5′-TAGAAC-3′. It was also found that tallimustine preferentially alkylates the N-7 position of guanines located on the periphery of these sequences. These findings suggest a cooperative binding model for tallimustine in which one molecule noncovalently resides in the DNA minor groove and locally perturbs the DNA structure, thereby facilitating alkylation by a second tallimustine of an exposed guanine on another side of the DNA. Tallimustine is a potent antitumor agent, but it causes significant myelotoxicity,[7] a common problem with many minor groove binding agents.[8] It proceeded to phase II studies in colorectal and small cell lung cancer patients but showed a low therapeutic index, which led to a halt in its development.

Brostallicin (PNU-166196) is a synthetic α-bromoacrylamido derivative of a four-pyrrole distamycin in which the amidine terminal function is replaced by a guanidine moiety. Unlike tallimustine, this compound showed a tolerable myelotoxicity and is now under clinical investigation,[9] being regarded as the most promising distamycin analog.

Brostallicin is inactive *in vitro*, and it requires the presence of glutathione and glutathione-*S*-transferase (GST) to behave as an alkylating agent.[10] The mechanism of alkylation involves an initial Michael attack of glutathione onto the brostallicin α,β-unsaturated carbonyl system, which uncovers an electrophilic alkyl bromide capable of DNA alkylation (Figure 6.6). This unique mechanism of action leads to sinergism with cisplatin because the latter drug increases the levels of GST in cancer cells.

Another derivative of distamycin that has entered clinical development is the sulfonated derivative PNU-145156E (FCE 26644). Despite its distamycin-type backbone, its main mechanism of action involves binding to the basic fibroblast growth factor, a pleiotropic cytokine that plays an important role in angiogenesis. Although it does not show a significant cytotoxcity on its own, PNU-145156E entered phase I trials in combination with other drugs,[11] without displaying a significant response.

Another interesting compound that interacts with DNA in AT-rich sequences is Hoechst 33258 (pibenzimol), initially designed as an antifilarial. Later studies on this compound showed antitumor activity, leading to phase I clinical studies, which were discontinued due to the development of hyperglycemia in some patients.[12] This observation led to a phase II study in patients with advanced carcinoma of the exocrine pancreas, but no relevant activity was observed.[13] When complexed to DNA, this

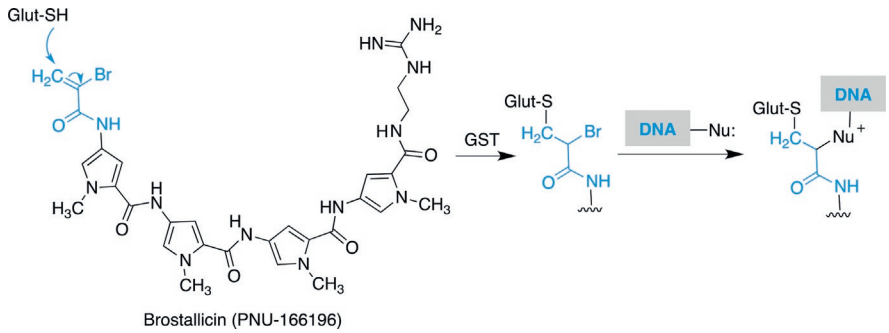
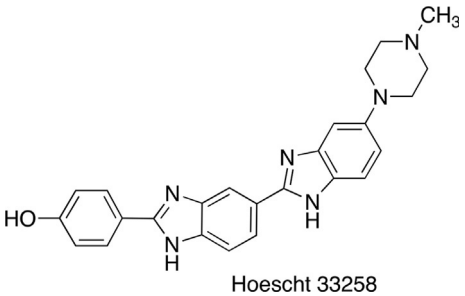


FIGURE 6.6

Bioactivation of brostallicin and DNA alkylation by its glutathione adduct.

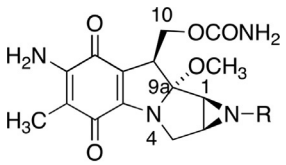
compound exhibits enhanced fluorescence under high ionic strength conditions, which allows its use for DNA quantitation.[14] The N–H groups of the benzimidazole rings in Hoechst 33258 can be considered as bioisosters of the amide N–H groups in distamycin, and they have been shown to lead to similar binding to DNA in X-ray diffraction studies.[15]



Hoescht 33258

3 MITOMYCINS

Mitomycin C (Mutamycin®)[16] is a naturally occurring antitumor quinone from *Streptomyces caespitosus* that contains quinone and aziridine units, although not directly linked. It has been used as a cytotoxic since the 1960s and is active against a variety of tumors, including breast, stomach, esophagus, and bladder,[17] as well as non-small cell lung cancer.[18] The *N*-methyl derivative of mitomycin C is also a natural product called porfiromycin, which has reached phase III clinical studies for treatment of head and neck cancer in combination with radiotherapy, with acceptable toxicity and encouraging activity.[19]



R = H Mitomycin C
R = Me Porfiromycin

Mitomycin C and porfiromycin can be considered as the prototype of reductively activated alkylating agents. The most common structural motif in these compounds is the quinone, which has reduction potentials similar to the substrates of reductases. These compounds are particularly useful for the treatment of hypoxic tumors because in these environments, the bioreduction to hydroquinones is not reversed by oxygen, and they can also act as radiosensitizers.[20] Hypoxia-based strategies for tumor-specific prodrug activation are discussed in more detail in Section 2.2 of Chapter 13.

Because of the presence of a quinone moiety in mitomycin C, a semiquinone intermediate **6.1** can be generated by addition of one electron with participation of a variety of flavoenzymes. Under aerobic conditions, this semiquinone can be oxidized back to the parent mitomycin, generating superoxide anion. The usual mechanism involving superoxide dismutation explains the formation of hydrogen peroxide, which, in the presence of trace metals, forms hydroxyl radicals (Figure 6.7). Although these

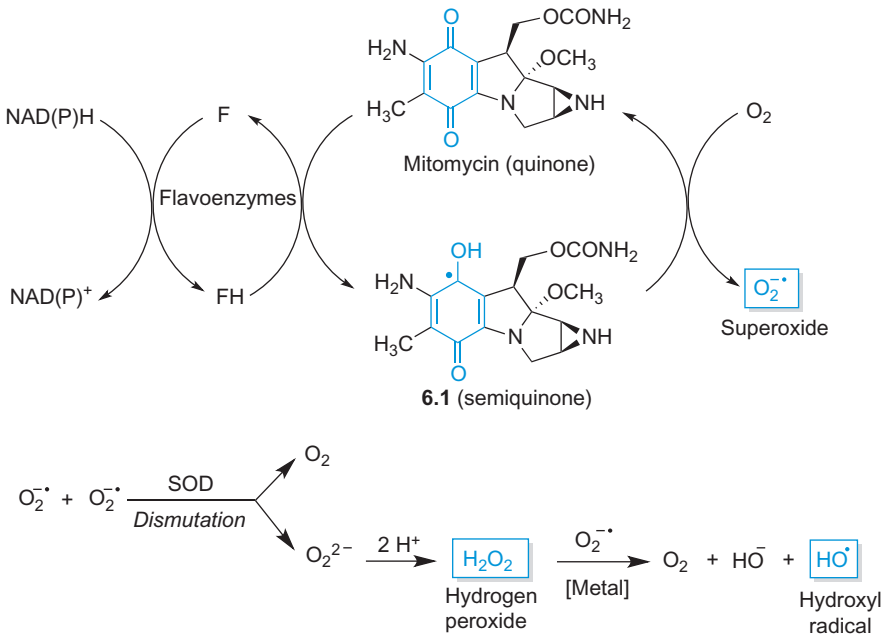


FIGURE 6.7

Generation of ROS by redox cycling of mitomycin.

reactive oxygen species can damage intracellular macromolecules, leading to oxidative stress, redox cycling has been ruled out as the primary mechanism of cytotoxicity of mitomycin C.[21]

The main mechanism of action of mitomycin is a characteristic example of an *in situ* bioreductive activation[22] leading to a cytotoxic species (Figure 6.8). It involves two consecutive one-electron reduction steps to the corresponding semiquinone **6.1** and then to hydroquinone **6.2**. Both forms can initiate the cascade of reactions leading to DNA alkylation, but available evidence indicates that hydroquinone is the active species.[23] Furthermore, human carcinoma cell lines with high levels of DT-diaphorase, an obligate two-electron reducing enzyme that cannot generate intermediate semiquinones, show greater susceptibility to mitomycin, which is inhibited by treatment with diaphorase inhibitors.[24] Spontaneous elimination of methanol from hydroquinone **6.2** gives the iminium derivative **6.3**; this reaction takes place only in aqueous solution, which suggests that protonation of the leaving group by water is essential.[25] A similar elimination reaction is not possible in mitomycin because the N-4 nitrogen lone pair is not available due to its conjugation with one of the quinone carbonyls, leading to a vinylogous amide structure. Indole derivative **6.4**, formed by deprotonation of **6.3**, contains two good leaving groups, namely the aziridine ring and the carbamate. Protonation of the aziridine nitrogen of **6.4** and subsequent elimination with concomitant opening of the aziridine ring affords quinone methide **6.5**. This highly reactive intermediate contains an electrophilic position that reacts with nucleophilic groups on DNA through a Michael-type reaction to give the unstable intermediate **6.6**. This reaction proceeds with absolute specificity toward certain sequences at the minor groove (see later) and involves the guanine N-2 amino group or N-7 position as nucleophiles. Most alkylation events due to mitomycin

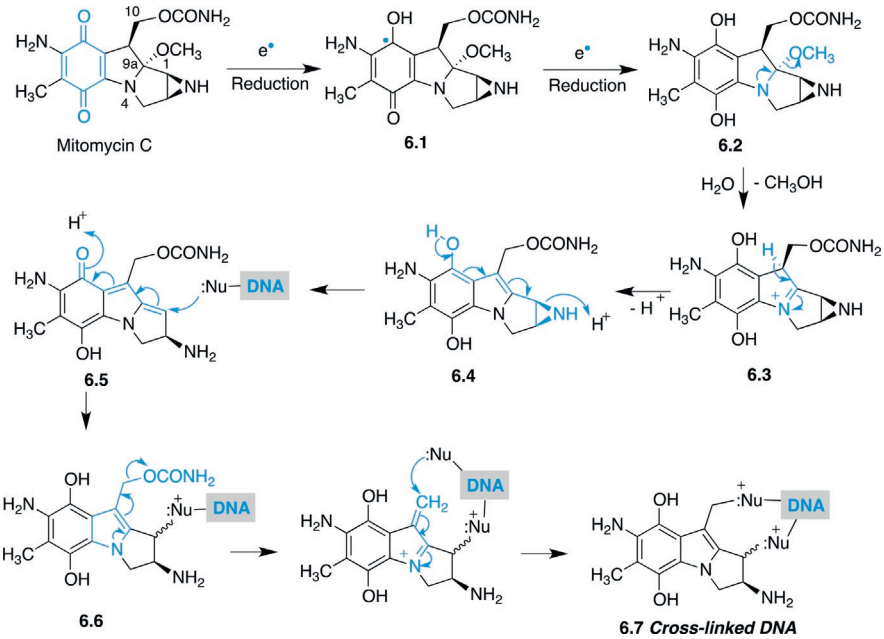


FIGURE 6.8

Bioreductive alkylation of DNA by mitomycin C.

are monoalkylations,[26] but in some cases elimination of the carbamate group generates an electrophilic iminium species, which undergoes a second alkylation by attack from a guanine 2-amino group and leads to DNA cross-linking (**6.7**).[27] Furthermore, mitomycin has been shown to also target rRNA, which may be another physiologically relevant cytotoxicity mechanism.[28]

DNA alkylation by mitomycin takes place preferentially at the minor groove, as shown in Figure 6.9.

Both inter- and intrastrand cross-linking by mitomycin have been observed, although the former is predominant. Interstrand and intrastrand cross-linking are specific, respectively, to 5′-CG[29] and 5′-GG[30] sequences in the minor groove.[31] This selectivity arises from the first alkylation event and has been explained in terms of hydrogen bonding between the guanine N-2 amino group[32,33] and one of the carbamate oxygens, as shown in the models in Figure 6.10, which are based on high-resolution nuclear magnetic resonance (NMR) and molecular modeling studies.[33]

The bioreductive alkylation process has been proposed to explain the interaction of mitomycin C with other nucleophilic biomolecules, providing additional mechanisms for its cytotoxicity. These nucleophiles include rRNA,[34] glutathione,[35] and thioredoxin reductase.[36] The proposed mechanism for the activation of mitomycin C to the active hydroquinone **6.2** by a dithiol is shown in Figure 6.11.

Intermediates similar to **6.2** are generated from the aziridine alkaloids FR-900482 and FR-69979, isolated from a culture broth of *Streptomyces sandaenis*. These compounds give interstrand cross-linking reactions with the same selectivity as mitomycin.[37] The cascade of reactions is initiated by bioreductive activation involving cleavage of the N–O bond to give the eight-membered ketone **6.8**, which

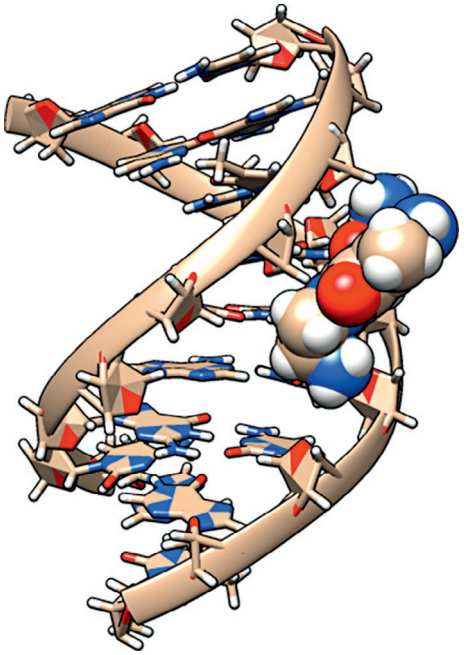


FIGURE 6.9

Structure of a monoalkylated mitomycin C–DNA complex. Generated from Protein Data Bank reference 199D and displayed with Chimera 1.8.1.

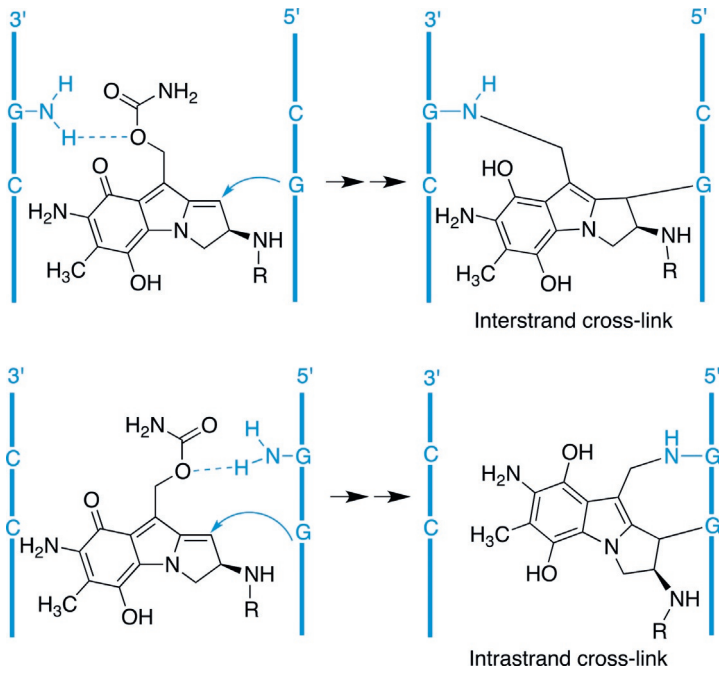


FIGURE 6.10

Inter- and intrastrand DNA cross-linking by mitomycin.

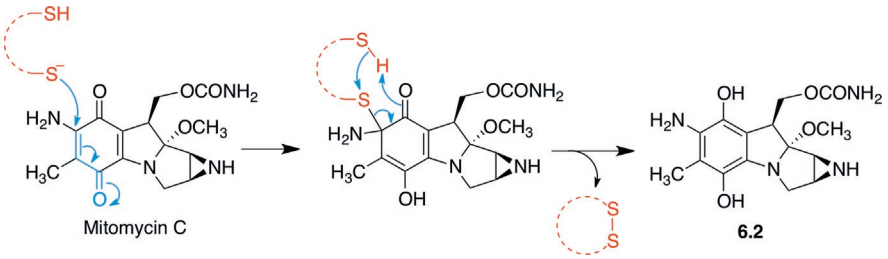


FIGURE 6.11

Activation of mitomycin by a biomolecule containing a dithiol structural fragment.

is transformed into **6.9** by intramolecular nucleophilic attack of the amino group thus generated onto the ketone carbonyl. Evolution of this intermediate as described for **6.2** gives quinone methide intermediate **6.11**, which is very similar to mitomycin intermediate **6.5**, and leads to DNA cross-linking products by a similar mechanism involving amino groups at the guanine N-2 position (Figure 6.12).[38,39] Covalent cross-linking between the DNA minor groove and DNA-binding proteins, including a minor groove-binding oncoprotein, has been described.[40]

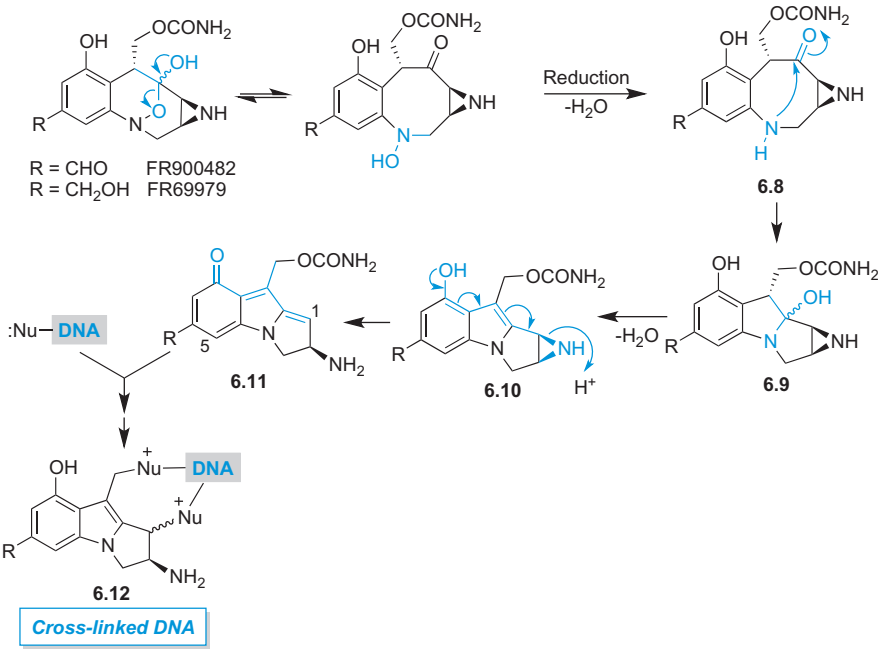


FIGURE 6.12

DNA cross-linking by other aziridine alkaloids.

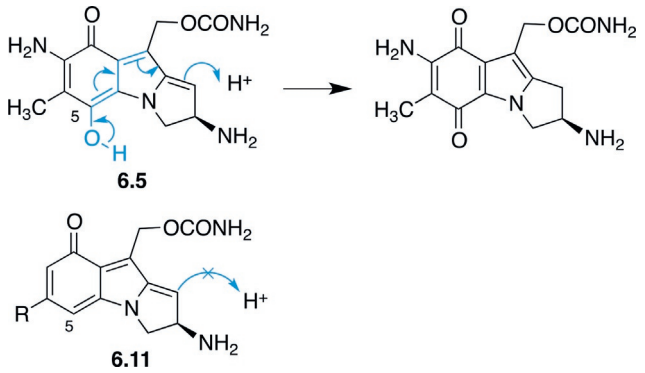
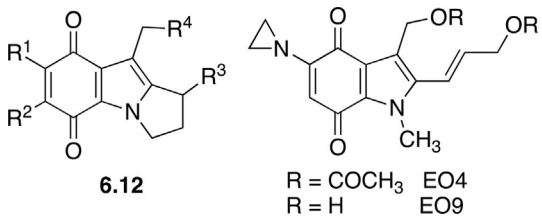


FIGURE 6.13

Differences in the chemical reactivity of the quinone methides derived from mitomycin C (**6.5**) and other aziridine alkaloids (**6.11**).

FR-900482 and FR-69979 are more efficient cross-linking agents than mitomycin. This can be explained in terms of the dual nucleophilic–electrophilic character of the quinone methide **6.5** generated from the latter, which facilitates its protonation at C-1,[41] a reaction that competes with nucleophilic attack from DNA (Figure 6.13). Despite their apparent similarity, intermediates **6.11** generated from the FR compounds lack nucleophilic character due to the absence of a C_5–OH group conjugated with the C-1 position.

The unique mechanism of action and clinical success of mitomycin, coupled with its high toxicity, has prompted the preparation of a large number of synthetic analogs, many of which belong to the mitosene group and have the general structure **6.12**. Some simpler indolequinone derivatives, such as EO4 and apaziquone (EO9), were also designed as mitomycin analogs.



The mechanism of DNA alkylation by the mitosenes is shown in Figure 6.14, using the compound known as WV15 (**6.13**) as an example. After reductive activation to **6.14**, elimination of an acetate generates iminium cation **6.15**, which is able to alkylate DNA to give **6.16**. A second elimination of a benzylic acetate group generates cation **6.17**, which can again act as a DNA alkylating species, leading to the bis-adduct **6.18**. The order of reactivity of the C-1 and C-10 positions of the mitosenes

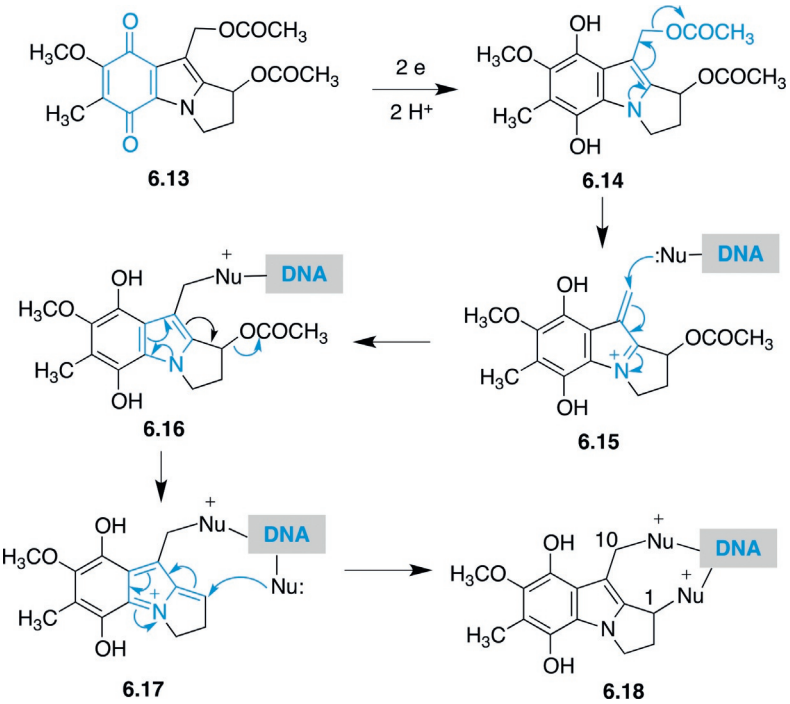


FIGURE 6.14

DNA alkylation by the mitosenes.

is apparently reversed with regard to that of mitomycin C, and the mitosene C-10 position is covalently bonded to the guanosine 2-amino[42] and adenosine 6-amino positions.[43]

The aziridinylquinone apaziquone (EO9, EOquin®)[44] has undergone extensive clinical trials due to its good activity against hypoxic cells and its lack of bone marrow toxicity in preclinical models. However, despite achieving partial responses in phase I studies, it showed no antitumor activity in phase II trials for breast, colon, pancreatic, gastric, and non-small lung cancers. The reasons for this failure can be the very short half-life of the drug, due to fast elimination following intravenous administration, and its poor tissue penetration. These shortcomings prevent its systemic use, but they are actually advantageous for local administration. Thus, EO9 was assayed for treatment of early stage superficial bladder cancer by intravesical administration,[45] where it showed good activity and the absence of major organ toxicity.[46] Apaziquone may be the first new drug to be approved for bladder cancer in more than 20 years; its approval by the U.S. Food and Drug Administration (FDA) is pending phase III clinical trial results.

The EO compounds were designed to alkylate DNA after reduction via formation of quinone methide species. As shown in Figure 6.15, reduction of the drug molecule yields the hydroquinone

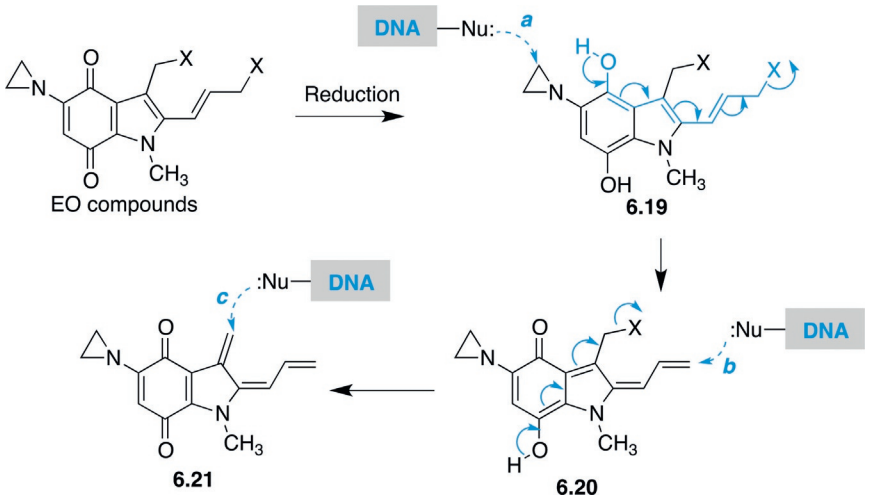


FIGURE 6.15

Bioreductive activation of the EO compounds.

6.19, activating the aziridine ring for nucleophilic attack by DNA (***a***). When X is a good leaving group, two elimination reactions afford highly electrophilic quinone methide intermediates **6.20** and **6.21**, allowing two other sites for DNA alkylation (***b*** and ***c***). EO4 has been shown to give cross-linked DNA adducts involving the ***a*** and ***c*** modes of attack,[47] whereas in the case of EO9, both monoalkylation at the aziridine ring[47] and cross-linking[48] have been described.

One of the main limitations of mitomycin and the mitosenes is the need for reductive activation, which renders them less active in tissues in which the bioreduction can be reverted, especially in the presence of oxygen. In the search for mitomycin analogs with activity in nonhypoxic cells, a number of semisynthetic compounds have been designed that are activated by processes other than reduction. Structurally, these compounds are characterized by the presence of a aminoethylene disulfide side chain as exemplified by KW-2149, which has been examined in clinical trials,[49] although serious pulmonary toxicity was observed.[50] KW-2149 causes interstrand DNA cross-links and DNA–protein cross-links, resulting in single-strand DNA breaks and inhibition of DNA synthesis. The mechanism proposed to account for these observations is summarized in Figure 6.16 and involves liberation of thiol **6.22** by reaction of the drug with a mercapto group contained in glutathione. Compound **6.22** can be activated by reductases through the standard mechanism, involving the formation of **6.23** and subsequent DNA alkylation by a mechanism related to that proposed for the case of mitomycin. On the other hand, *in vitro* studies have shown that **6.22** exists predominantly as its spiro isomer **6.24**; this intermediate is proposed to react with intracellular thiols to give **6.25**, thus providing an alternative route for hydroquinone generation that is not dependent on reductase activity.[51] Due to this mechanism, KW-2149 is active in nonhypoxic tumor cells and in cell lines that express low levels of DT-diaphorase and are therefore resistant to mitomycin.[52]

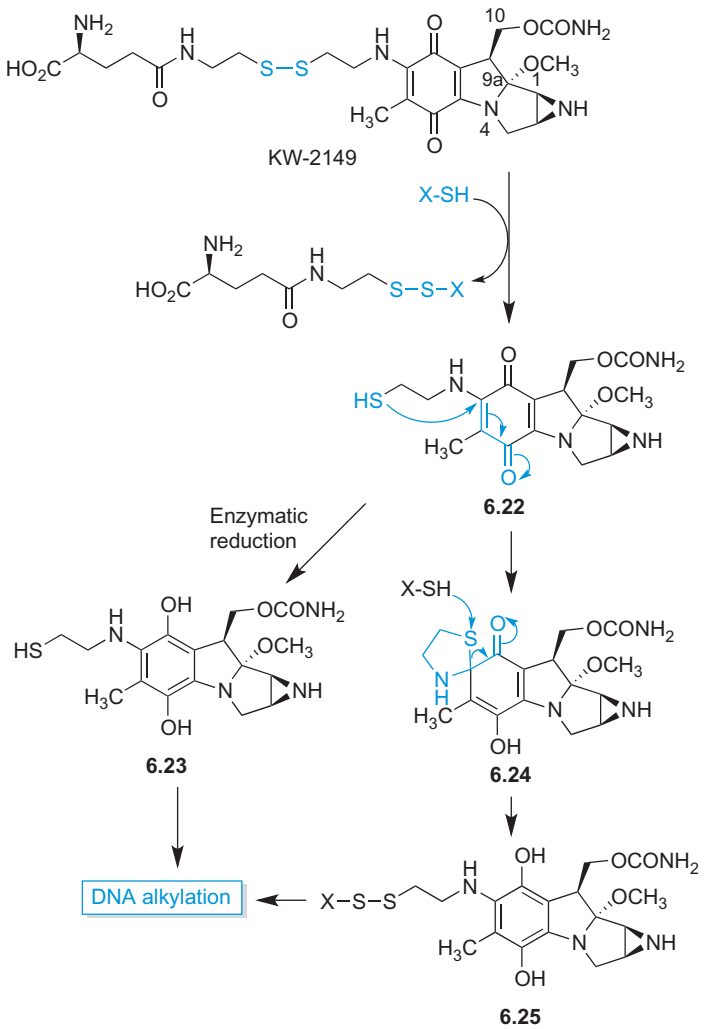


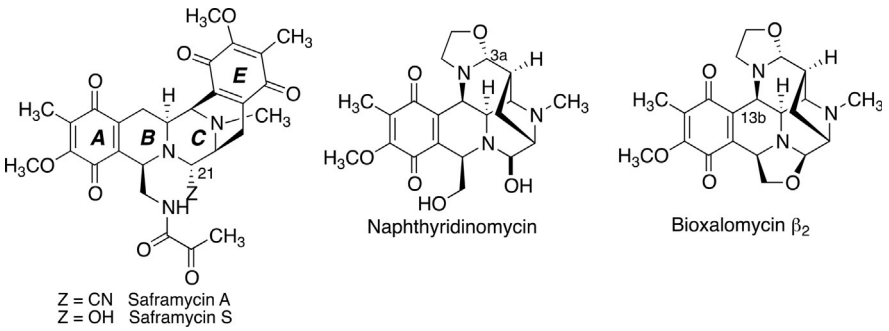
FIGURE 6.16

Nonreductive bioactivation of KW-2149, a mitomycin analog.

4 TETRAHYDROISOQUINOLINE ALKALOIDS

Antitumor natural products belonging to the tetrahydroisoquinoline family[53] have been under study for more than 35 years, starting with the isolation of napthyridinomycin. These compounds normally bind to DNA by alkylation of specific nucleotide sequences in the minor groove. Most of these alkaloids contain quinone moieties and act by reductive alkylation mechanisms and also by generation of oxygen radicals via their one-electron reduction to a semiquinone species. The presence of either a nitrile or a

hydroxy group on the position of the pyrazine ring α to the isoquinoline nitrogen allows the generation of an intermediate iminium species that is essential for DNA alkylation.



Recognition of the saframycins by the DNA minor groove shows some specificity toward 5′-GGG and 5′-GGC sequences,[54] and it is followed by alkylation. Saframycin S, one of the most active saframycins, is active in the quinone form, which has been explained through the formation of iminium cation **6.26** and subsequent covalent binding to DNA involving attack by guanine amino groups to give aminal **6.27** (Figure 6.17).[55]

In the case of saframycin S, there is a second type of covalent binding mechanism involving its previous reduction to a dihydroquinone, which facilitates the formation of the alkylating iminium species.[56] The less reactive saframycin A only alkylates DNA in its hydroquinone form,[57] and indeed several hydroquinone analogs of saframycin A have been shown to be up to 20-fold more active than the parent quinone.[58] The mechanism of DNA alkylation by these hydroquinones (**6.29**) is proposed to involve B-ring opening with assistance from the phenolic hydroxyl group to give quinone methides **6.30**, which subsequently cyclize again to iminium derivatives **6.31**, the actual DNA alkylating agents. The redox equilibrium between the saframycins and their semiquinones **6.28**, as intermediates in the reduction of the natural products to hydroquinones **6.29**, is also involved in the generation of cytotoxic oxygen radicals (Figure 6.18).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key transcriptional coactivator necessary for entry into S phase due to its involvement in the maintenance and/or protection of telomeres, has been identified as another protein target for several members of the saframycin class. This enzyme forms a ternary complex with saframycin-related compounds and DNA that induces a toxic response in cells.[59]

A mechanism very similar to the one summarized in Figure 6.14 accounts for DNA alkylation by naphthyridinomycin,[60] although a second mode of alkylation at C-3a after quinone reduction and

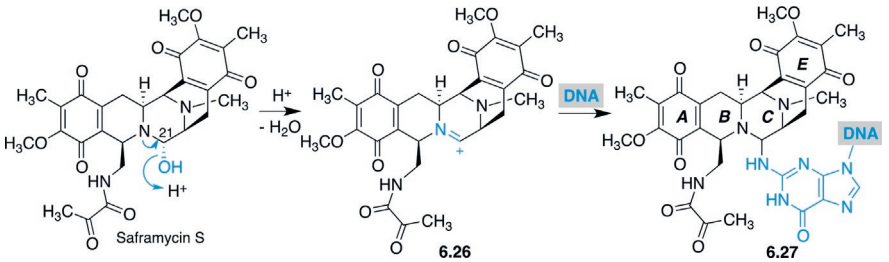


FIGURE 6.17

Iminium ion-mediated alkylation of DNA by saframycin S.

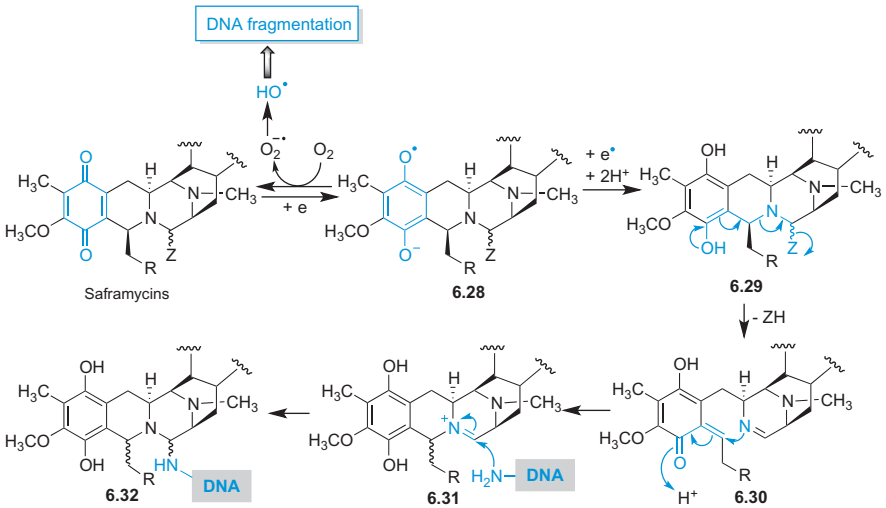


FIGURE 6.18

Bioreductive activation of the saframycins.

opening of the oxazolidine ring has been suggested following molecular modeling studies.[61] In the case of bioxalomycins, it has been shown that the reduced form yields DNA interstrand cross-links with 5′-CpG-3′ selectivity[62] involving alkylation at C-7 following the usual mechanism and also at C-13b, as shown in Figure 6.19.

The ecteinascidins are structurally complex alkaloids, formed by two fused tetrahydroisoquinoline rings linked to a 10-membered lactone bridge through a benzylic sulfide linkage and containing in most

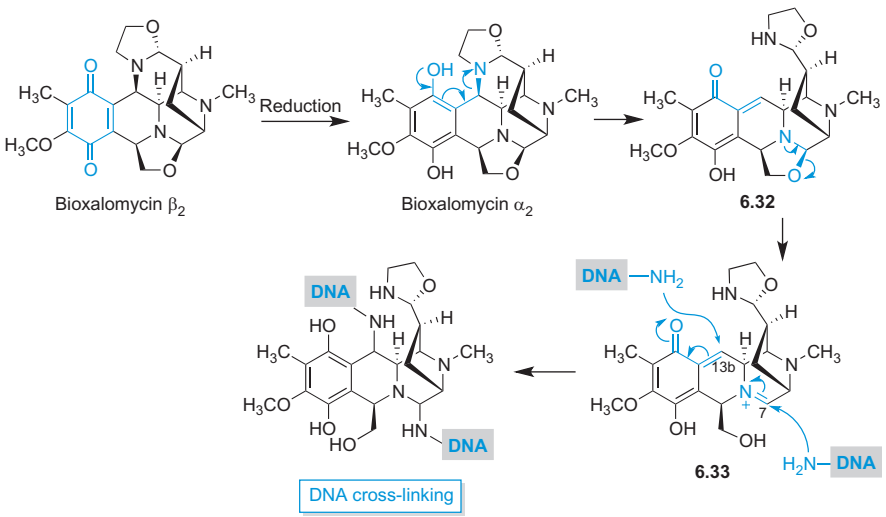
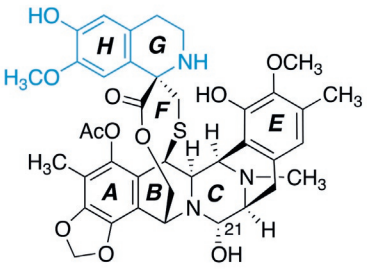


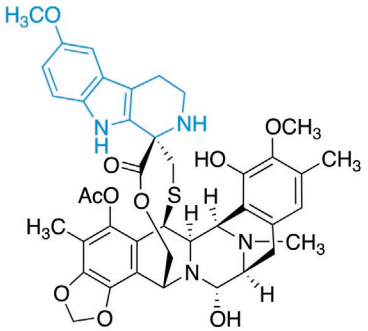
FIGURE 6.19

Bioreductive activation of bioxalomycin β_2.

cases an additional tetrahydroisoquinoline or tetrahydro-β-carboline ring forming a spiro system with the rest of the structure.[63] They are broad-spectrum antitumor agents that are several orders of magnitude more potent than other tetrahydroisoquinoline alkaloids. The first one to be developed was trabectedin (ecteinascidin 743, ET-743, Yondelis®), which was originally isolated from the marine tunicate *Ecteinascidia turbinata* and obtained by mariculture techniques during the first stages of clinical development, but which it is now produced by semisynthesis from safracin B.[64] Lurbinectedin (tryptamicidin, PM01183) is a related alkaloid in which the spiro-tetrahydroisoquinoline fragment (rings G and H) has been replaced by a tetrahydro-β-carboline unit. Phase II clinical trials have shown significant results in patients with ovarian cancer resistant to platin drugs.



Trabectedin (ecteinascidin-743, ET-743)



Lurbinectedin (tryptamicidin, PM01183)

Trabectedin was granted the status of orphan drug for the treatment of soft tissue sarcoma and ovarian cancer by both the European Medicines Agency (EMA) and the FDA and, after extensive clinical studies,[65,66] was approved by the EMA for the treatment of advanced soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer (in combination with doxorubicin/liposomal doxorubicin). Other clinical studies with trabectedin for the treatment of different cancers are ongoing. Furthermore, it is currently being tested in phase II or III for breast and pediatric sarcomas, as well as for soft tissue sarcoma as first-line treatment.[67]

Recognition of trabectedin by the DNA minor groove is specific for certain sequences having a central guanine, including TGG, CGG, AGC, and GGC sequences, but not CGA.[68] Also, it involves hydrogen bonding with certain functional groups of trabectedin, as shown in Figure 6.20.[69,70]

DNA alkylation involves attack of the amino group of the central guanine onto an iminium species generated at C-21 by loss of the hydroxyl group. NMR studies have shown that the covalent adduct is protonated at N-12, and this has led to the proposal that iminium generation is assisted by proton transfer from N-12 to the hydroxyl acting as a leaving group (Figure 6.21a). The resulting adduct receives additional stabilization from van der Waals interactions and at least one hydrogen bond between rings A and B of the drug and neighboring nucleotides in the same or opposite strands of the DNA double helix, thus creating the equivalent to interstrand cross-links (Figure 6.21b).[71]

Minor groove alkylation by trabectedin has been studied using gel electrophoresis and ^{1}H-NMR experiments, which have shown it to be reversible. It has been proposed that the differences in rate of the reverse reaction are responsible for the observed sequence specificity because nonfavored sequences (e.g., 5′-AGT) are dealkylated at an enhanced rate, allowing migration of trabectedin to the favored ones (e.g., 5′-AGC). Due to hydrogen bonding, the drug forms a stable and tight complex

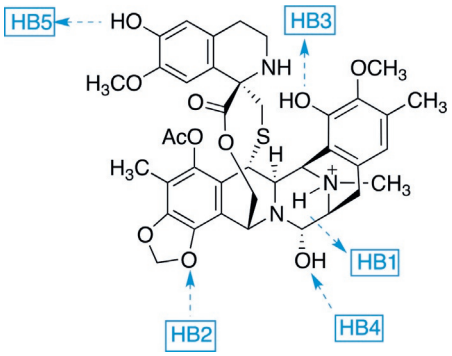


FIGURE 6.20

Hydrogen bonds (HB) involved in the recognition of trabectedin by the DNA minor groove. The arrows are oriented from hydrogen donor to hydrogen acceptor groups.

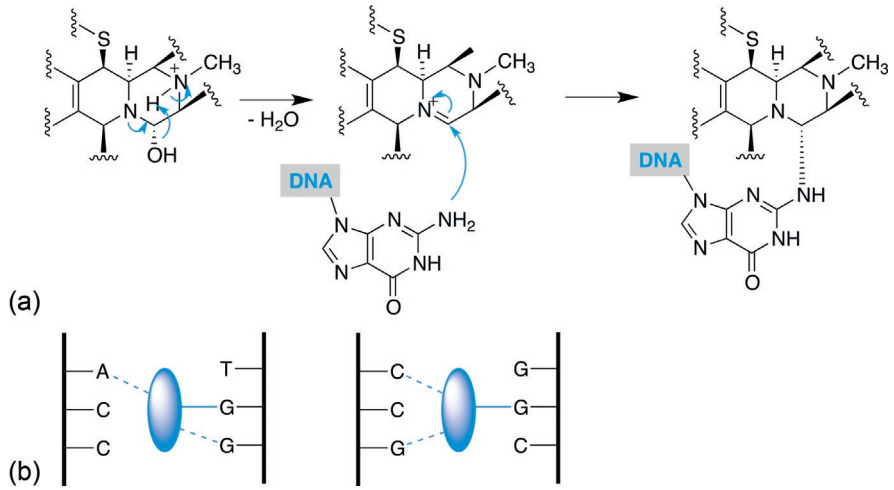


FIGURE 6.21

(a) DNA alkylation by trabectedin. (b) Interstrand "cross-linking" by trabectedin, involving a combination of covalent bonds and hydrogen binding.

at the 5′-AGC target sequence where the covalent linkage is less accessible to attack by a water molecule. In the case of trabectedin–AGT adducts, the complex is less stable and has more dynamic motion, leading to a higher conformational flexibility that renders it more accessible to solvation, with the consequent increase in the rate of the reverse reaction, as shown in Figure 6.22. In summary, the site selectivity of trabectedin depends on the rate of reversibility of the covalent adducts and not on the rate of the covalent bond-forming reaction.

The mechanism of action of trabectedin and related compounds is complex, and indeed they can be viewed as multitarget drugs.[72] X-ray crystallography and NMR studies, supported by computational

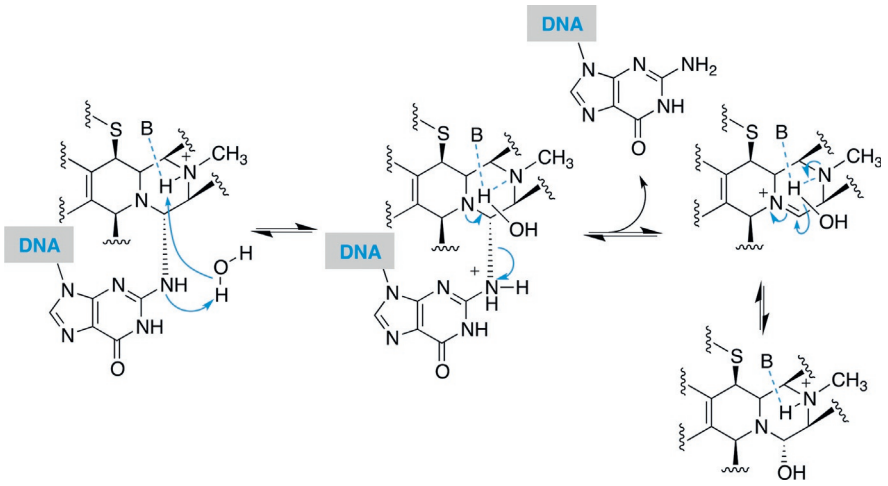


FIGURE 6.22

Reversibility of the covalent DNA–trabectedin complex.

studies, show that trabectedin binding into the minor groove of DNA induces widening of the minor groove and bending of the helix toward the major groove, leading to a bending of the DNA molecule that is characteristic of this family of compounds (Figure 6.23).[73] This trabectedin-induced distortion of the helix would normally trigger nucleotide excision repair (NER), in which the damaged part of the sequence is cut out by endonucleases and repaired by DNA polymerase (see Section 9 of Chapter 7 and Section 4 of Chapter 14). However, trabectedin, in a unique mechanism of action, reverses NER, causing the endonuclease components to create lethal single strand breaks in the DNA rather than repairing it.[74,75] NER requires the recruitment of various factors to the damaged site, and a molecular modeling study suggested the formation of a ternary complex between one of these factors, DNA and trabectedin, involving the formation of hydrogen bonds between the two oxygen atoms in trabectedin ring H and an arginine residue of the factor.[76] Nevertheless, the antiproliferative activity of PM00128 (ET-673), lacking this ring, was similar to that of trabectedin.[77]

DNA double-strand break is also relevant to the mechanism of action of trabectedin because it has been shown that cells deficient in the homologous repair (HR) mechanism are approximately 100 times more sensitive to trabectedin.

At biological concentrations, the trabectedin–DNA adduct also interacts with some DNA transcription factors, especially the NF-Y factor.[79] A molecular modeling study has shown that the DNA–ET-743 complex is superimposable with the minor groove of DNA bound to the zinc finger of the transcription regulator EGR-1, suggesting that ET-743 may target chromosome sites where zinc fingers of transcription factors interact with DNA.[80] Furthermore, trabectedin is also active on promoters regulated by transcription factors that bind to the major groove. At low concentrations, trabectedin inhibits the transcription by monocytes and macrophages of the pro-inflammatory mediator CCL2, which has an important role in monocyte recruitment at tumor sites, and interleukin-6, a growth factor for several tumors.

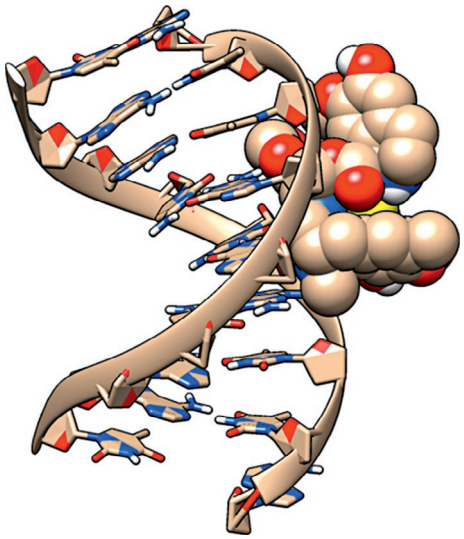


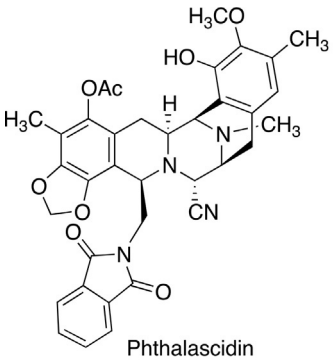
FIGURE 6.23

Structure of the trabectedin–DNA complex. Generated from Protein Data Bank reference 1EZH[78] and displayed with Chimera 1.8.1.

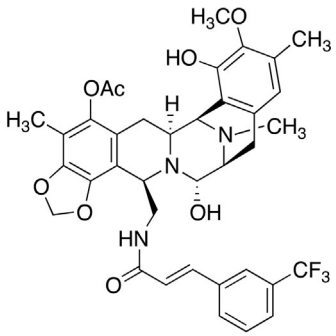
In addition to the previously mentioned effects on DNA repair and DNA transcription, trabectedin has significant effects on a number of biochemical processes. Thus, like taxol, it disrupts the microtubule network of tumor cells,[81] and at doses higher than therapeutic, it forms a cross-link between DNA and topoisomerase I by interaction of its spirotetrahydroisoquinoline subunit with the protein.[82]

Lurbinectedin (PM01183) binds at the DNA minor groove in a similar way as trabectedin, showing selectivity for triplets that have a central guanine that allows covalent adduct formation (AGC, CGG, AGG, and TGG). The formation of these adducts induces double-strand breaks, accumulation of cells in the S phase, and apoptosis.[83] It is under clinical trials, including a phase I study to evaluate dose-limiting toxicities[84] and several studies in combination with paclitaxel or gemcitabine in patients with advanced solid tumors and non-small cell lung cancer, respectively.[85]

Due to the complexity of the trabectedin structure, extensive studies have been carried out on the preparation of simpler analogs. One of them is phthalascidin,[86] with an activity similar to that of the natural product and in which the phthalimino group plays a similar role as the spirotetrahydroisoquinoline unit in trabectedin.[87] Another related compound is PM00104 (Zalypsis®), an analog of the marine natural product jorumycin that blocks the minor groove of DNA after binding covalently to the amino group of several guanine residues, giving a complex that is further stabilized by hydrogen bonding with nucleotides in the opposite strand and van der Waals interactions.[88] This compound is in phase II of clinical trials for the treatment of multiple myeloma, bladder cancer, and Ewing sarcoma, as well as in early stage studies for a variety of additional solid and hematological tumor cell lines.[89]



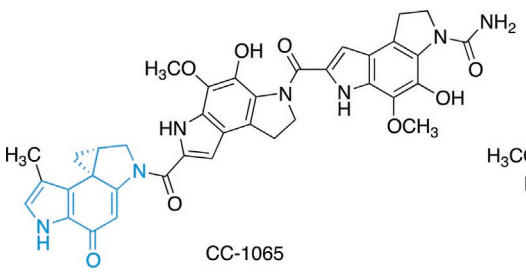
Phthalascidin



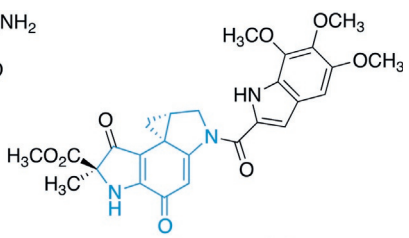
PM-00104 (Zalypsis®)

5 CYCLOPROPYLINDOLE ALKYLATING AGENTS

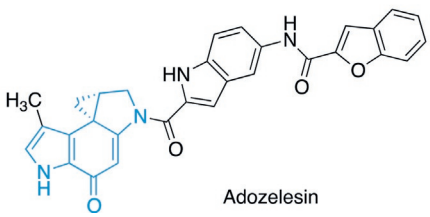
This name, although incorrect from the standpoint of chemical nomenclature, is usually employed to design a number of antitumor compounds that contain a cyclopropane ring fused to an indole system. The first member of this class was the natural product CC-1065, an extremely active cytotoxic agent (100- to 400-fold more potent than doxorubicin), isolated in trace quantities from the culture of *Streptomyces zelensis* in 1978 and whose unique structure was confirmed by single-crystal X-ray diffraction in 1981. Despite its very high *in vitro* antitumor activity, CC-1065 cannot be used in humans because it caused deaths in experimental animals due to its delayed hepatotoxicity.[90] The duocarmycins[91,92] are a family of simplified related natural products, first isolated from *Streptomyces* bacteria in 1988 and displaying also a very high cytotoxicity. The study of these alkaloids led to conclusions about their pharmacophore that were employed in the search for synthetic compounds with better antitumor selectivity and DNA sequence specificity. The first to enter clinical trials was adozelesin, which retained the very potent cytotoxicity of CC-1065 while lacking its hepatoxicity, but the study had to be stopped at phase II because of the low activity observed.



CC-1065



Duocarmycin A

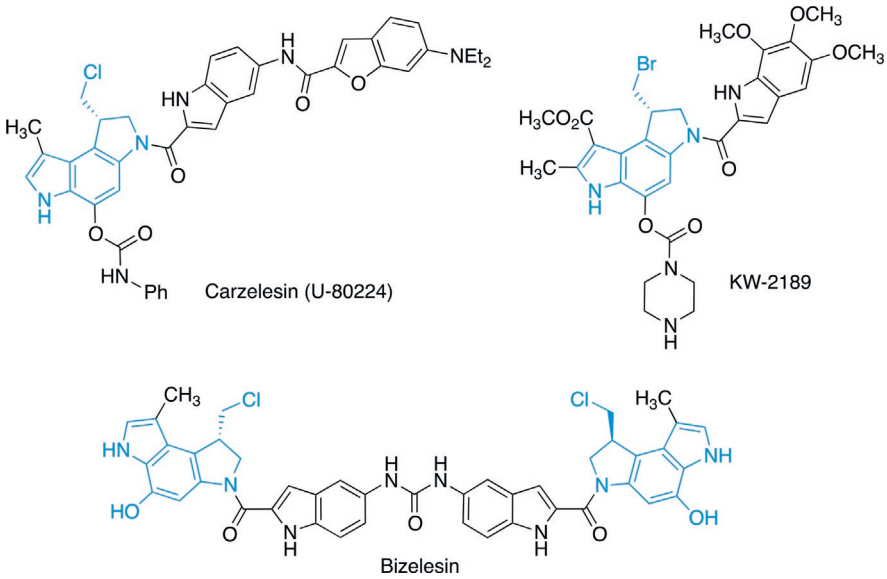


Adozelesin

The structure of CC-1065 and their analogs fits the DNA minor groove curvature, where they bind specifically to AT-rich sequences, followed by irreversible alkylation of adenine N-3 (Figure 6.24).

The cyclopropane ring opening needs to be assisted by the electron-withdrawing effect of the carbonyl group. Prior to interaction with DNA, this assistance is prevented by the conjugation between the carbonyl and the indole nitrogen atom, which form a vinylogous amide. However, the twist that the drug molecule needs to undergo in order to be accommodated into the deep and narrow minor groove AT regions forces the nitrogen atom out of the plane of the unsaturated carbonyl system and therefore out of conjugation (Figure 6.25).

A family of halomethyl prodrugs of the cyclopropylindoles, activated by carboxyl esterases, includes carzelesin (U-80224),[93] KW-2189,[94] and bizelesin.[95] Carzelesin was very efficient against xenografts from pediatric rhabdomyosarcomas, including those resistant to topotecan, and it entered clinical trials, but its efficiency was very poor. The extremely potent bizelesin, which is a symmetrical dimer of the alkylating subunit of CC-1065, is highly specific for the 50-T (A/T)(4)A-30 sequence. Preclinical studies indicated excellent activity in a variety of mouse tumors, but phase I clinical studies in humans failed to show any response. KW-2189 is a water-soluble double prodrug of duocarmycin B_2, and it has undergone phase II clinical trials in patients with advanced malignant melanoma.[96] Hybrid compounds containing the cyclopropylindole fragment or its precursors and minor-groove binding distamycin portions have also been prepared.[97]



The halomethyl prodrugs are activated by cyclization to a cyclopropane derivative after hydrolysis of any protection on the phenolic hydroxyl (Figure 6.26).

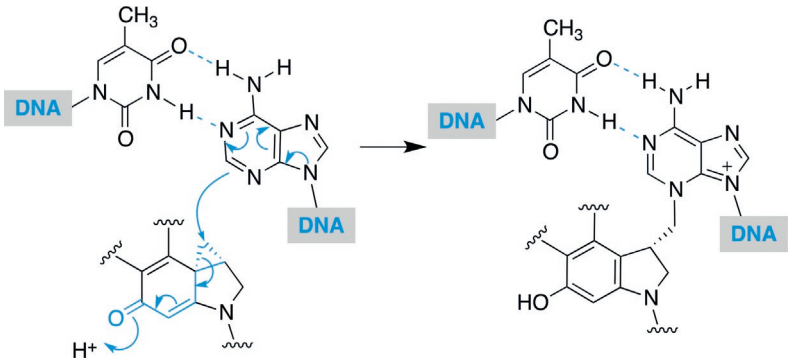


FIGURE 6.24

DNA alkylation by the cyclopropylindoles.

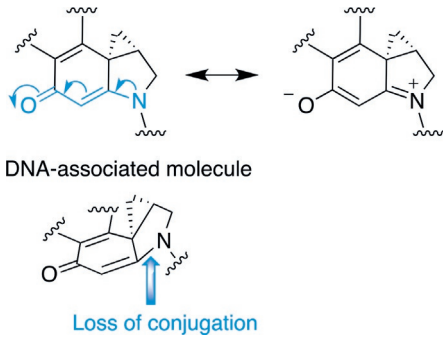


FIGURE 6.25

The role of conjugative effects in the activity of cyclopropylindoles.

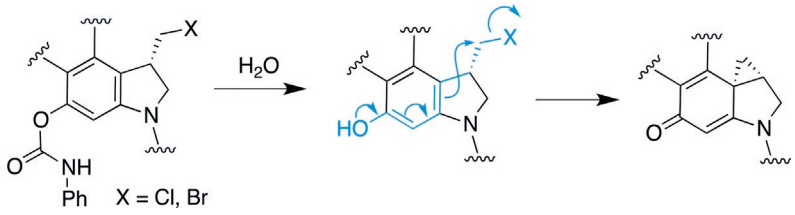
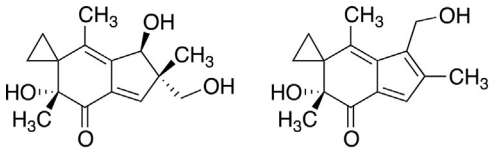


FIGURE 6.26

In vivo generation of the cyclopropane ring from halomethyl precursors.

6 IROFULVEN

Irofulven (hydroxymethylacylfulvene, HMAF, MGI-114) is an analog of the fungal toxin illudin S[98] that showed high potency in cell cultures, including those resistant to most other anticancer agents, and has undergone a large number of phase I and II clinical studies.[1a] Its activity seems to depend on bioactivation by the NADPH-dependent enzyme alkenal/one oxidoreductase (AOR), an enzyme that is highly expressed in many tumors. Unfortunately, in addition to the usual toxic effects shown by similar drugs, irofulven has shown an unexpected retinal toxicity.



Irofulven acts by bioreductive alkylation of DNA. Hydride transfer from NADPH to the enone fragment of the drug affords a reactive metabolite that is attacked by nucleophilic atoms of DNA, with opening of the cyclopropane ring and concomitant loss of the adjacent hydroxy group (Figure 6.27).[99] The driving force of this reaction is presumably the aromatization of the six-membered ring.

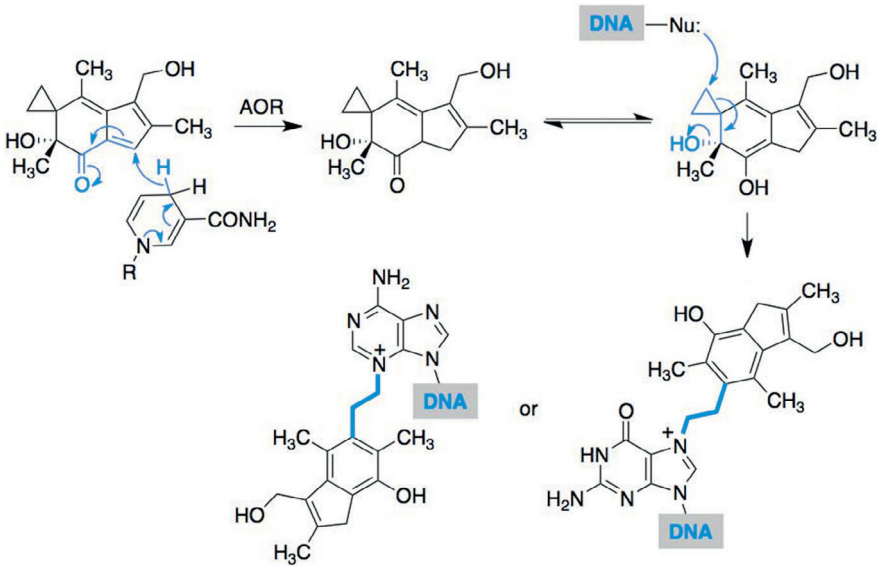
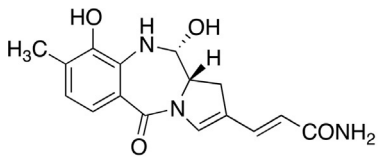


FIGURE 6.27

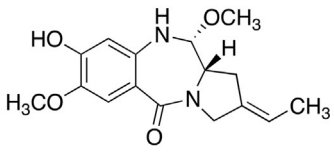
Bioreductive alkylation of DNA by irofulven.

7 PYRROLO[1,4]BENZODIAZEPINES

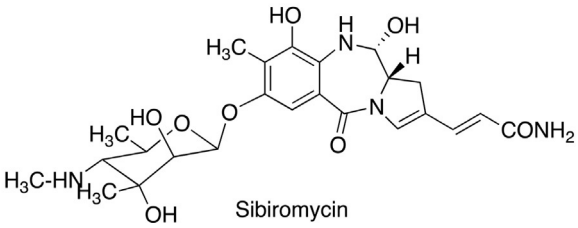
Anthramycin, tomaymycin, and sibiromycin are natural pyrrolo[1,4]benzodiazepine antitumor antibiotics that react with the minor groove of DNA to form covalently bound complexes.[100] They show activity toward several tumors, but their clinical use is limited by their cardiotoxicity and tissue necrosis induction.



Anthramycin



Tomaymycin



Sibiromycin

These compounds form a covalent bond with the 2-amino group of guanine, as shown by X-ray diffraction,[101] through the formation of an intermediate iminium cation (Figure 6.28).

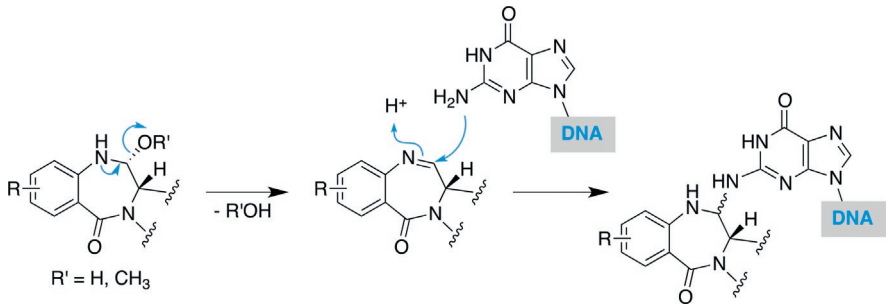


FIGURE 6.28

DNA alkylation by pyrrolo[1,4]benzodiazepines.

REFERENCES

1 For reviews of DNA MGBs, see. (a) Cai X, Gray PJ, Von Hoff DD. *Cancer Treatment Rev* 2009;**35**:437; (b) Khan GS, Shah A, ur-Rehman Z, Barker D. *J Photochem Photobiol B Biol* 2012;**115**:105
2 (a) Wartell RM, Larson JE, Wells RD. *J Biol Chem* 1974;**249**:6719; (b) Van Dyke MW, Hertzberg RP, Dervan PB. *Proc Natl Acad Sci U S A* 1982;**79**:5470.
3 Dolenc J, Oostenbrink C, Koller J, van Gusteren WF. *Nucl Acid Res* 2005;**33**:725.
4 Uytterhoeven K, Sponer J, Van Meervelt L. *Eur J Biochem* 2002;**269**:2868.
5 Baraldi PG, Núñez AC, Espinosa A, Romagnoli R. *Curr Topics Med Chem* 2004;**4**:231.
6 Sunavala-Dossabhoy G, Van Dyke MW. *Biochemistry* 2005;**44**:2510.
7 Viallet J, Stewart D, Shepherd F, Ayoub J, Cormier Y, Di Pietro N, et al. *Lung Cancer* 1996;**15**:367.
8 D'Incalci M, Sessa C. *Expert Opin Invest Drugs* 1997;**6**:875.
9 Gelderblom H, Blay JY, Seddon BM, Leahy M, Ray-Coquard I, Sleijfer S, et al. *Eur J Cancer* 2014;**50**:388.
10 Fedier A, Fowst C, Tursi J, Geroni C, Haller U, Marchini S, et al. *Br J Cancer* 2003;**89**:1559.
11 Sola F, Capolongo L, Moneta D, Ubezio P, Grandi M. *Cancer Chemother Pharmacol* 1999;**43**:241.
12 Kraut E, Malspeis L, Balcerzak S, Grever M. *Proc Am Soc Clin Oncol* 1988;**7**:62.
13 Kraut E, Fleming T, Segal M, Neidhart J, Behrens BC, MacDonald J. *Invest New Drugs* 1991;**9**:95.
14 Hard T, Fan P, Kearns DR. *Photochem Photobiol* 1990;**51**:77.
15 Teng M, Usman N, Frederik CA, Wang AH-J. *Nucleic Acid Res* 1988;**16**:2671.
16 For a review of the chemistry of mitomycinoid alkaloids, see Bass PD, Gubler DA, Judd TC, Williams RM. *Chem Rev* 2013;**113**:6816.
17 Teicher BA. In: DeVita VT, Hellman S, Rosenberg SA, editors. *Cancer: principles and practice of oncology*. 5th ed. Philadelphia, PA: Lippincott-Raven; 1997. p. 405.
18 Spain RC. *Oncology* 1993;**50**:35.
19 Haffty BG, Son YH, Wilson LD, Papac R, Fischer D, et al. *Radiat Oncol Investig* 1997;**5**:235.
20 Workman P, Stratford IJ. *Cancer Metastasis Rev* 1993;**12**:73.
21 Wang Y, Gray JP, Mishin V, Heck DE, Laskin DL, et al. *Mol Cancer Ther* 2010;**9**:1852.
22 Wolkenberg SE, Boger DL. *Chem Rev* 2002;**102**:2477.
23 For a comprehensive review of the molecular toxicology of mitomycin C, see Paz MM, Pritsos CA. *Adv Mol Toxicol* 2012;**6**:243.
24 (a) Siegel S, Gibson NW, Preusch PC, Ross D. *Cancer Res* 1990;**50**:7483; (b) Mikami K, Naito M, Tomida A, Yamada M, Sirakusa T, Tsuruo T. *Cancer Res* 1996;**56**:2823.
25 Danishefsky SJ, Ciufolini M. *J Am Chem Soc* 1984;**106**:6425.
26 Tomasz M, Palom Y. *Pharmacol Ther* 1997;**76**:73.
27 Tomasz M, Chawla AK, Lipman R. *Biochemistry* 1988;**27**:3182.
28 Snodgrass RG, Collier AC, Coon AE, Pritsos CA. *J Biol Chem* 2010;**285**:19068.
29 Sastry M, Fiala R, Lipman R, Tomasz M, Patel DJ. *J Mol Biol* 1995;**247**:338.
30 Bizanek M, McGuinness BF, Nakanishi K, Tomasz M. *Biochemistry* 1992;**31**:3084.
31 Norman D, Live D, Sastry M, Lipman R, Hingerty BE, Tomasz M, et al. *Biochemistry* 1990;**29**:2861.
32 Li V-S, Kohn H. *J Am Chem Soc* 1991;**113**:275.
33 Kumar S, Lipman R, Tomasz M. *Biochemistry* 1992;**31**:1399.
34 Snodgrass RG, Collier AB, Coon AE, Pritsos CA. *J Biol Chem* 2010;**285**:19068.
35 Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL, Nakanishi K. *Science* 1987;**235**:1024.
36 Paz MM, Zhang X, Lu J, Holmgren A. *Chem Res Toxicol* 2012;**25**:1502.
37 Williams RM, Rajski SR, Rollins SB. *Chem Biol* 1997;**4**:127.

38 Paz MM, Hopkins PB. *J Am Chem Soc* 1997;**119**:5999.
39 Paz MM, Sigursson ST, Hopkins PB. *Bioorg Med Chem* 2000;**8**:173.
40 (a) Rajski SR, Williams RM. *Bioorg Med Chem* 2000;**8**:1331; (b) Beckerbauer L, Tepe JJ, Cullison J, Reeves R, Williams RM. *Chem Biol* 2000;**7**:805.
41 Kohn H, Zein N. *J Am Chem Soc* 1983;**105**:4105.
42 Maliepaard M, de Mol NJ, Tomasz M, Gargiulo D, Janssen LHM, Duynhoven JPM, et al. *Biochemistry* 1997;**36**:9211.
43 Ouyang A, Skibo B. *Biochemistry* 2000;**39**:5817.
44 For a review, see Phillips RM, Hendriks HR, Peters GJ. *Br J Pharmacol* 2013;**168**:11.
45 Choudry GA, Stewart PAH, Double JA, Krul MRL, Naylor B, Flannigan GM, et al. *Br J Cancer* 2001;**85**:1137.
46 Aamdal S, Lund B, Koier I, Houten M, Wanders J, Verweij J. *Cancer Chemother Pharmacol* 2000;**45**:85.
47 Phillips RM. *Biochem Pharmacol* 1996;**52**:1711.
48 Bailey SM, Wyatt MD, Friedlos F, Hartley JA, Canox RJ, Lewis AD, et al. *Br J Cancer* 1997;**76**:1596.
49 Saijo N. *Chest* 1998;**113**:17S.
50 Schrijvers D, Catimel G, Highley M, Hopponer FJ, Dirix L, De Bruijn E, et al. *Anticancer Drugs* 1999;**10**:633.
51 Na Y, Wang S, Kohn H. *J Am Chem Soc* 2002;**124**:4666.
52 Dirix L, Catimel G, Koier I, Prove A, Schrijvers D, Joossens E, et al. *Anticancer Drugs* 1995;**6**:53.
53 For reviews, see. (a) Scott JD, Williams RM. *Chem Rev* 2009;**102**:1669; (b) Avendaño C, de la Cuesta E. *Chem Eur J* 2009;**16**:9722
54 Rao KE, Lown JW. *Chem Res Toxicol* 1990;**3**:262.
55 Lown JW, Joshua AV, Lee JS. *Biochemistry* 1982;**21**:419.
56 Ishiguro K, Takahashi K, Yazawa K, Sakiyama S, Arai T. *J Biol Chem* 1981;**256**:2162.
57 Hill GC, Remers WA. *J Med Chem* 1991;**34**:1990.
58 Myers AG, Plowright AT. *J Am Chem Soc* 2001;**123**:5114.
59 Xing C, LaPorte JR, Barbay JK, Myers AG. *Proc Natl Acad Sci U S A* 2004;**101**:5862.
60 Zmijewski MJ, Miller-Hatch K, Mikolajczak M. *Chem Biol Interact* 1985;**52**:361.
61 Hill GC, Wunz TP, McKenzie NE, Gooley PR, Remers WA. *J Med Chem* 1991;**34**:2079.
62 Williams RM, Herberich B. *J Am Chem Soc* 1998;**120**:10272.
63 Menchaca R, Martínez V, Rodríguez A, Rodríguez N, Flores M, Gallego P, et al. *J Org Chem* 2003;**68**:8859.
64 Cuevas C, Pérez M, Martín MJ, Chicharro JL, Fernández-Rivas C, Flores M, et al. *Org Lett* 2000;**2**:2545.
65 For reviews, see. (a) D'Incalci M, Erba E, Damía G, Galliera E, Carrasa L, Marchini S, et al. *Oncologist* 2002;**7**:210; (b) Schwartsmann G, Da Rocha AB, Mattei J, Lopes R. *Expert Opin Investig Drugs* 2003;**12**:1367; (c) D'Incalci M, Jimeno J. *Expert Opin Invest Drugs* 2003;**12**:1843; (d) Schöffski P, Dumez H, Wolter P, Stefan C, Wozniak A, Jimeno J, et al. *Expert Opin Pharmacother* 2008;**9**:1609; (e) Brodowicz T. *Future Oncol* 2014;**10**:s1; (f) Blay J-Y. *Future Oncol* 2014;**10**:s7, s13.
66 For a summary of clinical studies of trabectedin in tabular form, see reference 1a.
67 http://www.pharmamar.com/yondelis.aspx.
68 Zewail-Foote M, Hurley LH. *J Am Chem Soc* 2001;**123**:6485.
69 Seaman FC, Hurley LH. *J Am Chem Soc* 1998;**120**:13028.
70 Marco E, García Nieto R, Mendieta J, Manzanares I, Cuevas C, Gago F. *J Med Chem* 2002;**45**:871.
71 Moore RM, Seman FC, Wheelhouse RT, Hurley RH. *J Am Chem Soc* 1998;**120**:2490.
72 For reviews, see. (a) Manzanares I, Cuevas C, García-Nieto R, Gago F. *Curr Med Chem Anticancer Agents* 2001;**1**:257; (b) D'Incalci M, Galmarini CM. *Mol Cancer Ther* 2010;**9**:2157; (c) Incalci M. *Future Oncol* 2014;**10**:5
73 (a) Zewail-Foote M, Hurley LH. *J Med Chem* 1999;**42**:2493; (b) Hurley LH, Zewail-Foote M. *Adv Exp Med Biol* 2001;**500**:289.

74 Takebayashi K, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, et al. *Nat Med* 2001;**7**:961.
75 Zewail-Foote M, Ven-Shun L, Kohn H, Bearss D, Guzmán M, Hurley LH. *Chem Biol* 2001;**135**:1.
76 Herrero AB, Martín-Castellanos C, Marco E, Gago F, Moreno S. *Cancer Res* 2006;**66**:8155.
77 Erba E, Cavallaro E, Damia G, Mantovani R, Di Silvio A, Di Francesco AM, et al. *Oncol Res* 2005;**14**:579.
78 García-Nieto R, Manzanares I, Cuevas C, Gago F. *J Am Chem Soc* 2000;**122**:7172.
79 Bonfanti M, La Valle E, Fernández-Sousa J-M, Faircloth G, Caretti G, Mantovani R, et al. *Anticancer Drug Des* 1999;**14**:179.
80 García-Nieto R, Manzanares I, Cuevas C, Gago F. *J Med Chem* 2000;**43**:4367.
81 García-Rocha M, García-Grávalos MD, Ávila J. *Br J Cancer* 1996;**73**:875.
82 Takebayashi K, Pourquier P, Yoshida A, Kohlhagen G, Pommier Y. *Proc Natl Acad Sci U S A* 1999;**96**:7196.
83 Leal JFM, Martínez-Díez M, García-Hernández V, Moneo V, Domingo A, Bueren-Calabuig JA, et al. *Br J Pharmacol* 2010;**161**:1099.
84 Elez ME, Tabernero J, Geary D, Macarulla T, Kang SP, Kahatt C, et al. *Clin Cancer Res* 2014;**20**:2205.
85 http://www.mskcc.org/cancer-care/trial/13-170.
86 Martínez EJ, Owa T, Schreiber SL, Corey EJ. *Proc Natl Acad Sci U S A* 1999;**96**:3496.
87 Martínez EJ, Corey EJ, Owa T. *Chem Biol* 2001;**8**:1151.
88 Leal JFM, García-Hernández V, Moneo V, Domingo A, Bueren-Calabuig JA, Negri A, et al. *Biochem Pharmacol* 2009;**78**:162.
89 http://www.pharmamar.com/zalypsis-fase-ii.aspx.
90 McGovren JP, Clarke GL, Pratt EA, DeKoning TF. *J Antibiot* 1984;**37**:63.
91 (a) Boger DL. *Pure Appl Chem* 1994;**66**:837; (b) Boger DL, Johnson DS. *Proc Natl Acad Sci U S A* 1996;**92**:3642; (c) Boger DL, Boyce CW, Garbaccio RM, Golberg JA. *Chem Rev* 1997;**97**:787.
92 Cacciari B, Romagnoli R, Baraldi PG, Da Ros T, Spalluto G. *Expert Opin Ther Patents* 2000;**10**:1853.
93 Li LH, DeKoning TF, Kelly RC, Krueger WC, McGovern JP, Padbury GE, et al. *Cancer Res* 1992;**52**:4904.
94 Ogasawara H, Nishio K, Takeda Y, Ohmori T, Kubota N, Funayama Y, et al. *Jpn J Cancer Res* 1994;**85**:418.
95 Schwartz GH, Patnaik A, Hammond LA, Rizzo J, Berg K, von Hoff DD, et al. *Ann Oncol* 2003;**14**:775.
96 Markovic SN, Suman VJ, Vukov AM, Fitch TR, Hillman DW, Adjei AA, et al. *Am J Clin Oncol* 2002;**25**:308.
97 Baraldi PG, Tabrizi MA, Preti D, Fruttarolo F, Avitabile B, Bovero A, et al. *Pure Appl Chem* 2003;**75**:187.
98 Shavit E. *Fungi* 2008;**1**:18.
99 Neels JF, Gong J, Yu X, Sturla SJ. *Chem Res Toxicol* 2007;**20**:1513.
100 For a review of pyrrolobenzodiazepines as sequence-selective DNA binding agents, see Kamal A, Reddy MK, Srivastava AK, Srikanth YVV. In: Ekinci D, editor. *Medicinal chemistry and drug design*. Rijeka, Croatia: InTech; 2012.
101 Kopka ML, Goodsell DS, Baikalov I, Grzeskowiak K, Cascio D, Dickerson RE. *Biochemistry* 1994;**33**:13593.

CHAPTER

OTHER ANTICANCER DRUGS TARGETING DNA AND DNA-ASSOCIATED ENZYMES

7

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00007-7

1 DNA INTERCALATION AND ITS CONSEQUENCES

In general, intercalation can be defined as the reversible inclusion of a molecule into a compound with a layered structure. In biochemistry, intercalation refers to a mode of interaction of small organic molecules with DNA.

Many anticancer drugs in clinical use interact with DNA through intercalation, which can be defined as the process by which compounds containing planar aromatic or heteroaromatic ring systems are inserted between adjacent base pairs perpendicularly to the axis of the helix and without disturbing the overall stacking pattern due to Watson–Crick hydrogen bonding. Because many typical intercalating agents contain three or four fused rings that absorb light in the ultraviolet (UV)-visible region of the electromagnetic spectrum, they are usually known as chromophores. In addition to the chromophore, other substituents in the intercalator molecule may highly influence the binding mechanism, the geometry of the ligand–DNA complex, and the sequence selectivity, if any.

The intercalation process[1,2] starts with the transfer of the intercalating molecule from an aqueous environment to the hydrophobic space between two adjacent DNA base pairs. This process is thermodynamically favored because of the positive entropy contribution associated with disruption of the organized shell of water molecules around the ligand (hydrophobic effect). To accommodate the ligand, DNA must undergo a conformational change involving an increase in the vertical separation between the base pairs to create a cavity for the incoming chromophore. The double helix is thereby partially unwound,[3] which leads to distortions of the sugar–phosphate backbone and changes in the twist angle between successive base pairs (Figure 7.1). Once the drug has been sandwiched between the DNA base

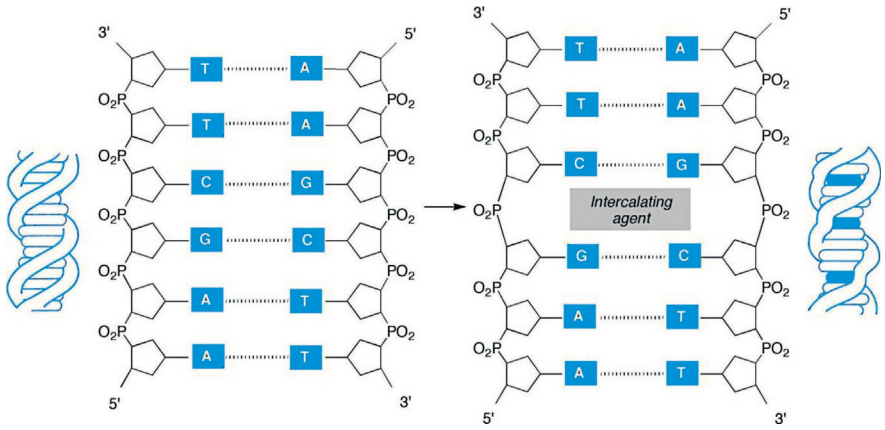


FIGURE 7.1

Deformation of DNA by intercalating agents.

pairs, the stability of the complex is optimized by a number of noncovalent interactions, including Van der Waals and π-stacking interactions,[4] reduction of coulombic repulsion between the DNA phosphate groups associated with the increased distance between the bases due to helix unwinding, ionic interactions between positively charged groups of the ligand and DNA phosphate groups, and hydrogen bonding. In general, cationic species are more efficient DNA intercalators because they interact better with the negatively charged DNA sugar–phosphate backbone in the initial stages and also because intercalation releases counterions associated with phosphate groups such as Na^+, leading to the so-called *polyelectrolyte effect*. This is a very important driving force for intercalation because it diminishes repulsive interactions between the closely spaced charged counterions. In fact, most intercalating agents are either positively charged or contain basic groups that can be protonated under physiological conditions.

The interaction of a typical intercalating agent, ethidium bromide, with DNA is shown in Figure 7.2. The planar chromophore establishes Van der Waals and π-stacking interactions, and the two amino moieties bind to phosphate groups in the DNA backbone via ionic interactions.

DNA intercalators are less sequence-selective than minor groove binding agents and, in contrast with them, show a preference for G–C regions. This selectivity is mainly due to complementary hydrophobic or electrostatic interactions of substituents attached to the chromophore within the major or minor grooves. DNA intercalation is also governed by the nearest-neighbor exclusion principle, which states that both neighboring sites on each site of the intercalation remain empty—that is, they bind, at most, between alternate base pairs.[5] This is an example of a negative cooperative effect, whereby binding to one site induces a conformational change that hampers binding to the adjacent base pair.

Intercalation of a drug molecule into DNA is only the first step in a series of events that eventually lead to its biological effects.[6] Structural changes induced in DNA by intercalation lead to interference with recognition and function of DNA-associated proteins such as polymerases, transcription factors, DNA repair systems, and, especially, topoisomerases. The role of topoisomerases in the design of antitumor drugs is discussed in Sections 4–7.

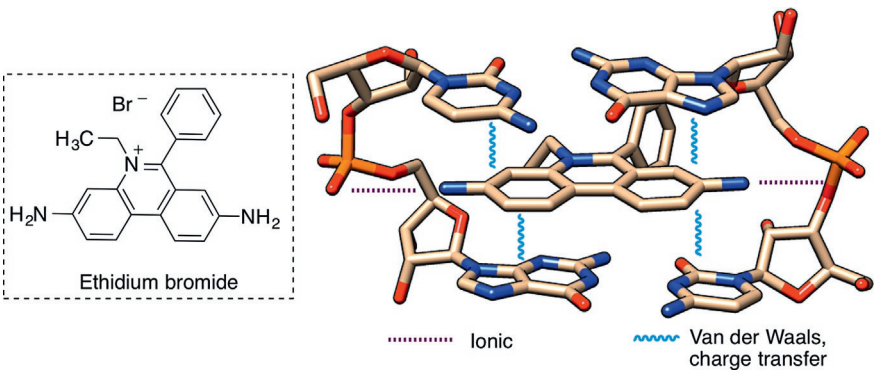


FIGURE 7.2

Ethidium bromide, a prototype intercalating agent, and its interaction with DNA. The three-dimensional structure was generated from Protein Data Bank reference drb007 and displayed with Chimera 1.8.1.

2 MONOFUNCTIONAL INTERCALATING AGENTS

2.1 ELLIPTICINE AND ITS ANALOGS

Ellipticine, an alkaloid isolated from the leaves of *Ochrosia elliptica* and other Apocynaceae plants, is the prototype of intercalators based on the pyridocarbazole system. It has potent anticancer properties, and several of its derivatives have been the subject of clinical trials.[7] These compounds are multimodal anticancer agents because they exert their biological activity via several modes of action, with intercalation with DNA and topoisomerase II inhibition being the best established. Intercalation studies showed that although at physiological pH ellipticine can exist as a neutral species and a monocation (Figure 7.3), it is the latter form that is responsible for DNA intercalation. A crystal structure determination of ellipticine in complex with the hexanucleotide $d(CGATCG)_2$ showed the intercalation of two ellipticine molecules, with the pyridine nitrogen orientated toward the major groove. The preference of GC base pairs was evident because the AT–TA site remained empty.[8]

Ellipticine binds to topoisomerase II (Top2) and to the DNA–Top2 complex in its deprotonated form, and it is considered a catalytic inhibitor instead of a poison of this enzyme (see Sections 5 and 6).[9] Inhibition of Top2 is associated with ellipticine-induced DNA strand breaks.

In vitro experiments employing a peroxidase–H_2O_2 oxidizing system showed that some cytochrome P450 (CYP)-dependent metabolites of ellipticine are able to bind covalently to DNA,[10] although it is not clear whether this process is responsible for the cytotoxicity of ellipticines.[11,12]

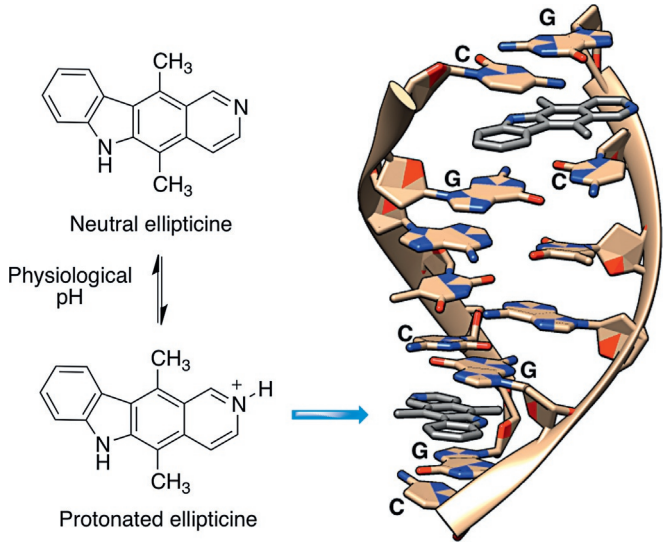


FIGURE 7.3

Neutral and protonated forms of ellipticine and intercalation of the latter into DNA. The structure of the DNA–ellipticine complex was generated from protein Data Bank reference 1Z3F and displayed with Chimera 1.8.1.

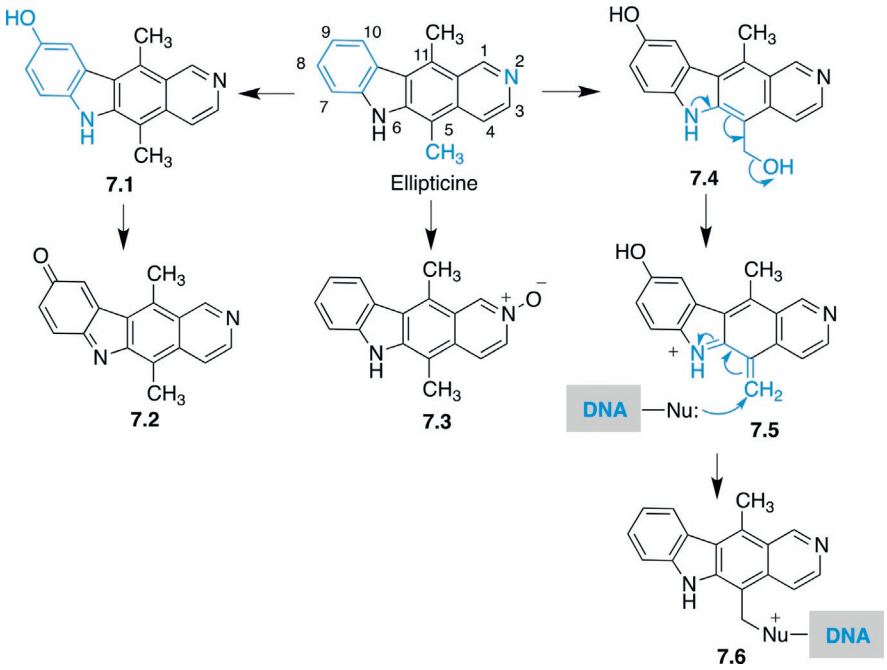


FIGURE 7.4

Ellipticine metabolites and their binding to DNA.

It has been proven that the 9-hydroxy ellipticine derivative **7.1** is oxidized to quinonimine **7.2**,[13] which, despite its high electrophilicity, is unable to establish covalent bonds with DNA. DNA binding is associated with other metabolites, including the *N*-oxide **7.3** and the hydroxymethyl derivative **7.4**, which can be tentatively assumed to react through the intermediacy of stabilized cation **7.5** to give the DNA-alkylated product **7.6**, as shown in Figure 7.4.[14]

Due to the higher efficiency of cations as intercalating agents, some N-2 quaternized ellipticine analogs were assayed, among which the most interesting was *N*-methyl-9-hydroxyellipticinium (NMHE). Its quinonimine **7.7**, which is more reactive than the previously mentioned compound **7.2** due to the presence of the strongly electron-withdrawing cationic heterocyclic nitrogen atom, reacts with a variety of biologically relevant nucleophiles at its C-10 position to give adducts **7.9** (Figure 7.5).

Although compounds related to NMHE have been employed as the basis for the design of bis-intercalating compounds (see Section 4), a correlation between the *in vivo* antitumor activity of NMHE and formation of covalent adducts has not been established. In fact, it has been shown that the extent of irreversible binding to DNA is similar in NMHE-sensitive and -resistant cell lines.[15]

Celiptium®, the acetate salt of NMHE, demonstrated clinical activity for the treatment of breast cancer, but its clinical use was hampered by serious toxicities.[16] Other ellipticinium analogs progressed

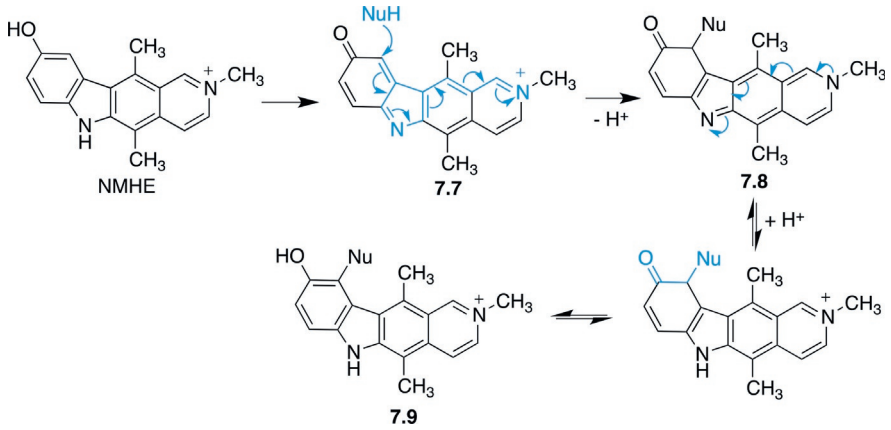
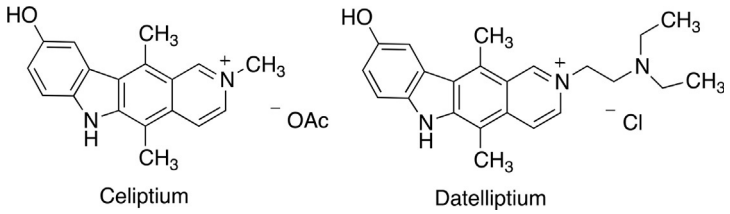


FIGURE 7.5

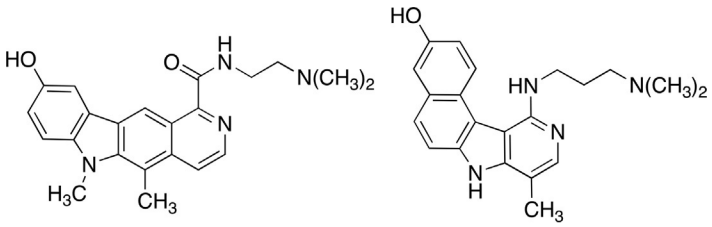
Reaction of NMHE with nucleophilic biomolecules.

to clinical trials. In 1992, 2-(diethylamino-2-ethyl)-9-hydroxyellipticinium chloride (Datelliptium®) was found to be active in previously treated metastatic breast cancer and to lack the toxicities reported for 9-hydroxy-*N*-methylellipticinium acetate, but it revealed unexpected hepatotoxic effects in humans that had not been observed in animals.



The olivacine derivative S-16020 is another important antitumor pyridocarbazole derivative whose (dimethylamino)ethylcarboxamide side chain increases its DNA intercalating ability, leading to potent activity as a stimulator of Top2-mediated DNA cleavage. Despite its close similarity with ellipticine, both compounds show little cross-resistance. Phase I[17] and phase II clinical trials[18] have indicated limited antitumor activity in head and neck cancer.

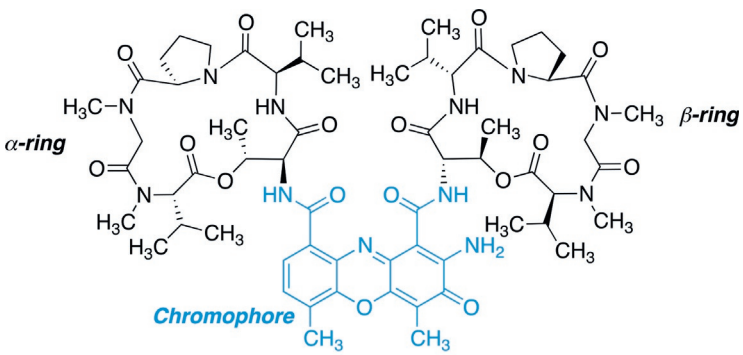
Intoplicine, an intercalating compound that can be considered as structurally related to the ellipticines, behaves as a dual topoisomerase I and II poison at cleavage sites different from those of other known topoisomerase inhibitors.[19] Because of the high activity of intoplicine in preclinical cancer models, its original mechanism of action, and an acceptable toxicity profile, it was further evaluated in several phase I studies.[20] In these trials, patients developed serious liver toxicity at dose levels below those believed to be necessary for antitumor activity, although a new dosing regimen was tested in patients with various tumors.



In recent years, patent applications for ellipticine-based cancer treatments have continued to be submitted. Additional mechanisms of action established more recently,[21] such as kinase inhibition, interaction with the p53 transcription factor, bio-oxidation, and adduct formation, showed that compounds of the ellipticine family are multitarget anticancer agents.

2.2 ACTINOMYCINS

Actinomycin D (dactinomycin, Cosmegen®) is a member of the actinomycin family of compounds that was isolated from several *Streptomyces* strains. It contains a phenoxazine chromophore attached to two cyclic depsipeptides containing five amino acid residues, and it can be considered as a hybrid compound that behaves both as a DNA intercalator and as a minor groove binding agent. Although it differs from most intercalating drugs in that it lacks a positive charge, it has been suggested that this is compensated by its high dipole moment, arising from a nonsymmetrical distribution of polar substituents.[22] Dactinomycin is used alone or in combination to treat sarcomas, pediatric solid tumors (e.g., Wilm's tumor, a type of renal tumor), germ cell cancers (testicular cancer), and choriocarcinoma. Its ability to generate superoxide radicals was discussed in Section 5 of Chapter 4.



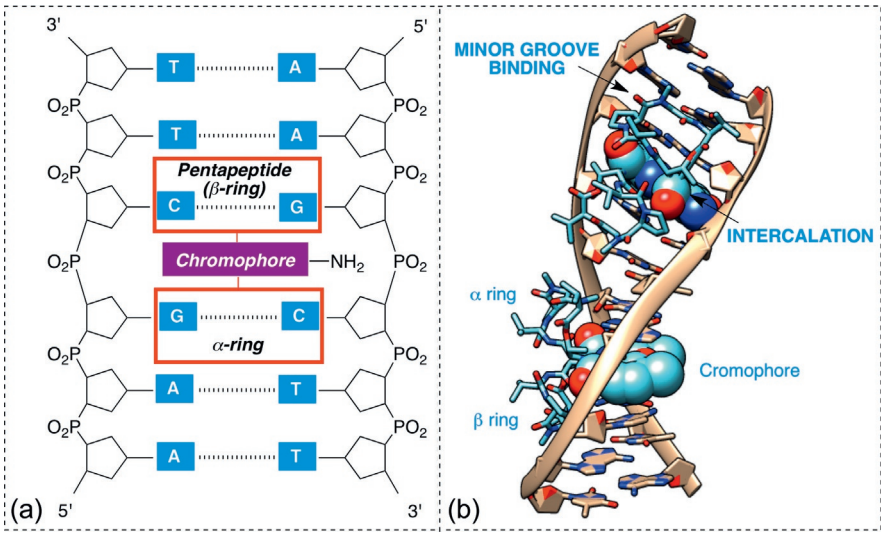


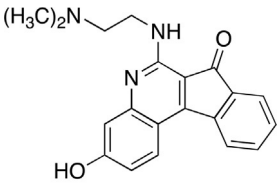
FIGURE 7.6

DNA intercalation by actinomycin D. The three-dimensional structure showing two molecules of actinomycin D intercalated to a ATGCTGCAT sequence was generated from Protein Data Bank reference 1MNV and displayed with Chimera 1.8.1.

Actinomycin D is the paradigm of intercalating compounds with sequence selectivity. X-ray diffraction[23] and molecular modeling studies[24] have been extensively employed to characterize its complex with several forms of DNA.[25] The actinomycin chromophore selects guanine–cytosine pairs and is therefore inserted between the G–C step. Hydrogen bonds are established between the guanine 2-amino group and the carbonyl oxygen of a threonine residue of the pentapeptide side chains, and also between the guanine N-3 atom and the NH group of this residue. The proline, sarcosine, and methylvaline residues are involved in further hydrophobic interactions with the DNA minor groove (Figure 7.6). Several proposals have been put forward regarding the nature of the preferred flanking base sequences adjacent to the GC intercalation site.[26] The formation of this very stable actinomycin–DNA complex prevents the unwinding of the double helix, which leads to inhibition of the DNA-dependent RNA polymerase activity and hence transcription.[27] As in the case of other intercalating agents, Top2 inhibition may also be one of the causes of cytotoxicity.

2.3 FUSED QUINOLINES

Among these compounds, TAS-103 showed a marked efficacy against various lung metastatic tumors and a broad antitumor spectrum in human xenografts and reached clinical trials for the treatment of solid tumors.[28] DNA binding and unwinding assays indicated that this drug intercalates into DNA, although spectroscopic studies showed that outside binding is also important.[29]

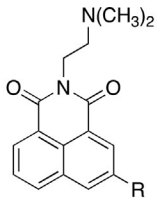


TAS-103

In addition to being an intercalating agent, TAS-103 is also considered a dual Top1–2 inhibitor, although other studies have indicated that cellular susceptibility to TAS-103 is not correlated with Top2 expression. A search for other proteins able to bind this drug showed that it is recognized by the signal recognition particle, a universally conserved ribonucleoprotein that directs the traffic of proteins within the cell and allows their secretion.[30]

2.4 NAPHTHALIMIDES AND RELATED COMPOUNDS

Naphthalimide derivatives bearing an aminoalkyl side chain, such as mitonafide[31] and amonafide,[32] have shown interesting cytotoxic activity[33] that is due to intercalation and Top2 inhibition.[34] Both compounds have been extensively tested in clinical trials, but although they have been used as leads in the design of bis intercalators (see later), they have not been employed in therapeutics.



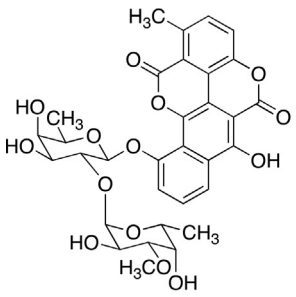
R = NO_2 Mitonafide
R = NH_2 Amonafide

2.5 CHARTREUSIN, ELSAMICIN A, AND RELATED COMPOUNDS

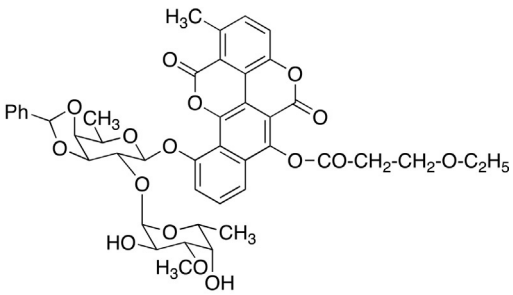
Chartreusin and elsamicin A are structurally related antitumor antibiotics that were isolated from *Streptomyces chartreusis* and from an unidentified actimomycete strain, respectively. Both compounds bind to GC-rich tracts in DNA, with a clear preference for B-DNA over Z-DNA conformation. They also inhibit RNA synthesis and cause single-strand scission of DNA through formation of free radicals (see Chapter 4, Section 6). Elsamicin A binding to the P1 and P2 promoter regions of the *c-Myc* oncogene, which is mutated in many types of cancer, inhibits the binding of the transcription factor specificity protein 1 (Sp1), thus inhibiting transcription.[35]

Chartreusin suffers from unfavorable pharmacokinetic properties (slow oral absorption and biliar excretion), which prevented its clinical development. Among semisynthetic chartreusin analogs with

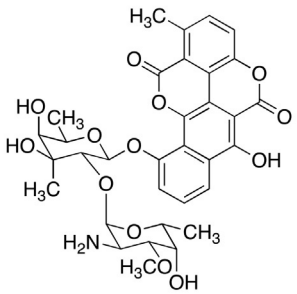
improved pharmacokinetics, IST-622 entered phase II clinical trials for the oral treatment of breast cancer.[36] Phase I clinical studies with elsamicin A for relapsed or refractory non-Hodgkin's lymphoma showed an activity that was modest, but it was considered promising because of the absence of myelosuppression.[37]



Chartreusin



IST-622



Elsamicin A

2.6 OTHER MONOFUNCTIONAL INTERCALATING AGENTS

Other intercalating agents (acridines, anthracyclines) are discussed in Section 6, which deals with Top2 poisons.

3 BIFUNCTIONAL INTERCALATING AGENTS

In efforts to increase the binding constant of intercalating compounds, bifunctional or even polyfunctional compounds were designed. Bifunctional intercalators (bis intercalators) contain two intercalating units, normally cationic, separated by a spacer chain that must be long enough to allow double intercalation taking into account the neighbor exclusion principle (Figure 7.7).

Ditercalinium is an interesting bis intercalator derived from ellipticinium with a novel mechanism of action different from that of its monomer because Top2 inhibition is not involved. It causes inhibition of enzymes that locate and repair damaged DNA sites, especially the nucleotide excision repair

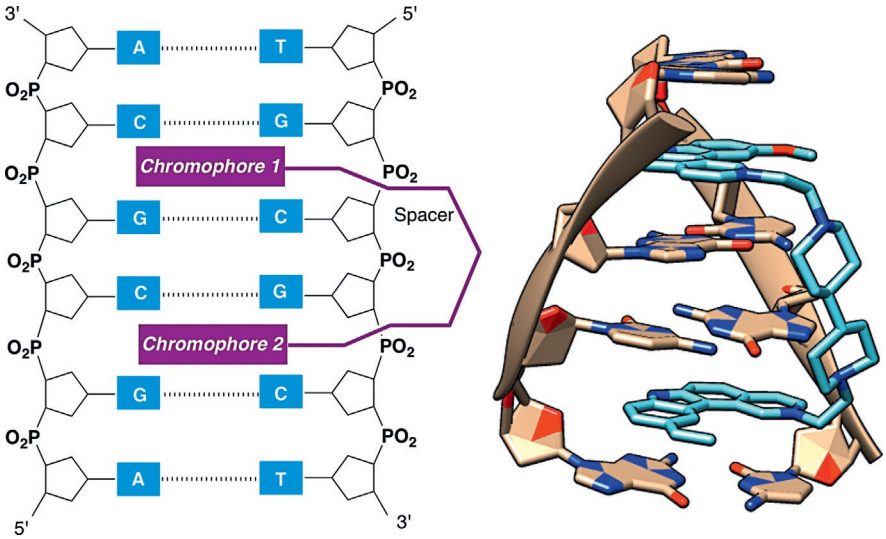
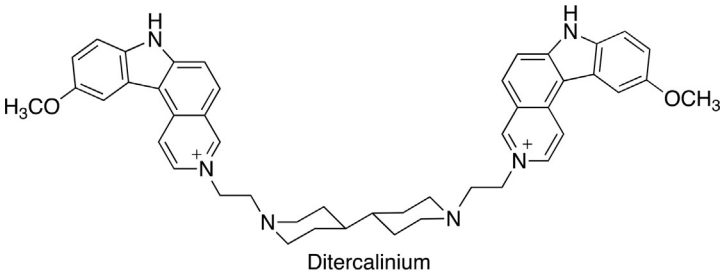


FIGURE 7.7

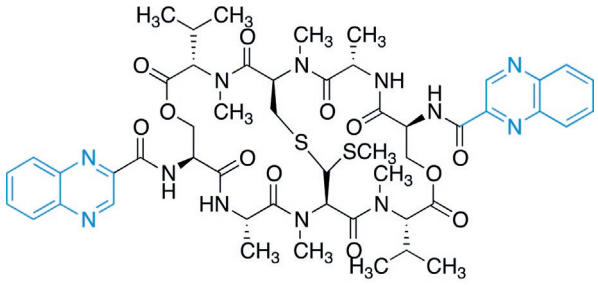
Interaction of bis-intercalating agents with DNA. The three-dimensional structure corresponds to the binding of TOTO, a bis-intercalating fluorescent probe, and was generated from Protein Data Bank reference 108D[38] and displayed with Chimera 1.8.1.

system,[39] due to the unstacking and bending that it induces on DNA because of the rigidity of the linker chain.[40] In addition, it is assumed to associate with mitochondrial DNA, inhibiting its replication.[41]

Elinafide (LU 79553) is a bis intercalator derived from the naphthalimide pharmacophore[42] that exhibited excellent antitumor activity and reached phase I clinical trials,[43] showing anti-neoplastic activity in ovarian cancer, breast cancer, and mesothelioma. Many mechanistic studies on elinafide and its analogs have been undertaken,[44] but this drug has neuromuscular dose-limiting toxicity that has halted its clinical development.



Echinomycin is an antitumor antibiotic isolated from *Streptomyces echinatus* that consists of two quinoxaline chromophores attached to a cyclic octadepsipeptide ring, with a thioacetal cross-bridge. Because of its potent antitumor activity, this compound advanced to several phase II clinical studies,[45,46] although it was eventually withdrawn from further clinical trials because it showed a high toxicity without any marked therapeutic benefit. Recently, echinomycin has been characterized as a very potent inhibitor of the binding of hypoxia-inducible factor 1 (HIF-1) to DNA. This is an interesting feature because HIF-1 is a transcription factor that controls genes involved in processes important for tumor progression and metastasis, including angiogenesis, migration, and invasion.[47]



Echinomycin

Several studies have proven that both echinomycin quinoxaline rings bis intercalate into DNA, with CG selectivity, while the inner part of the depsipeptide establishes hydrogen bonds with the DNA bases of the minor groove region of the two base pairs between the chromophores (Figure 7.8).[48] A calorimetric study proved that the binding reaction is entropically driven, showing that the complex is predominantly stabilized by hydrophobic interactions, although direct molecular recognition between echinomycin and DNA, mediated by hydrogen bonding and van der Waals contacts, also plays an important role in stabilizing the complex.[49]

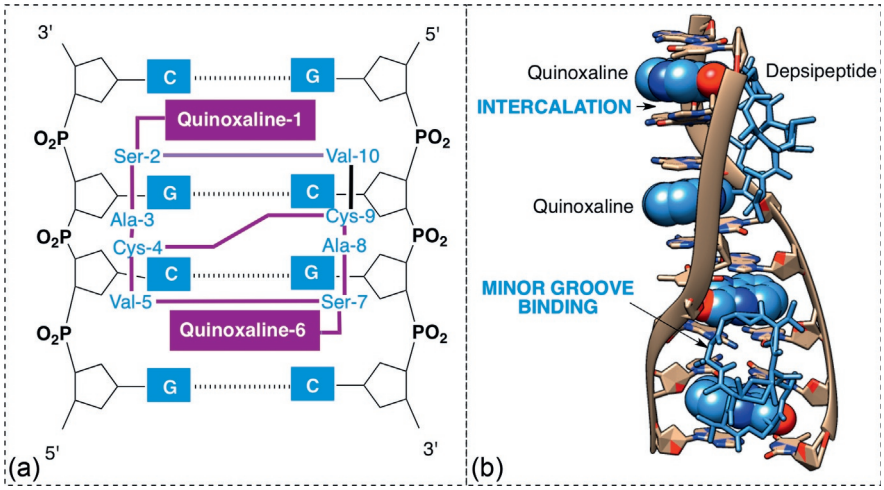


FIGURE 7.8

Interaction of echinomycin with a DNA fragment. The three-dimensional structure of the echinomycin-d (ACGTACGT) duplex was generated from Protein Data Bank reference 3G03[50] and displayed with Chimera 1.8.1.

4 INDIRECT DNA DAMAGE BY DNA TOPOISOMERASE INHIBITORS

The discovery of DNA topoisomerases in the 1970s solved the topological problem posed by DNA replication. These enzymes are major elements in cellular life and the target of a plethora of antibiotics and antitumor compounds. Their inhibitors are among the most efficient inducers of apoptosis[51] and include some of the most widely used anticancer drugs.[52]

Topoisomerases regulate the three-dimensional geometry (topology) of DNA, leading to the interconversion of its topological isomers and to its relaxation. Identical loops of DNA having different numbers of twists are topoisomers—that is, molecules with the same formula but different topologies—and their interconversion requires the breaking of DNA strands. Regulation of DNA supercoiling is essential to DNA transcription and replication, when the DNA helix must unwind to permit the proper function of the enzymatic machinery involved in these processes.

Among the various topoisomerases,[53] we briefly discuss the roles of Top1 and Top2. In eukaryotic cells, Top1 breaks a single DNA strand, whereas Top2 breaks both strands and requires ATP hydrolysis and Mg^{2+} for full activity. Both proteins introduce transient single-strand breaks in the DNA molecule and store the energy gained during the cleavage reaction in a transient covalent linkage between DNA and a tyrosine of the protein in order to use it later for their ligase activity. The catalytic mechanism in both cases consists of two transesterification steps beginning with a nucleophilic attack of a DNA phosphodiester bond by a tyrosyl residue from the topoisomerase active site. The resulting covalent attachment of the tyrosine to the DNA phosphate is either at the 3′ end of the broken DNA, in the case of nuclear and mitochondrial enzymes Top1 and Top1mt, or at the 5′ end of the broken DNA for the other topoisomerases. These are known as the "cleavable complex" because they are transient and have easily reversible linkages, which formation permits the DNA relaxation. In the religation step, a hydroxyl group from deoxyribose attacks the previously formed tyrosine phosphate, and the end result is a DNA molecule that is chemically unchanged but closed in a different topology.

Topoisomerases are crucial for the several DNA functions that require the DNA to be unraveled, a process that generates tension and entanglement in DNA. Removing positive supercoils is required for replication and transcription progression; otherwise, their accumulation hinders the melting of the DNA duplex by helicases and consequently polymerase translocation along the DNA template.

On the other hand, topoisomerase poisons may induce genetic instability.[54] In this connection, some alarming studies have been published suggesting that maternal exposure to low doses of dietary Top2 poisons, including bioflavonoids such as genistein or quercetin, may contribute to the development of infant leukemia.[55]

4.1 TOPOISOMERASE I MECHANISM

Topoisomerase I is located in areas of active RNA transcription in order to release superhelical stress generated during mRNA synthesis. As previously mentioned, in the case of eukariotic Top1, a single strand is attacked and a 3′-phosphotyrosyl linkage is formed. Religation takes place through attack of the 5′ hydroxyl to the previously formed phosphate group (Figure 7.9).

After making a transient break ("nick") of a single strand of DNA, the DNA relaxation mechanism is originated by "controlled rotation" rather than by "strand passage." In other words, Top1 enzymes relax DNA by letting the 5′-hydroxyl end rotate around the intact strand (Figure 7.10).

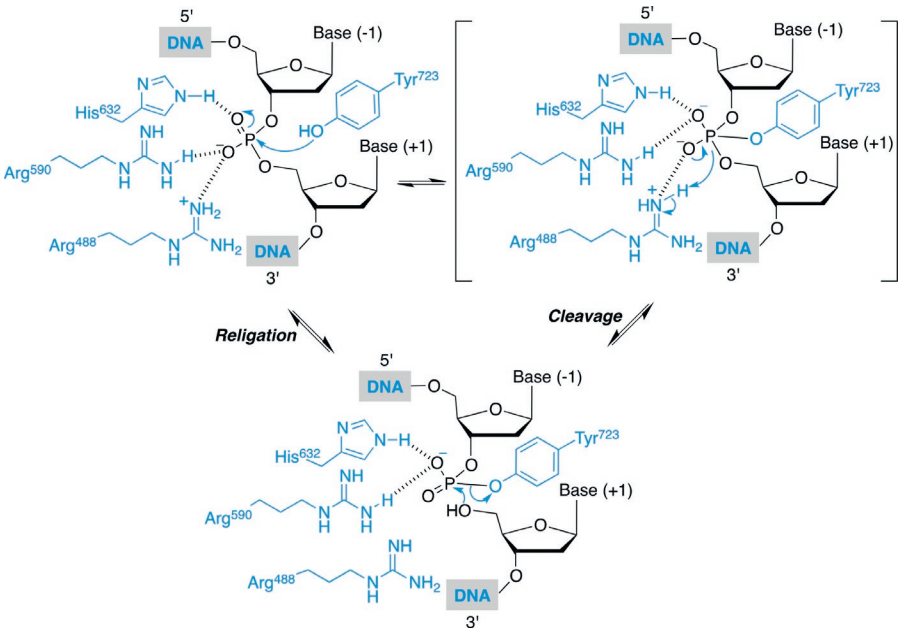


FIGURE 7.9

The catalytic cycle of topoisomerase Ib.

The cytotoxicity of Top1 inhibitors is due to trapping of cleavable complexes (Top1cc) rather than to the inhibition of Top1 catalytic activity, because the cleavable complexes lead to DNA damage by DNA replication and transcription. This trapping takes place during apoptosis induced by anticancer drugs such as Top1 inhibitors, the Top2 inhibitors etoposide, doxorubicin, and amsacrine, and the tubulin inhibitors vinblastine and taxol, being considered a general process of programmed cell death caused by alterations of the DNA structure induced by caspases and reactive oxygen species.[56]

4.2 TOPOISOMERASE II MECHANISM

Eukariotic Top2 is a homodimeric enzyme that makes a transient DNA double-strand break, where the tyrosines from the active sites of both monomers attack the phosphodiester bond to the 5′ side of the phosphate, leading to a covalent 5′-phosphotyrosyl linkage in each strand. This mechanism is shown in Figure 7.11, in comparison to the one previously described for Top1.

These breaks between the strands are not directly opposite to each other; instead, they are separated by a four-base pair overhang, generating a space through which another region of intact DNA can be passed (Figure 7.12). In other words, in the case of Top2 enzymes, a full DNA duplex, known as the T (transported) strand, goes through the double-strand break made by the enzyme homodimers.

The full catalytic cycle of Top2 is complex and is summarized in Figure 7.13, together with the names of drugs that have steps of this cycle as targets.[57] The enzyme assumes two different

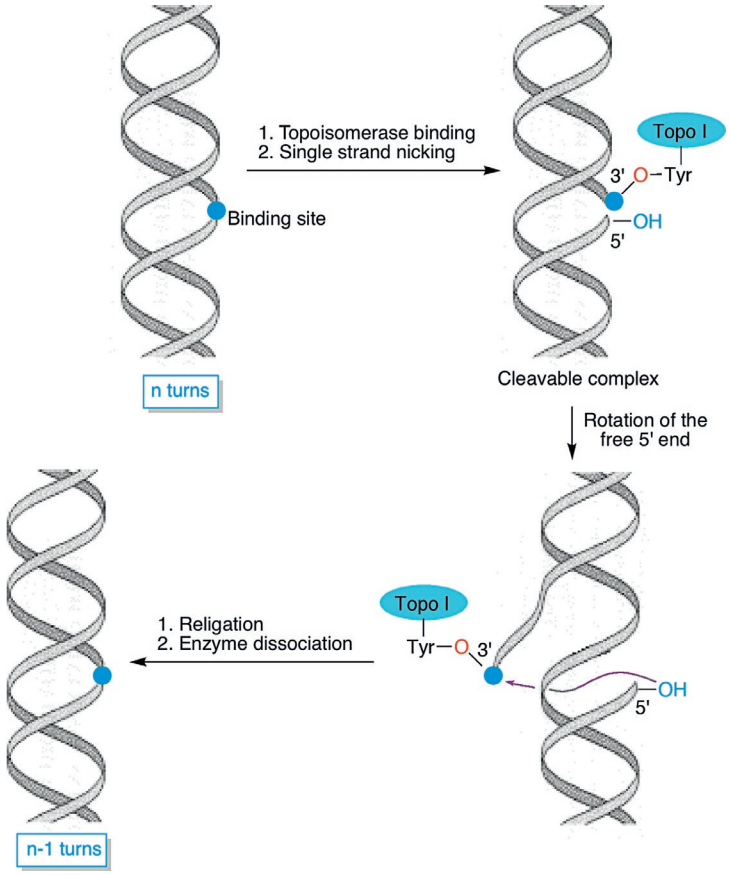


FIGURE 7.10

Mechanism of DNA unwinding by topoisomerase I.

conformations, resembling an open clamp in the absence of ATP and a closed clamp in the presence of ATP. The open conformation can bind two segments of DNA, forming the pre-cleavage complex. One of these segments will be nicked by the enzyme (G segment), and another will be transported (T segment). Afterwards, two ATP molecules are bound, leading to the dimerization of the ATPase domains and hence to a conformational change from the open to the closed clamp structure. The nucleophilic reactions that break both strands of the G segment of DNA then take place, generating the post-cleavage complex. This allows the passage of the T segment through the gap thus produced, which requires the hydrolysis of one molecule of ATP. The broken ends of the G segment are then ligated, and the remaining ATP molecule is hydrolyzed. Upon dissociation of the two ADP molecules from ATP hydrolysis, the T segment is transported through the opening at the C-terminal part of the enzyme, which is then closed. Finally, the enzyme returns to the open clamp conformation, liberating the G segment.

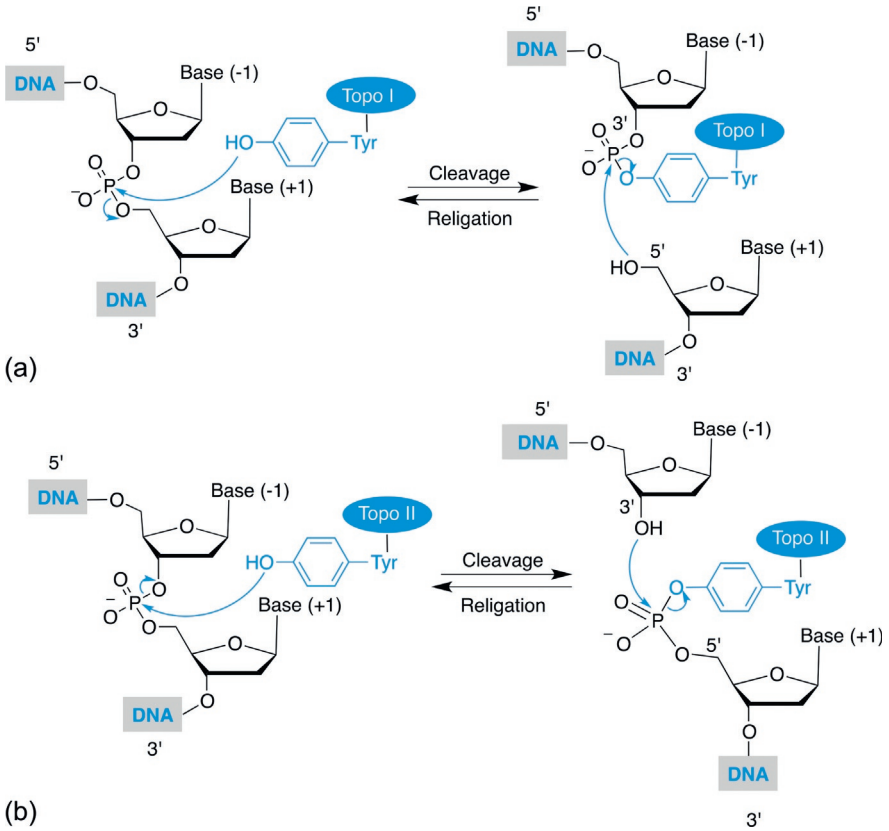


FIGURE 7.11

Comparison of the cleavage and religation events catalyzed by topoisomerase I (a) and topoisomerase II (b).

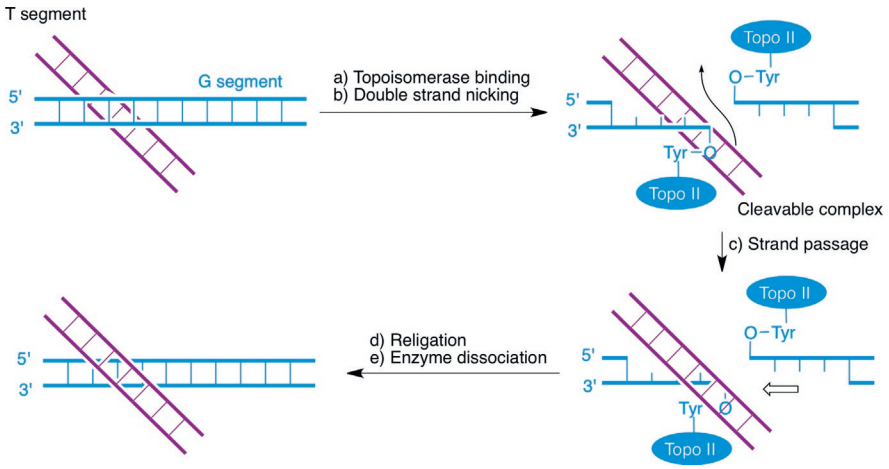


FIGURE 7.12

Mechanism of DNA unwinding by topoisomerase II.

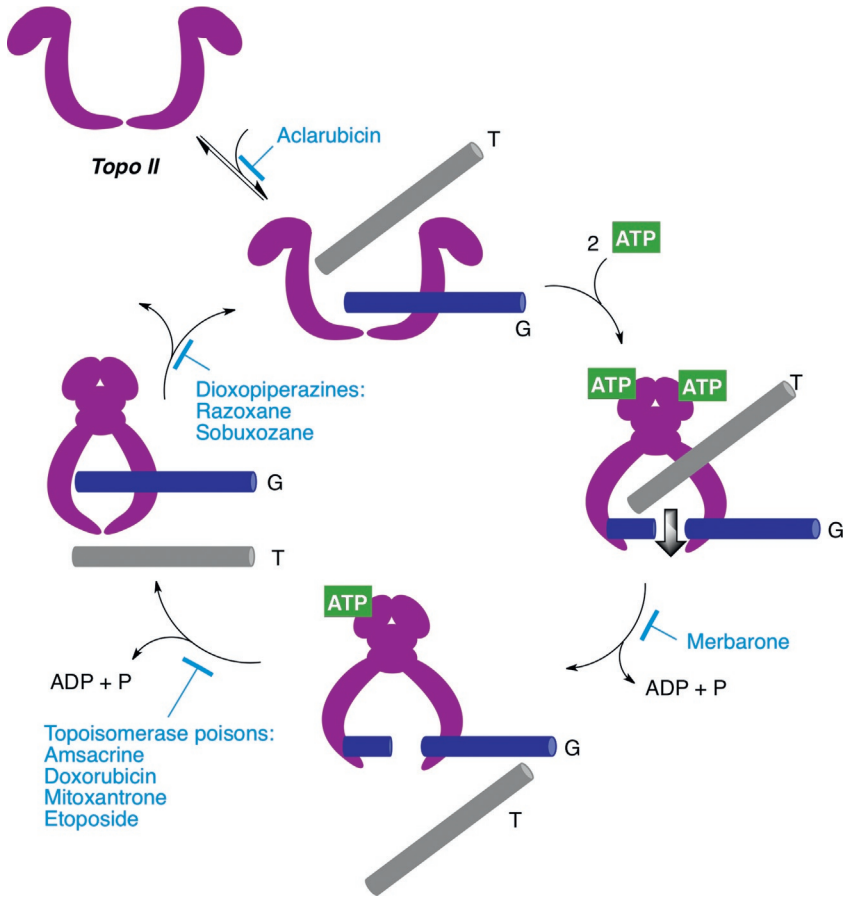


FIGURE 7.13

Catalytic cycle of topoisomerase II and its main inhibitors.

Some antitumor drugs acting at the topoisomerase level have inhibition of enzymatic activity as their primary mode of action, and these are known as "catalytic topoisomerase inhibitors" or "topoisomerase suppressors."[58,59] Other drugs targeting the topoisomerases, including intercalating drugs, interfere with the enzyme's cleavage and rejoining activities by trapping the cleavable complex and thereby increasing the half-life of the transient topoisomerase-catalyzed DNA break. Some of the most clinically useful anticancer drugs are of the latter type and are normally referred to as "topoisomerase poisons" because they convert the topoisomerase enzyme into a DNA-damaging agent. The behavior of these types of inhibitors is summarized in Figure 7.14, which shows that topoisomerase poisons trap the cleavable complex with increasing efficiency as drug concentration increases, whereas suppressors inhibit the formation of the cleavable complex. Finally, topoisomerase inhibitors that act by DNA intercalation enhance the formation of the cleavable complex at low compound levels but inhibit it at higher concentrations.[60]

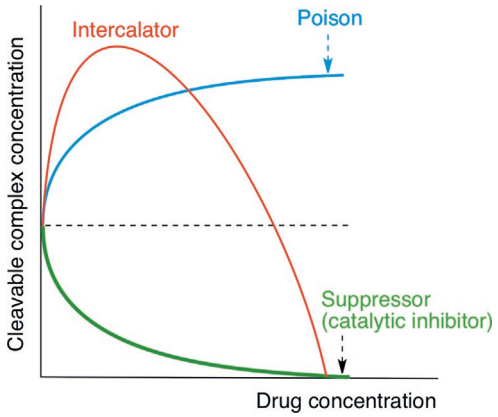


FIGURE 7.14

Modes of topoisomerase inhibition.

Because the level and time course of expression of these enzymes vary in different cell types, and the development of resistance to one type of inhibitor is often accompanied by a concomitant rise in the level of the other enzyme, there is increasing interest in drugs that can act as dual Top1/2 poisons.[61,62]

5 SPECIFIC TOPOISOMERASE I INHIBITORS

Compounds that inhibit TopI[63] can be divided into two categories:

1. Topoisomerase I suppressors, which are those compounds that inhibit the enzyme but do not stabilize the intermediate DNA–Top1 covalent complex.
2. Topoisomerase I poisons, which act after DNA cleavage by inhibiting religation. This can be achieved through three different mechanisms involving (1) binding of the enzyme to the previously formed drug–DNA binary complex, (2) recognition of the enzyme–DNA binary complex by the drug, or (3) interaction of DNA with the drug–enzyme complex.[64]

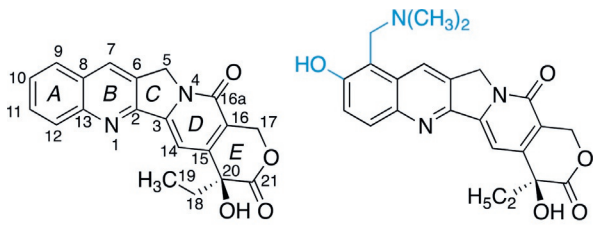
It is interesting to note that although Top1 seems not to be essential for cell survival because other topoisomerases can (at least temporarily) play its role, its inhibition nevertheless leads to cell death. This means that the cause of apoptosis is not the suppression of the catalytic activity but, rather, the series of molecular events that take place upon trapping of the DNA–Top1 complex, and these are not known in full detail.[65]

5.1 CAMPTOTHECINS

Camptothecin (CPT) is an alkaloid that was isolated in 1966 from the bark of the Chinese tree *Camptoteca acuminata* as a potent anticancer drug, although its therapeutic development was initially limited by its poor solubility and unacceptable toxicity. Identification of Top1 as its sole

target validated the inhibition of this enzyme as a goal for cancer chemotherapy and prompted the search for water-soluble, more active, less toxic analogs.[66,67] Structure–activity relationship studies showed that substituents at ring A and at the C-7 position of ring B were allowed, whereas the ring E lactone was essential for activity. Because these Top1-targeted drugs are S-phase specific, they achieve optimal inhibitory activity when the tumor is continuously exposed to the drugs for long periods of time and are adequate for tumors with a high proportion of proliferating cells but unsuitable for those tumors that have high numbers of noncycling cells in the G_1 phase, such as prostate and kidney cancer.

The main problem associated with CPT is its very poor water solubility, which hampers its formulation. Two CPT analogs that solve this problem by the introduction of basic substituents that allow the preparation of salts, namely topotecan and irinotecan (CPT-11), were introduced into clinical trials in the 1980s and gained regulatory agency approval for the treatment of various cancers in the 1990s. Topotecan (Hycampin®) is used for the treatment of fluoropyrimidine-refractory ovarian and small cell lung cancers,[68] although hematological toxicity is a common side effect due to the destruction of bone marrow progenitors.



Camptothecin Topotecan

Irinotecan (Camptosar®), which received accelerated approval by the U.S. Food and Drug Administration (FDA) in 1996 and full approval in 1998, is a prodrug that needs to be hydrolyzed by a carboxylesterase[69] to its active metabolite SN-38 (Figure 7.15). It is used in colorectal cancer, showing synergism with cisplatin.[70] Several studies have underscored the importance of pharmacogenetic considerations in its clinical application[71] because there is a considerable degree of polymorphism in the main enzyme involved in its hepatic metabolism, namely uridinediphosphate glucuronosyl transferase 1A1 (UGT1A1).[72] Simmitecan is a closely related prodrug, whose active form is known as chimmitecan, and is being studied in phase I for the treatment of advanced solid tumors.[73]

One of the main limitations of all CPT derivatives is their spontaneous and rapid inactivation (within minutes) by opening of the lactam function in the E ring. This reaction is reversible and both species are present at physiological pH, but the carboxylic form binds readily to serum albumin, thereby shifting the lactone–carboxylate equilibrium toward the inactive species (Figure 7.16).[74] Any factor hampering the binding to albumin favors activity; for instance, the higher potency of topotecan and irinotecan with regard to CPT has been attributed to interference of their substituents with binding to albumin.[75] Furthermore, a higher E ring stability leads to lower bladder toxicity, one of the main problems associated with the use of CPT derivatives. The reason is that the relatively low pH of urine prompts the cyclization of the secreted carboxylate form, leading to the local formation of high amounts of the cytotoxic lactone species.[76]

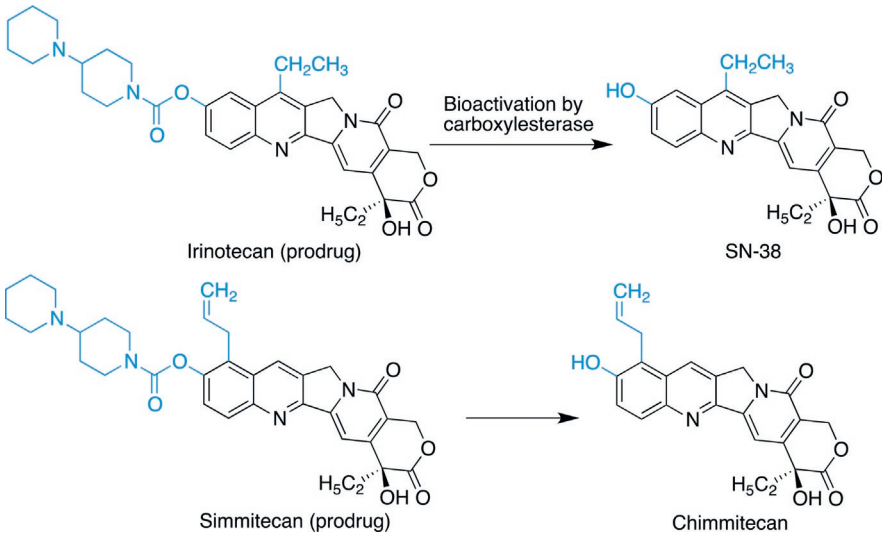


FIGURE 7.15

Camptothecin-related prodrugs with improved aqueous solubility.

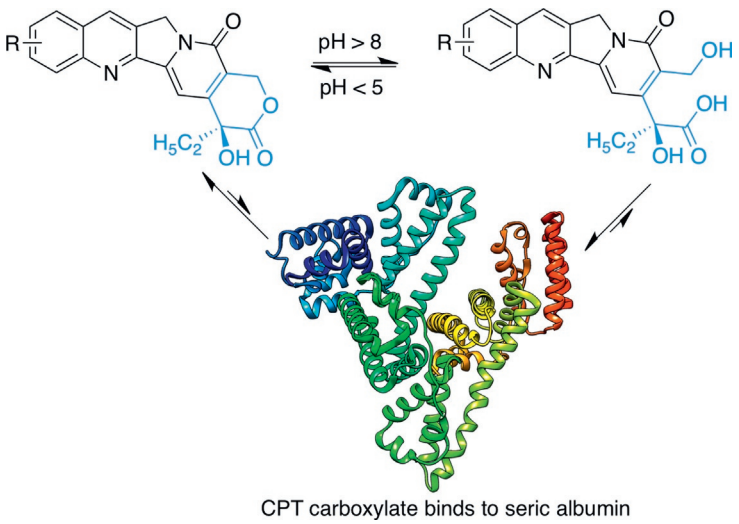
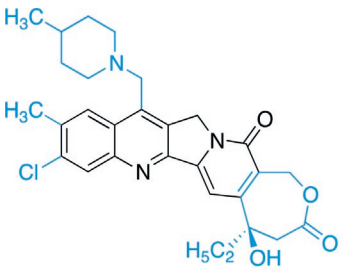


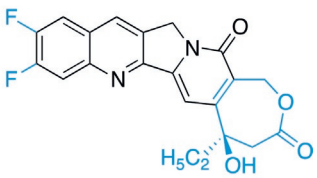
FIGURE 7.16

Camptothecin E-ring opening and association of the carboxylic form with serum albumin.

One approach to overcome the E ring lactone instability is to enlarge it by one carbon atom, which limits its opening but also prevents its reclosure.[77] The resulting synthetic compounds are known as homocamptothecins.[78] The most promising of these compounds are elomotecan (BN80927)[79,80] and diflomotecan (BN80915),[81,82] which have been tested in clinical trials for colon, breast, prostate, and lung cancer.

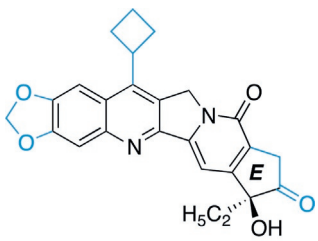


Elomotecan (BN 80927)



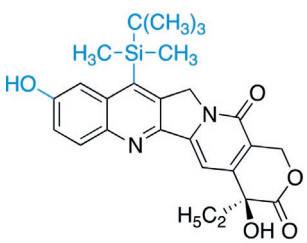
Diflomotecan (BN 80915)

A second approach to stabilize the CPT E ring involves replacement of the lactone function by a ketone, as in the case of the stable compound S39625.[83]

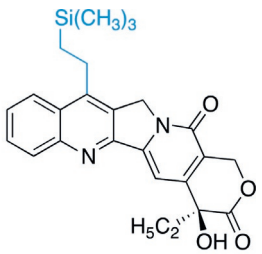


S39625

In a third approach, it was reasoned that the introduction of sufficiently lipophilic substituents would promote the partitioning of the drug into the lipid bilayer, thereby affording protection from hydrolysis.[84] The most advanced compounds designed according to this strategy are the silyl CPTs silatecan (AR67)[85] and karenitecin (BNP1350).[86]



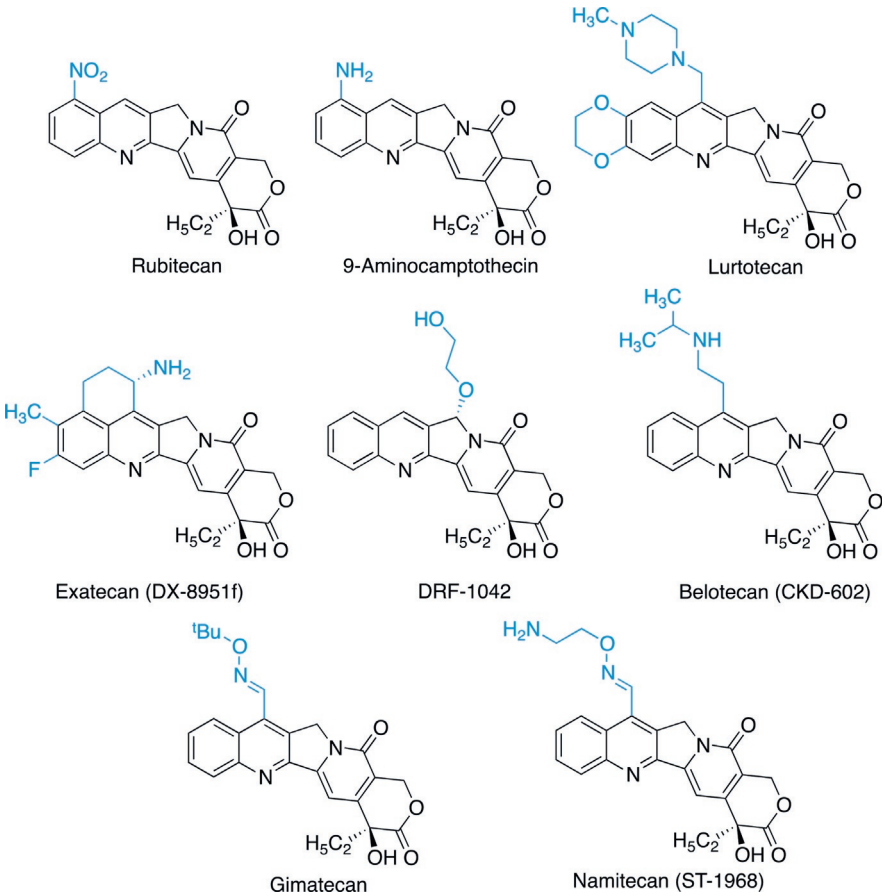
Silatecan (AR67)



Karenitecin (BNP1350)

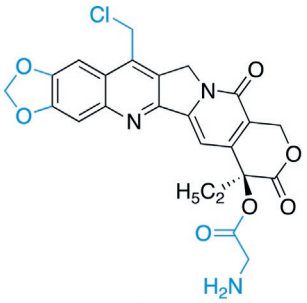
A number of additional second-generation CPT analogs have entered clinical trials.[87,88] Examples of A ring-substituted compounds include rubitecan[89] and its active metabolite 9-aminocamptothecin.[90] Among B ring-substituted compounds are lurtotecan,[91] exatecan (DX-8951f),[92,93] DRF1042,[94] and belotecan (CKD-602, Camtobell®). The latter compound gained approval in some countries for the treatment of ovarian and small cell lung cancer.[95]

Gimatecan and namitecan (ST1968) belong to a subclass of B ring-modified camptothecins by introduction of *O*-substituted oxime substituents at C7. Gimatecan, an orally active lipophilic CPT derivative, was developed to provide rapid uptake and enhanced accumulation. It received an orphan drug designation by the European Medicines Agency (EMA) for the treatment of glioma and is also being studied for epithelial ovarian and other cancers.[96] The water-soluble namitecan (ST1968) exhibited an acceptable toxicological profile[97] and entered clinical studies, showing benefit in patients with a number of tumors, including bladder and endometrium carcinomas.

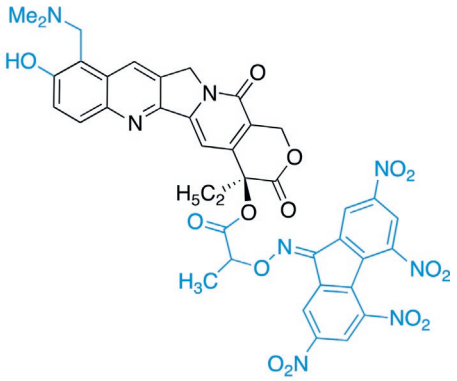


The E ring hydroxyl substituent is essential for CPT binding (see later), but it can be used for the generation of derivatives with improved properties. For instance, CMMD-Gly is a water-soluble glycine

ester of a CPT derivative that has undergone promising preclinical studies.[98] TLC388 (Lipotecan®) was designed as a multitarget drug because it contains a molecule of topotecan and another of a tetranitrofluorene oxime, with the two active fragments linked by a molecule of lactic acid. The E ring modification stabilizes the lactone moiety, and the presence of the nitro substituents makes the compound a strong radio- and chemosensitizer. This compound demonstrated significant effectiveness in phase I and II clinical trials on patients with hepatocellular carcinoma, and in 2010 it was granted orphan drug designation for this indication by both the EMA and the FDA. In 2013, it was included in the "Green Path" program by the Chinese State Food and Drug Administration (SFDA).



CMMD-Gly



TLC388 (Lipotecan)

The mode of action of CPT and other Top1 inhibitors is very different compared to that of other enzyme inhibitors. They do not bind to DNA or to Top1 by themselves because they require the presence of both Top1 and DNA associated in a cleavable complex. This observation led to the hypothesis that CPT binds at the interface of both Top1 and DNA in a ternary complex. This hypothesis, which was confirmed by the determination of the crystal structure of a ternary Top1 cleavable complex with topotecan,[99] converted these drugs into the paradigm for interfacial inhibitors, which differ from orthosteric and allosteric inhibitors in that they bind at the interface of two or more macromolecules (Figure 7.17). This mode of drug–target interaction is uncommon, but it has also been observed for other anticancer drugs that can only bind to certain points ("hot spots") of the interfaces formed between two biological macromolecules, including Top2 inhibitors (adriamycin, etoposide, and dexrazoxane) and tubulin binders (paclitaxel, vinblastine, and colchicine). The following are the main characteristics of interfacial inhibitors:[100]

1. Their target is a biological system formed by two macromolecules (proteins or nucleic acids).
2. They couple to the interface generated upon binding of these macromolecules to each other. The precise binding site is generated by the movements of the macromolecules upon, for instance, cleavage of DNA or bending of the microtubule filament.
3. Drugs normally bind reversibly by hydrogen bonding, π-stacking, or metal chelation. Drug binding to these "hot spots" tends to be highly enantiospecific.

The very deep penetration of CPT into its site leads to a thermodynamically favorable increase in entropy upon binding due to the liberation of a large number of molecules of hydration water, as shown in Figure 7.18.[101]

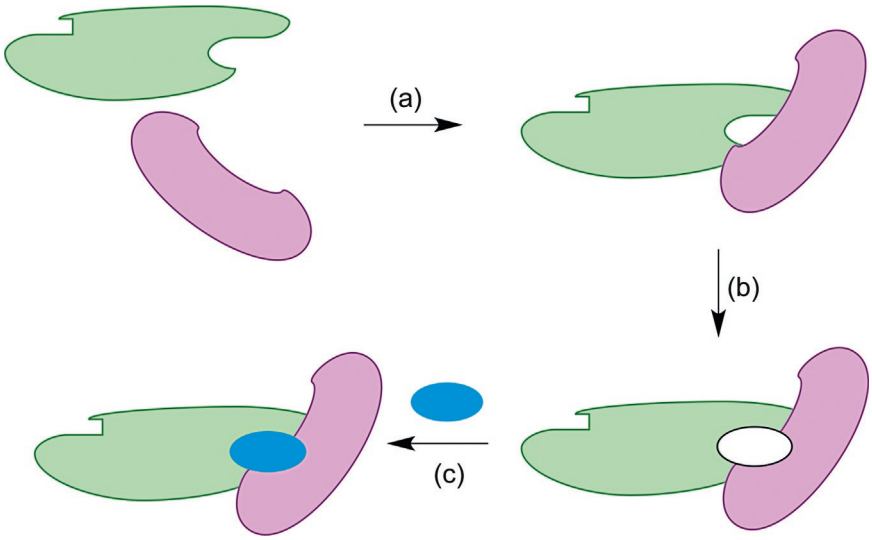


FIGURE 7.17

The interfacial binding process. (a) The two target macromolecules bind to each other. (b) Molecular movements generate the drug binding site at the molecule–molecule interface. (c) The drug binds, giving rise to a ternary complex.

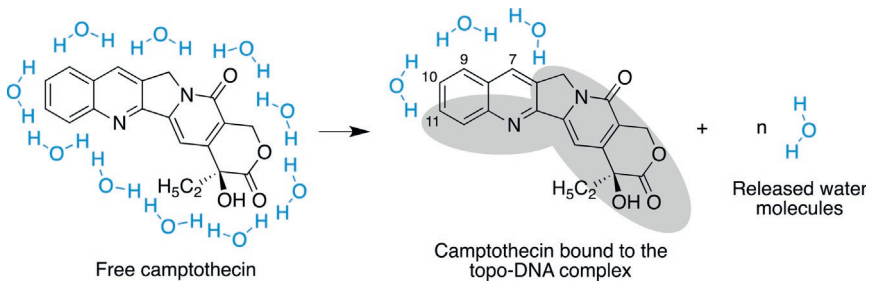


FIGURE 7.18

Favorable activation entropy for the formation of the camptothecin–Top1–DNA ternary complex.

The fine details of CPT binding have been the subject of much research. The first proposal, due to Pommier, involved an intercalation of CPT completely parallel to the base pairs, with the concave side of the molecule toward the major groove and ring E toward the cleavable strand (Figure 7.19a). On the other hand, Hol proposed an intercalation mode with the concave side of CPT toward the cleavable strand and ring E toward the minor groove, and with the G (+1) guanine displaced out of the double helix and stacked with rings A and B of CPT (Figure 7.19b). X-ray diffraction studies of the ternary complex formed by CPT[102] and topotecan[103] support the parallel alignment proposed by Pommier (Figure 7.20).

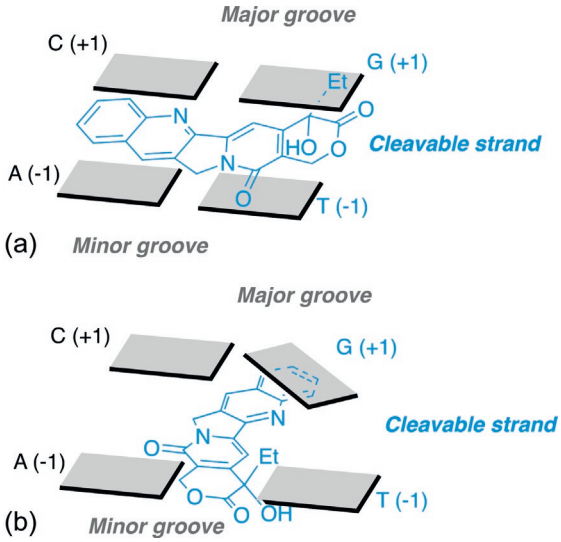


FIGURE 7.19

Initial models for the binding of camptothecin to DNA proposed by Pommier (a) and Hol (b).

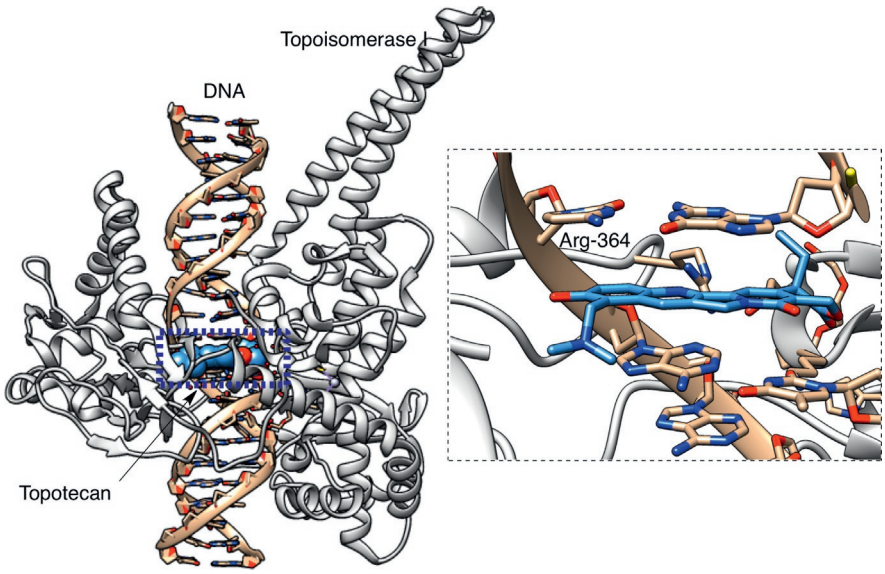


FIGURE 7.20

Structure of the ternary complex formed by topotecan with human topoisomerase I and a 22-base pair DNA duplex. Generated from Protein Data Bank reference 1K4T and displayed with Chimera 1.8.1.

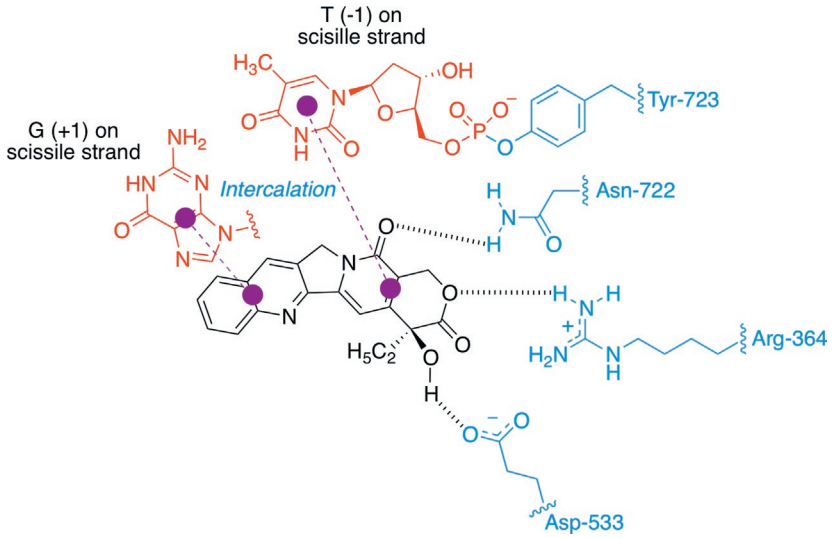


FIGURE 7.21

Main interactions in the camptothecin–DNA–Top1 ternary complex.

The main interactions between CPT at its binding site are summarized in Figure 7.21[99] and include stacking interactions with the G (+1) and T (–1) bases on the scissile strand and hydrogen bonding between the functional groups at the D and E rings of CPT and the Arg-364, Asp-533, and Asn-722 residues of Top1. As expected, the catalytic Tyr-723 residue is covalently attached to the T (–1) nucleotide.

Additional approaches to the optimization of the CPT derivatives, which include prodrug strategies, pegylation, nanoparticles as vehicles, and immunoconjugation, are discussed in Chapter 13.

5.2 NON-CAMPTOTHECIN TOPOISOMERASE I INHIBITORS

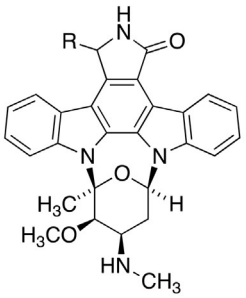
To date, three chemical families have been described that show this activity: indolocarbazoles, indenoisoquinolines, and phenanthridine derivatives. Indolocarbazoles were the first non-CPT Top1 inhibitors in clinical development and the more advanced of the three mentioned chemical families,[104] but they appear to hit other cellular targets besides Top1.[105] This ring system is present in several structurally related compounds that can target DNA, Top1, and several protein kinases.[106] The first of these compounds is staurosporine, a natural product originally isolated in 1977 from *Streptomyces staurosporeus* that has a wide range of biological activities but is best known as an ATP-competitive broad-spectrum kinase inhibitor. Currently, several staurosporine analogs are in advanced clinical trials as anticancer agents.[107] The related UCN-01, also a natural product isolated from *Streptomyces* cultures, is currently undergoing clinical studies.[108] Both compounds are discussed in Sections 4 and 5 of Chapter 10.

Rebeccamycin is also a natural product isolated from the actinomycete *Saccharothrix aerocoligenes*, with a dual Top1 and Top2 inhibiting activity. This compound showed an impressive cytotoxicity *in vitro* but could not be developed further because of poor water solubility. Among the many

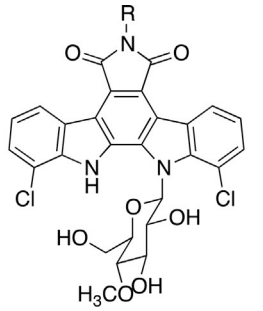
water-soluble rebeccamycin analogs that have been developed, compound NSC-655649 (BMY-27557-14) entered phase II clinical trials for renal cancer.[109] Interestingly, the presence of the aminoethyl side chain in this compound led to specific Top2 inhibitory activity.

The analog NB-506 has been characterized as a Top1 inhibitor that enhances DNA cleavage mediated by this enzyme. Because it shows cross-resistance with CPT, it has been suggested that they share a common binding site in the Top1–DNA complex, although NB-506 probably targets other additional cellular processes.[110] Intercalation is apparently not required to stabilize its complex with Top1–DNA, and in fact, a regioisomer of NB-506 without capacity to intercalate into DNA is an extremely potent Top1 poison.[111] Clinical studies on NB-506 started in 1994, and it has shown particular good activity in ovarian and breast cancer.

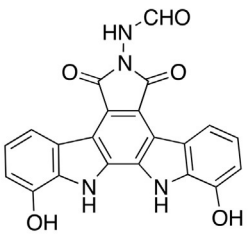
The related hydroxy derivative edotecarin (J-107088) is more active *in vitro* than NB-506 or CPT in the induction of Top1 cleavage complexes.[112] This glycoside has been studied clinically and has shown potent activity against lung and prostate cancers with a wider therapeutic window than many established drugs.[113] It has also shown activity in clinical trials for colon, breast, and other cancers.[114] The larger size of the imide nitrogen substituent in this compound hampers imide ring opening and glucuronidation, and it leads to an increased half-life.



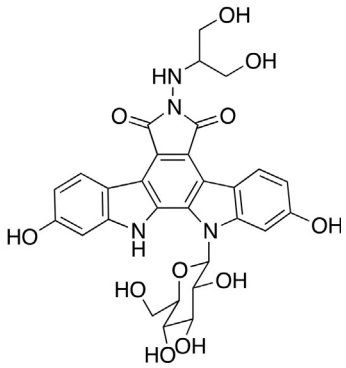
Compound	R
Staurosporine	H
UCN-01	OH



Compound	R
Rebeccamycin	H
NSC 655649 (BMY-27557-14)	$(CH_2)_2NEt_2$



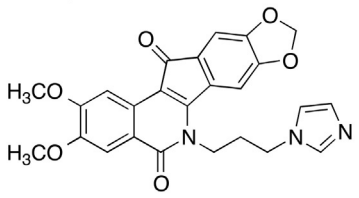
NB-506



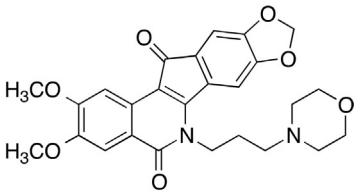
Edotecarin
(PHA-782615, J-107088)

Indenoisoquinolines NSC-725776 and NSC-724998 are in preclinical development[115] and under review for clinical trials.[116] The phenanthridine derivatives ARC-111 (topovale) and Genz-644282 have also emerged as promising antitumor candidates. On the basis of preclinical activity and safety,[117] Genz-644282 was selected for development and is currently undergoing phase I clinical trials. The antiproliferative activity of indenoisoquinolines is similar to or greater than that of CPTs, but they trap the cleavable complex at different sites. Phenanthridines share many of the same advantages as the indenoisoquinolines, which is not surprising considering their chemical similarities.

Indenoisoquinolines

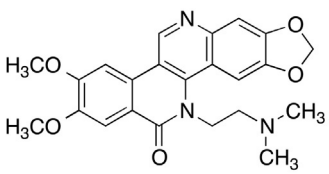


NSC 725776

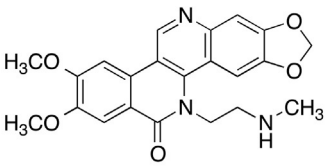


NSC 724998

Phenanthridines



ARC-111 (topovale)

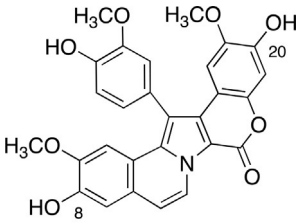


Genz-644998

Another promising class of Top1 inhibitors are the lamellarins,[118] isolated from marine organisms such as mollusks from the *Lamellaria* genus and *Didemnum* ascidians. This family of hexacyclic pyrrole alkaloids display promising antitumor activity because they induce apoptotic cell death through multitarget mechanisms, including inhibition of Top1, interaction with DNA, and direct effects on mitochondria. They are weak intercalating agents, and although their cationic derivatives are more potent in this regard, no correlation exists in these compounds between their intercalating activity and their cytotoxicity. Lamellarins also inhibit several protein kinases relevant to cancer, such as cyclin-dependent kinases, dual-specificity tyrosine phosphorylation activated kinase 1A, casein kinase 1, glycogen synthase kinase-3, and PIM-1.[119] Several members of the family, especially lamellarin I, reverse multidrug resistance (MDR) by direct inhibition of the P-gp-mediated efflux.[120,121] This pharmacological profile opens the possibility of their use as antitumor agents in resistant cells as well as their use as modulators of the MDR phenotype in combination with other antitumor compounds. Structure–activity relationship studies in the lamellarins[122] showed little tolerance toward changes in the substitution pattern in the natural products and underscored the importance of the methoxy and hydroxyl groups.

Lamellarin D, one of the most potent compounds of the series, promotes DNA cleavage through stabilization of DNA–Top1 cleavable complexes,[123] displays potent cytotoxic activities against multidrug-resistant tumor cell lines, and is highly cytotoxic to prostate cancer cells. It is being considered for clinical development together with other analogs. Although Top1 is not the only target of the

lamellarins, a molecular modeling study of the binding of lamellarin D to the DNA–Top1 complex has revealed the presence of hydrogen bonding interactions of the hydroxyls at C-8 and C-20 with the Glu-356 and Asn-722 residues of the enzyme.[124]



Lamellarin D

Despite the clinical successes of Top1 inhibitors, inherent resistance to these drugs has been reported. Because Top1 inhibitors induce cytotoxic DNA lesions, the repair of this damage is an important determinant in the cellular response. One DNA lesion can emerge from the abortive activity of DNA Top1, resulting in a DNA strand break that is encumbered with a 3′ protein adduct. If not repaired, such breaks can result in the development of more dangerous double-strand breaks (DSBs) that can lead to chromosome loss, translocations, or truncations. The versatile base excision repair (BER), which requires several enzymes such as tyrosyl-DNA phosphodiesterase (Tdp1), has been identified as one of the pathways responsible for repairing Top1-mediated DNA damage.[125] Consequently, inhibitors of the DNA repair enzymes have been foreseen as an adjunct therapy (see Chapter 13).[126]

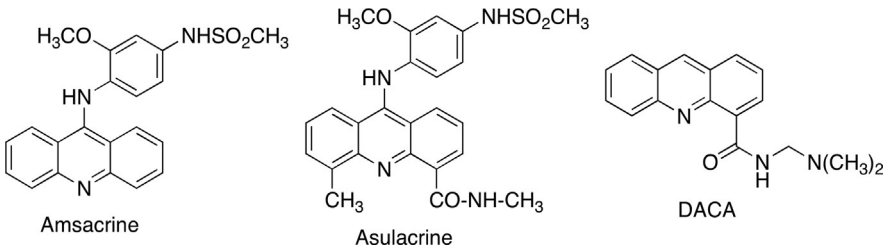
6 TOPOISOMERASE II POISONS

This class of Top2 inhibitors increases the levels of Top2–DNA cleaved DNA complexes by trapping the G strand–enzyme intermediates, thus blocking religation and enzyme release and leaving the DNA with a permanent DSB.[127] All Top2-targeted anticancer drugs that are currently used in the clinic are interfacial Top2 poisons for which several interaction models have been proposed. One intercalation model suggests that interactions with Top2 would mediate their entry into the ternary complex because intercalative poisons display strong affinities for Top2 even in the absence of DNA. Alternately, intercalation may precede DNA cleavage by Top2 with sites of cleavage mainly determined by the intercalator–DNA interactions.

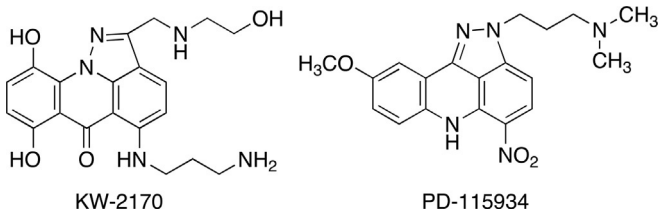
6.1 ACRIDINE DERIVATIVES

The intercalation concept was first introduced to explain the noncovalent binding of some acridine derivatives to DNA. Interest in these intercalators led to the development of a number of drugs,[128] starting with amsacrine (m-AMSA, Amsidyl®, Amsidine®, Amerkin®),[129] a drug used in the treatment of malignant lymphomas and acute non-lymphocytic leukemia[130,131] that was initially described in 1974, entered into clinical evaluation under National Cancer Institute (NCI) sponsorship in 1976, and approved for cancer therapy in several countries. The main mechanism of action of m-AMSA is the formation of a ternary complex with DNA and Top2, trapping the cleavable complex and inhibiting the religation step.[132] In addition to amsacrine, a large number of natural and synthetic acridines have been

tested as anticancer agents, and to date, a few molecules have entered clinical trials and have been approved for chemotherapy.[133] Asulacrine is a close analog with a broader spectrum of activity in experimental tumors but without improved clinical antitumor activity. DACA (XR5000) is an acridinecarboxamide and a mixed topoisomerase I/II poison that has undergone extensive clinical trials.[134,135] Hybrid compounds have also been designed that combine the acridine intercalating moiety with other groups that provide secondary interactions with the DNA minor groove.

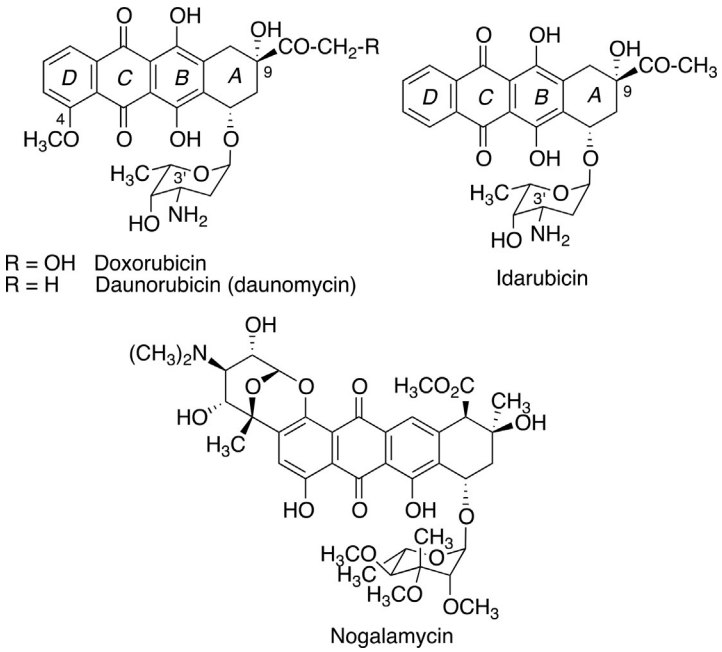


The pyrazoloacridone KW-2170 is a Top2 inhibitor of synthetic origin that entered phase II clinical trials.[136,137] The related pyrazoloacridine PD-115934, a dual Top1–Top2 inhibitor, also entered phase II clinical trials for several cancers.[138]



6.2 ANTHRACYCLINES AND RELATED COMPOUNDS

The anthracycline antibiotics, which were discussed as precursors of radical species in Section 3 of Chapter 4, represent the largest group of approved Top2 targeting agents. However, much of their earlier clinical investigation preceded the demonstration of their ability to promote topoisomerase-mediated DNA breaks, a property that does not fully explain the activity of the antracyclines unless other mechanisms are taken into account.[139] The first drugs targeting Top2 that were approved by the FDA were the anthracyclines doxorubicin (Adriamycin®, Rubex®, 1974) and daunomycin (Cerubidine®, 1979). The absence of a methoxy group in idarubicin (Idamycin®, 4-demethoxydaunorubicin) increased its liposolubility and cellular uptake. This compound is a potent bone marrow suppressant that was approved by the FDA in 1990. In 2009, it was also approved for the treatment of acute myeloid leukemia in adults.[140] Other anthracyclines that act primarily as Top2 catalytic inhibitors, such as aclarubicin, are mentioned in Section 7.1. The use of polymer conjugates and nanoparticles as vehicles for delivery of the anthracyclines is discussed in Chapter 13.



Anthracyclines are typical intercalating agents, and their tetracyclic A–D chromophore is oriented with its long axis perpendicular to the long axis of adjacent base pairs at the intercalation site. The daunorubicin–DNA complex is stabilized by the stacking interactions of rings B and C and by hydrogen bonding involving the hydroxyl group at C-9 of ring A, which acts as a donor to N-3 of guanine and as an acceptor from the amino group of the same guanine. Ring D protrudes into the major groove, and the amino sugar moiety lies in the minor groove and also takes part in hydrogen bonding with DNA (Figure 7.22).

As other antitumor intercalating agents, anthracyclines are Top2 poisons because of the formation of a stable drug–DNA–Top2 ternary complex and the consequent inhibition of replication and transcription. The sugar unit is crucial for the stabilization of this complex, and suppression of the C-4 methoxy and C-3′ amino groups increases Top2 inhibition.[141] In the case of nogalamycin, the presence of two sugar residues at both ends of the chromophore leads to a special way of interacting with DNA called threading intercalation,[142] in which one of the sugar units is located at the minor groove and the other at the major groove (Figure 7.23). The structure of the nogalamycin–DNA complex has been studied by X-ray diffraction.[143]

Mitoxantrone (Novantrone®) is a simplified analog of the anthracyclines that has a complex mechanism of action, including the generation of a stable drug–DNA–Top2 ternary complex. It has been approved for leukemias and advanced hormone refractory prostate cancer and is the only Top2 poison that has been approved for a noncancer indication, multiple sclerosis.[144] Isosteric substitution of one or more carbons of the benzene rings by nitrogen atoms has been employed as a strategy for the design of mitoxantrone analogs with geometries similar to those of the parent compounds but with increased affinity for DNA due to the presence of sites suitable for hydrogen bonding or ionic interactions. This increased affinity allows the suppression of the phenolic hydroxyls of mitoxantrone, which are responsible

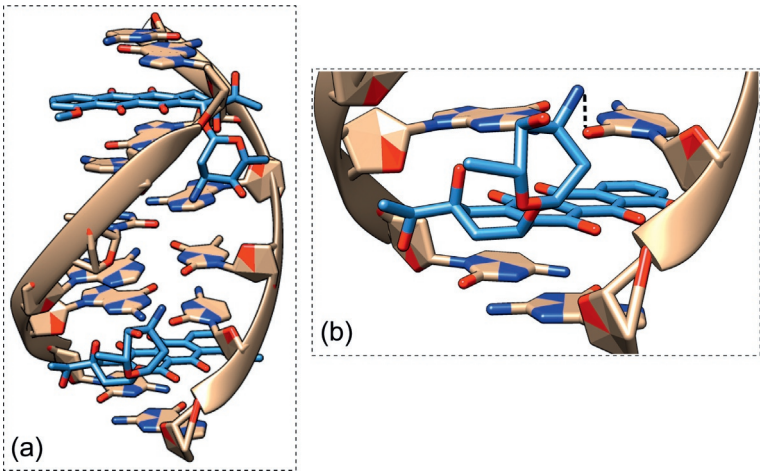


FIGURE 7.22

Intercalation of daunorubicin into DNA. Generated from Protein Data Bank reference 1D12 and displayed with Chimera 1.8.1.

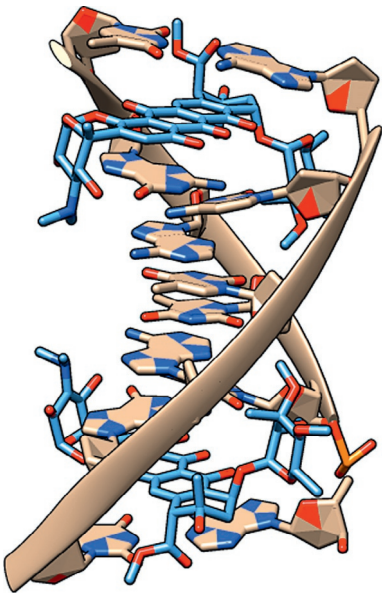
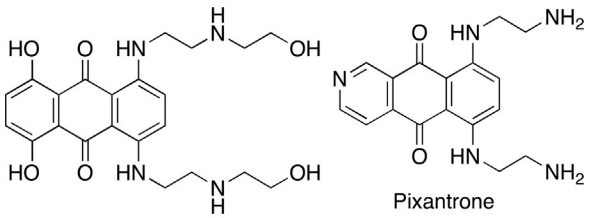


FIGURE 7.23

Interaction of nogalamycin with DNA, illustrating the threading intercalation process. Generated from Protein Data Bank reference 182D and displayed with Chimera 1.81.

for its chelating properties and therefore for its cardiotoxicity through oxygen radicals generated through Fenton chemistry. Based on this idea, some aza-bioisosters related to the anthracene-9,10-diones have been synthesized and screened *in vitro* and *in vivo* against a wide spectrum of tumor cell lines.[145,146]

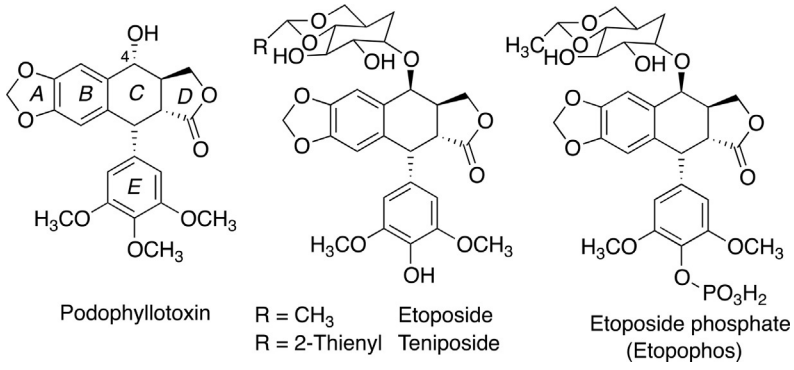
Among these compounds, pixantrone (Pixuvri®) has a high level of activity in blood-related tumors and entered phase III trials for the treatment of non-Hodgkin's lymphoma.[147,148] The FDA rejected its application in 2011 and required further clinical studies, but in May 2012 this drug received conditional marketing authorization from the European Commission. Interestingly, pixantrone was curative in some models of lymphoma and leukemia where currently marketed anthracyclines only prolonged survival, but it showed no measurable cardiotoxicity compared to them at equi-effective doses in animal models.[149] Another potential application of this drug is as an immunosuppressant in multiple sclerosis patients.[150] The mechanism of action of pixantrone involves intercalation with DNA and interaction with Top2, causing breaks in DNA strands.[151]



6.3 NON-INTERCALATING TOPOISOMERASE II POISONS

6.3.1 Etoposide and Its Analogs

The most important non-intercalating interfacial Top2 poisons are related to the natural lignan podophyllotoxin. Podophyllin resin, obtained by precipitating an alcoholic tincture of the rhizome of *Podophyllum peltatum*, has been used as a folk medicine for centuries. Its main active ingredient is a toxin lignan called podophyllotoxin (PPT, podofilox).[152] In the 1950s, a search was initiated to identify a more effective podophyllotoxin derivative[153] that eventually resulted in the development of a new class of anti-neoplastic agents that target Top2. The most important of these compounds are etoposide, teniposide, and etoposide phosphate (Etopophos®), which are semisynthetic derivatives of 4-epipodophyllotoxin that, in the absence of this enzyme, display little (if any) affinity for DNA.



Etoposide (VP-16-213)[154] is used mainly to treat testicular cancer that does not respond to other treatment and as a first-line treatment for small cell lung cancers, but it is also used to treat chorionic carcinomas, Kaposi's sarcoma, lymphomas, and malignant melanomas. A phosphate prodrug of etoposide (Etopophos®) has been used for antibody-directed enzyme prodrug therapy and is discussed in Section 2.4 of Chapter 13.

Initial nuclear magnetic resonance and binding studies of the binary enzyme–etoposide complex coupled with DNA functional studies (DNA cleavage) in the ternary complex suggested that the binding of etoposide to Top2 involved mainly interactions with the A, B, and E rings, whereas interactions with DNA in the ternary complex were due primarily to the D ring, with a contribution from the sugar moiety. Recently, the crystal structure of a large fragment of human Top2b complexed to DNA and to etoposide has been published (Figure 7.24). This study showed the rather different set of interactions depicted in Figure 7.25.[155] In agreement with this mode of binding, removal of the C-4 glycoside has little effect on induced DNA cleavage, and in fact, this group can be replaced by a polyamino side chain (see later discussion of TOP-53 and F14512).

Teniposide (Vumon®) is used less frequently, especially to treat lymphomas. DNA religation inhibition by etoposide seems to be due to inhibition of the release of ADP from the hydrolysis of ATP[156] and to its activation through oxidation–reduction reactions to produce derivatives that bind directly to DNA. It has been shown that the *O*-demethylated metabolite of etoposide **7.10**, which has the same potency as the parent drug, is subsequently oxidized to an *ortho*-quinone metabolite **7.12**, which is also a potent inhibitor of the Top2–DNA cleavable complex.[157] It has been proposed that the presence of free radical intermediates such as semiquinone **7.11** contributes to DNA strand breakage, which seems to be supported by the fact that the 4′-OH group of etoposide is essential for its activity as shown by the inactivity of its 4′-OMe derivative. On the other hand, etoposide is a substrate of myeloperoxidase, an enzyme with tyrosinase activity that catalyzes a one-electron oxidation to form the phenoxyl radical **7.13** (Figure 7.26). However, the formation of radicals **7.11** and

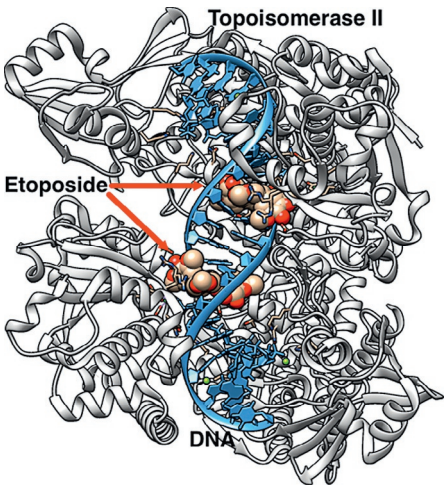


FIGURE 7.24

Structure of the ternary complex formed between human topoisomerase IIβ, DNA, and etoposide.

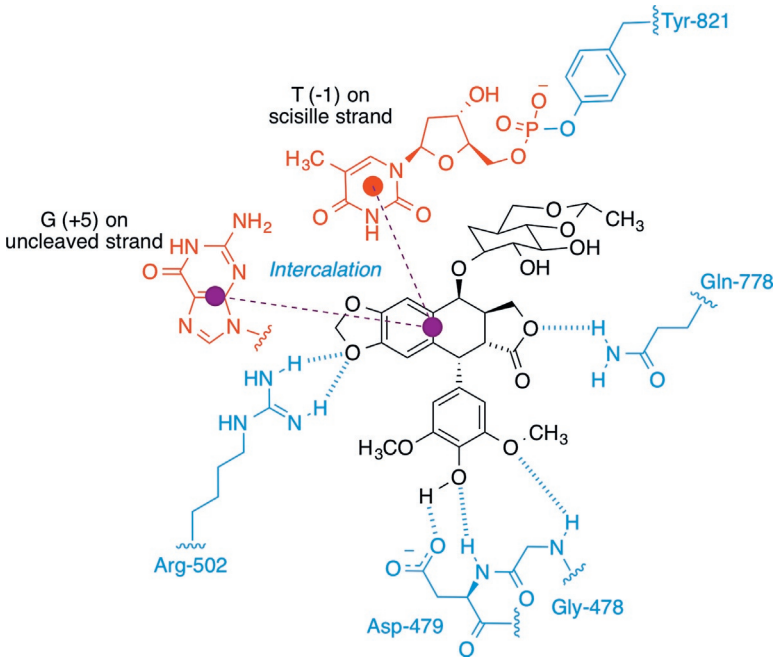


FIGURE 7.25

Main interactions in the etoposide–Top2–DNA ternary complex.

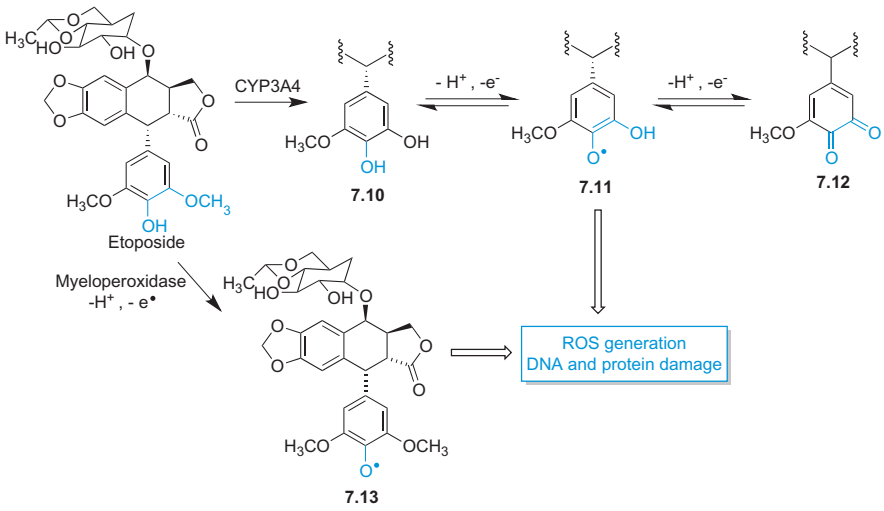


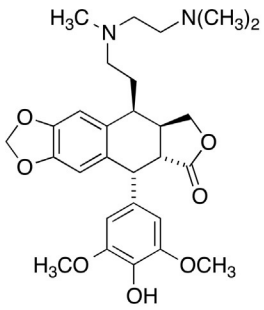
FIGURE 7.26

Reactive species generated in the metabolism of etoposide.

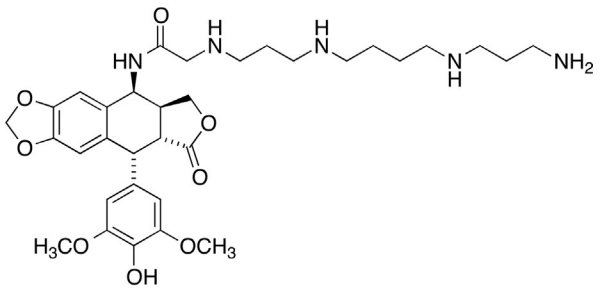
7.13 has been proposed to be related to the increased risk of secondary myeloid acute leukemia induced by long-term etoposide treatment.[158,159]

The presence of a polyamino side chain in the epipodophyllotoxin framework, as in the case of TOP-53,[160] turns etoposide into a DNA-binding drug[161] and enhances its potency.[162] Furthermore, the basic aminoalkyl side chain improves its solubility and allows drug association with phosphatidylserine, resulting in selective accumulation in lung. This drug is in phase I trials. In the case of F14512, the spermine moiety acts as a delivery vector into cancer cells via the polyamine transport system, an energy-dependent machinery frequently overactivated in cancer cells with a high demand for polyamines.[163] This compound is a potent antitumor agent targeting Top2 that entered phase I clinical studies in patients with relapsed or refractory acute myeloid leukemia.

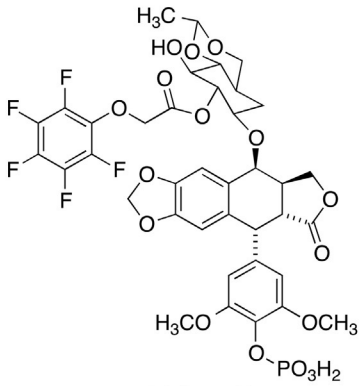
The phosphate prodrug tafluposide[164] is a lipophilic perfluorinated epipodophyllotoxin that has a dual Top1/2 inhibitory activity and high *in vivo* activity, which has progressed to phase II clinical trials for solid tumors.



TOP-53



F14512

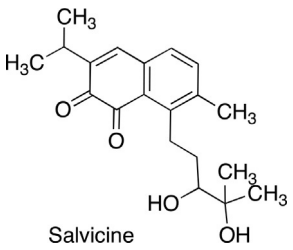


Tafluposide

6.3.2 Salvicine

Salvicine is a semisynthetic diterpenoid quinone compound obtained by structural modification of a natural lead isolated from the Chinese medicinal herb *Salvia prionitis*. It is a non-intercalative Top2 poison with a potent, broad-spectrum *in vitro* and *in vivo* antitumor activity and is in phase II

clinical trials. Salvicine is also an inhibitor of several resistance mechanisms, including MDR by down-regulating the expression of MDR-1 mRNA via the activation of c-jun and also DNA repair by the DNA protein kinase enzyme (see later).



Salvicine

Salvicine stabilizes DNA strand breaks through interactions with the enzyme by trapping the DNA–Top2 complex.[165] Molecular modeling studies predicted that salvicine binds to the ATP pocket in the ATPase domain and superimposes on the phosphate and ribose groups. Competition with ATP was confirmed experimentally.[166]

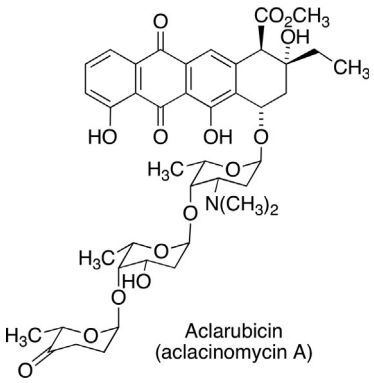
7 TOPOISOMERASE II CATALYTIC INHIBITORS

These compounds differ from topoisomerase poisons in that they do not stabilize the cleavable complex but, rather, act on other steps of the catalytic cycle. Catalytic inhibitors and Top2 poisons can exert synergic or antagonistic effects, depending on the treatment schedule. When cells are treated with high concentrations of drugs for short periods of time, competition is observed between both types of inhibitors because all available enzyme molecules are occupied by one of them, which brings about competition for the other. However, synergistic effects are observed after continuous exposure of cells to low concentrations of both types of inhibitors because under these conditions, not all the available enzyme molecules are occupied by one of the drugs, and some of them are therefore available to the other. These results resemble those observed under clinical conditions, and for this reason additive or synergistic effects are normally observed for both types of inhibitors under clinical settings. The therapeutic use of catalytic Top2 inhibitors as anticancer agents is limited to aclarubicin and the bis (dioxopiperazine) sobuzoxane.

7.1 INHIBITORS OF THE BINDING OF TOPOISOMERASE II TO DNA

7.1.1 Aclarubicin

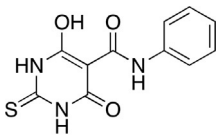
Although most anthracyclines act as specific Top2 poisons, some of them, such as aclarubicin (aclacinomycin A, Aclacin®), are Top2 catalytic inhibitors. This drug, which is clinically used in the treatment of acute myelocytic leukemia, is also a Top1 inhibitor at biologically relevant concentrations.[167]



Aclarubicin
(aclacinomycin A)

7.1.2 Merbarone

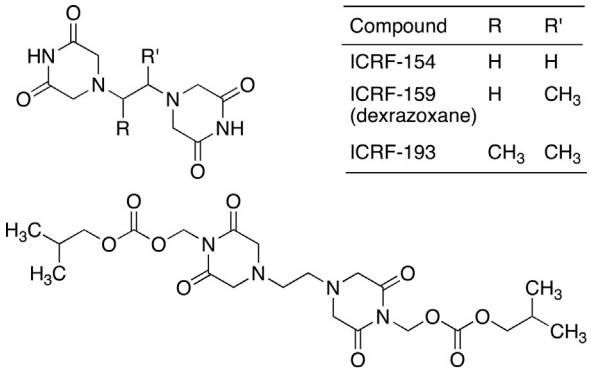
Merbarone is a derivative of thiobarbituric acid that was discovered during the course of a study of a large number of barbituric acid analogs by the NCI. This compound has been shown to inhibit the induction of DNA–Top2 cleavable complexes and has been tested clinically against a large number of tumors,[168] although it showed nephrotoxicity and poor anticancer activity.



Merbarone

7.1.3 Bis(dioxopiperazines)

These drugs[169] were introduced as chelating agents because they behave as prodrugs to EDTA amides, being useful cardioprotectors when associated with anthracyclines. Subsequently, it was shown that they also inhibit Top2 at a point upstream from the formation of the cleavable DNA–enzyme complex by stabilizing the closed-clamp form of Top2 as a post-passage complex.[170] This is achieved after interaction with the enzyme N-terminal domain by inhibiting its ATPase activity. The use of dexrazoxane hydrochloride (Totec®, Savene®) as cardioprotector against the cardiotoxic side effects of anthracyclines has been restricted because of its association with secondary malignancies.[171] This cardiomyocyte protection has been traditionally associated with the iron chelating activity displayed by the dexrazoxan hydrolysis product, as discussed in Chapter 4, but recent studies suggest that Top2 inhibition may have a role in this effect.[172] Sobuzoxane (MST-16, Perazolin®) has obtained approval in Japan for treatment of leukemia and lymphoma.



Compound	R	R'
ICRF-154	H	H
ICRF-159 (dexrazoxane)	H	CH_3
ICRF-193	CH_3	CH_3

Sobuzoxane (MST-16)

8 TELOMERASE INHIBITORS AND OTHER ANTICANCER APPROACHES TARGETING TELOMERES

Eukaryotic chromosomes are linear and have specialized ends called telomeres, which can be defined as regions of highly repetitive DNA at the end of a linear chromosome. Telomeric DNA consists of simple repetitive sequences with one strand G-rich relative to the other, C-rich, strand. Because DNA polymerases extend DNA chains in the 5′–3′ direction, the 3′–5′ chain (lead strand, which is replicated in the 5′–3′ direction) can be replicated to the end. However, the complementary chain (lagging strand) has to be replicated discontinuously, starting from a strand of nucleic acid called RNA primer that is built along the lagging strand's template. DNA polymerase is then able to use the free 3′-OH groups of the RNA primer to synthesize DNA in the 5′→3′ direction, resulting in a series of short fragments, called Okazaki fragments, containing the RNA primer attached to the 5′ end of a new DNA segment. The RNA primers are subsequently degraded and replaced by DNA segments (the gaps are filled in by polymerase), leading to the fusion of the successive segments to form a single DNA strand. At the end of this daughter strand, the RNA primer cannot be replaced by a new DNA segment because the required RNA primer has no place to bind the lagging strand's template and, when removed from polymerase, the daughter lagging strand is incomplete with loss of the final end (Figure 7.27a). Therefore, the telomeres of somatic cells are progressively shortened until they reach critically short lengths and cells enter p53- and Rb-dependent replicative senescence and, ultimately, apoptosis. This process is linked to aging. Telomerase is the ribonucleoprotein reverse transcriptase that creates single-stranded DNA using single-stranded RNA as a template. As previously mentioned, this enzyme adds a G-rich DNA sequence (in humans, AGGGTT repeats with an average of 5–15 kb) to the 3′ end of DNA lagging strands in the telomere regions, allowing their complete replication (Figure 7.27b).

Human telomerase is composed of at least two subunits, namely telomerase RNA (TR or TERC) and telomerase reverse transcriptase (TERT). The TERT subunit has a "mitten" structure that allows it

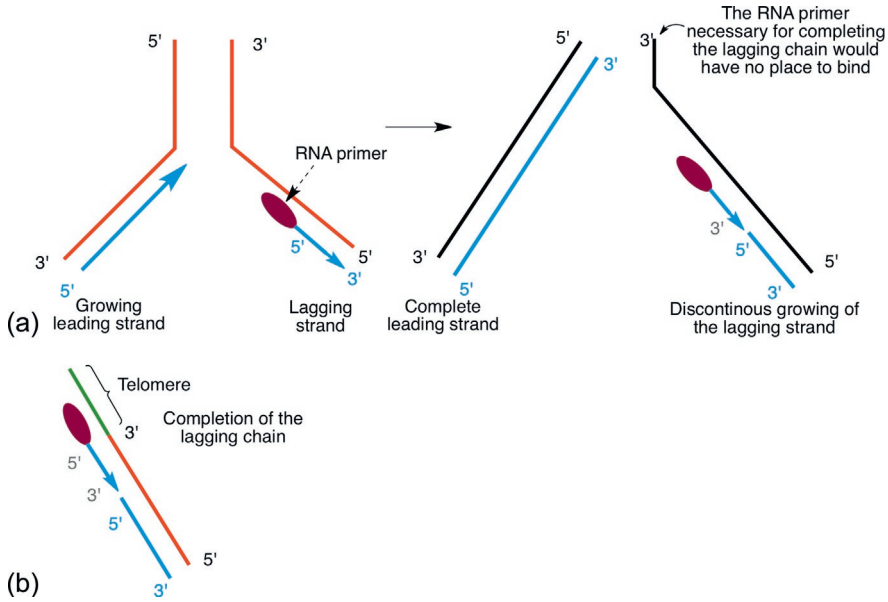


FIGURE 7.27

Addition of telomeres to the 3′ end of DNA by telomerase.

to wrap around the chromosome to add single-stranded telomere repeats, being able to confer infinite proliferative capacity on cells by extension of the G overhang. Cancer cells can exploit this unlimited proliferative potential that is a major and, to date, therapeutically unexploited phenotypic hallmark of cancer.[173–175]

The discovery of telomerase and its function in maintaining cellular immortalization by catalyzing telomere extension, together with the recognition of its selective expression in the majority of human cancers, stimulated interest in inhibiting its activity as an anticancer strategy, opening a new approach to DNA-targeted therapeutics. Telomerase-based cancer immunotherapy is a rapidly progressing field that has led to many compounds in advanced clinical trials for several cancers[176] and, in combination with other therapeutics, for drug-resistant tumors (see Chapter 12, Section 4.5).

Telomerase is expressed in 80–85% of cancer cells, whereas in most normal cells it is present in very low levels, if at all. In general, telomerase inhibition strategies may not be immediately effective in killing cancer cells, especially those with longer telomeres. The ideal conditions for telomerase inhibition to be useful are very short telomeres and high telomerase activity, as occurs in the aggressive prolymphocytic leukemia.

The main approaches that are being pursued for telomerase inhibition are as following:

1. Interaction with the telomerase substrate, namely the telomeric DNA template
2. Inhibition of the catalytic site of reverse transcriptase activity
3. Inhibition of the RNA domain template

Although many companies have developed major drug discovery programs screening for classical small-molecule inhibitors of telomerase activity, including focused sublibraries of reverse transcriptase inhibitors, a sufficiently potent and specific small-molecule inhibitor has not yet been found.

8.1 G-QUADRUPLEX LIGANDS

The substrate of telomerase is the single-stranded end of the telomeres, which must be in an unfolded, linear structure in order to fit the telomerase active site. The repeating G-rich sequences of telomeric DNA may form G-quadruplex structures in which four guanines are held in a planar rearrangement through the so-called Hoogsteen hydrogen bonding (Figure 7.28), with additional stabilization provided by a monovalent cation coordinated to the oxygen lone pairs. These secondary structures have been visualized with the selective antibody BG4.[177]

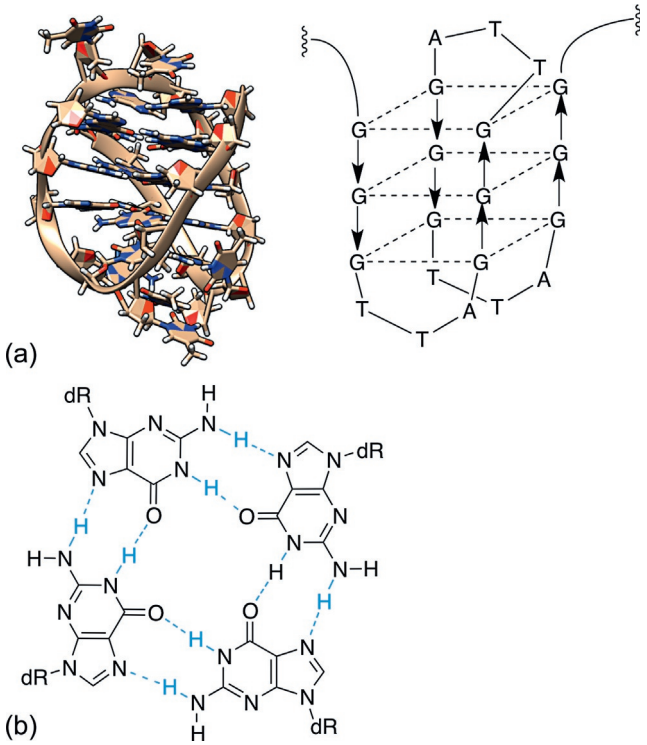
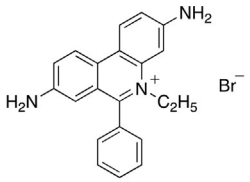


FIGURE 7.28

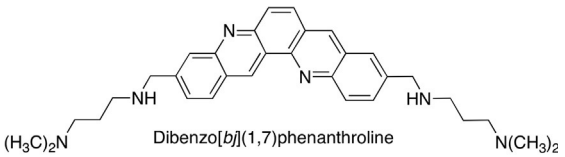
(a) Antiparallel DNA quadruplex. The three-dimensional structure was generated from Protein Data Bank reference 143D and displayed with Chimera 1.8.1. (b) Hydrogen bonding in DNA quadruplex.

G-quadruplex-forming sequences in gene promoters are linked to the transcriptional activity of the proximal gene, as has been reported for cancer-related genes such as *c-Myc*, *c-Kit*, *k-Ras*, *VEGF*, *PDGF*, *HIF1α*, *Bcl-2* and *RET*. Following these findings, any molecule capable of interacting with a specific G-quadruplex could modulate the transcriptional activity of the associated gene.[178]

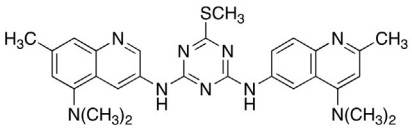
G-quadruplex ligands are often very similar to intercalating agents, and some of them, such as ethidium bromide, are prototype DNA intercalators. Nevertheless, the G-quadruplex nucleic acids have structural differences with the DNA double helix, and this provides a basis for selective recognition.[179] Most of the first-generation G-quadruplex ligands are polyaromatic molecules, such as dibenzophenanthroline and triazine derivatives, which interact by π-stacking and bear one or more substituents with positive charges, such as dibenzo[*bj*](1,7)phenanthroline and the quinoline-substituted compound 115405.[180] The natural product isolated from *Streptomyces anulatus* telomestatin is a potent G-quadruplex ligand that induces apoptosis of cancer cells through the displacement of POT1 (Protection of Telomere 1), a component of the protective telomeric protein shelterin[181] that modulates the activity of telomerase.[182] Shelterin is a protein complex with DNA remodeling activity that acts, together with several associated DNA repair factors, to change the structure of the telomeric DNA, thereby protecting chromosome ends. This complex is formed by six telomere-specific proteins that associate with the TTAGGG sequence. The shelterin subunits TRF1, TRF2, and POT1 recognize these repeats and are interconnected by shelterins TIN2, TPP1, and Rap1, forming a complex that allows cells to distinguish telomeres from sites of DNA damage. Without the protective activity of shelterin, telomeres are no longer hidden from the DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways. Many researchers suggest that shelterin dysfunction could play a major role in tumor formation. Specifically, induction of telomere uncapping due to abrogation of the TRF1 shelterin protein could be an alternative strategy to effectively kill cancer cells independently of the length of telomeres.[183]



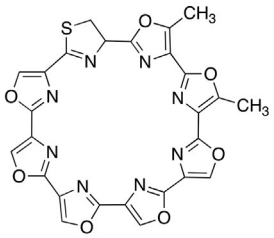
Ethidium bromide



Dibenzo[*bj*](1,7)phenanthroline

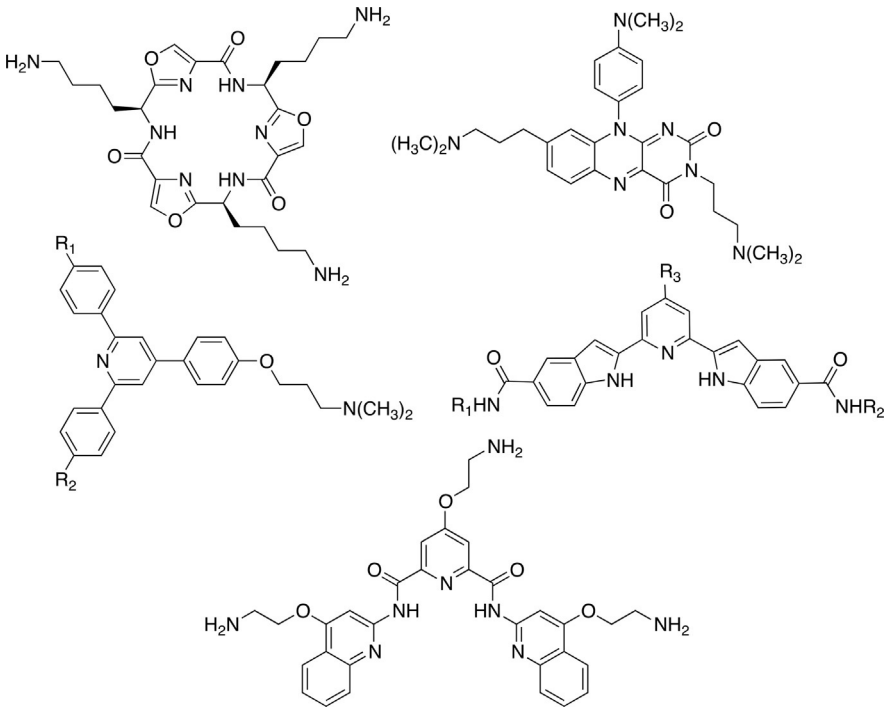


115405

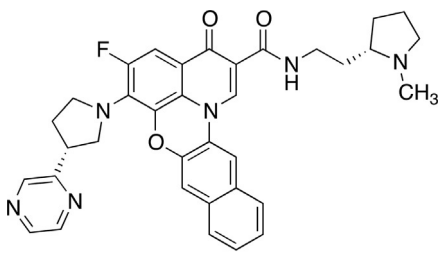


Telomestatin

It has been shown that between telomestatin and a G-quartet are located potassium or ammonium cations and, consequently, the rational design of G-quadruplex binding ligands would have to consider the monovalent cation coordination capabilities of the possible ligands.[184] Some examples of second-generation G-quadruplex stabilizers[185] are also shown here.



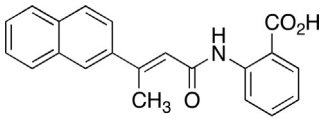
The fluoroquinolone derivative quarfloxin (quarfloxacin, CX-3543), originally derived from a group of fluoroquinolones that were shown to have dual Top2 and G-quadruplex interactions,[186] is the first therapeutic agent designed to target quadruplexes to enter clinical evaluation; it is in phase II trials for neuroendocrine tumors. Several powerful strategies to discover novel G-quadruplex ligands as anticancer drug candidates by screening of natural product extracts and structural optimization of previously identified typical compounds have been reviewed.[187]



Quarfloxin (Quarfloxazin, CX-3543)

8.2 INHIBITORS OF TELOMERASE REVERSE TRANSCRIPTASE

Because human telomerase reverse transcriptase (hTERT) acts as a reverse transcriptase, it is not surprising that some nucleosides that inhibit HIV-1 reverse transcriptase, such as AZT, are also telomerase inhibitors, although with poor activity and selectivity. BIBR1532 (Sirong®) is a mixed-type noncompetitive inhibitor with a binding site distinct from the sites for deoxyribonucleotides and the DNA primer, respectively. This compound defined a novel class of telomerase inhibitors with mechanistic similarities to non-nucleosidic inhibitors of HIV1 reverse transcriptase.[188] It inhibits cell proliferation in lung, breast, fibrosarcoma, and prostate cancer cells through induction of *p21*, coupled with down-regulation of *c-Myc* and *hTERT* transcription.[189] However, its lengthy lag period poses a problem because in some cases it may be greater than the life expectancy of the patient.

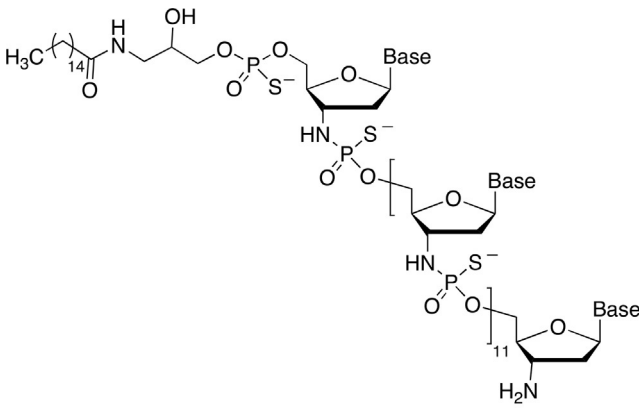


BIBR-1532

The selective inhibition of cancer cell growth through inhibition of hTERT by siRNA, antisense, or small-molecule inhibitors has been taken as a proof of principle that induction of telomere shortening is a viable therapeutic strategy in cancer.[190]

8.3 INHIBITORS OF THE RNA DOMAIN TEMPLATE

The human telomerase RNA component (hTR) has also been a target for antisense nucleotide approaches (for a more detailed discussion of antisense oligonucleotides, see Section 6 of Chapter 12). This hTR is not a messenger RNA and is not translated into a protein; therefore, these antisense oligomers will not have to compete with the ribosomal machinery and their toxicity will be low. Among the many antisense oligonucleotides targeted at hTR, the most advanced one is imetelstat (GRN-163 L), a 13-mer oligonucleotide belonging to the N3′–P5′ thiophosphoramidate (NPS) family that is covalently attached to a lipophilic palmitoyl moiety to enhance its potency.[191] Imetelstat has entered phase I and II clinical trials in patients with chronic lymphocytic leukemia, multiple myeloma (MM), and solid tumors,[192] but because of its hepatotoxicity, the FDA placed this drug on full clinical hold in 2014.



Imetelstat (GRN-163L)
Base sequence: 5'-Palm-TAGGGTTAGACAA-NH_2-3'

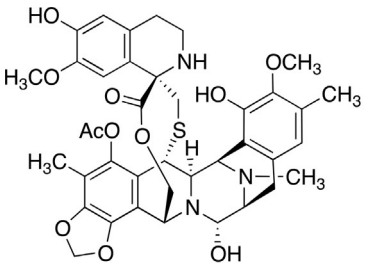
9 DNA REPAIR INHIBITORS

Several DNA repair pathways have evolved to maintain genomic integrity following endogenous or environmental DNA damage. In quiescent normal cells, these repair pathways are adequate to cope with the induced DNA damage before replication of the damaged DNA strand, but cancer cells proliferate rapidly, and this difference is partially responsible for the success of DNA-damaging anticancer therapy. Antitumor activity results from DNA lesions that persist during DNA replication (S phase of the cell cycle) and cause cell death or potentially lethal mutations. If DNA damage remains when cells divide (M phase), fatal chromosomal breakage takes place. These facts explain why chemotherapy is tolerable to most normal tissues but highly toxic to cancer cells. They also explain why the dose-limiting toxicities are frequently manifest in rapidly dividing normal cells such as the bone marrow and gut epithelium. Another explanation for the efficacy of DNA-damaging anticancer agents in specifically killing cancer cells is the imbalance of DNA damage signaling and repair pathways in cancer cells compared to normal cells. The DNA damage response (DDR) responsible for the signaling and repair of DNA lesions represents a barrier that is inactivated during tumor development, and loss of its elements, which is required to create the genomic instability to enable cancer to develop, renders tumors more susceptible to DNA damage.

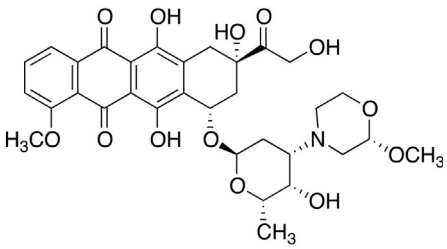
DNA repair inhibitors are promising as radio- or chemosensitizers and are discussed in detail in Chapter 14. We mention here the nucleotide excision repair (NER) process, which involves the action of at least 30 proteins in a "cut-and-paste"-like mechanism. DNA is repeatedly exposed in normal cells to exogenous (UV light, ionizing radiation, and chemicals) and to endogenous toxins produced as a consequence of natural metabolic processes. It is estimated that the average rate of damage is approximately 10^4 events per cell per day. Therefore, DNA repair systems are vital to preserve the integrity of the genome, but their protective effect can be a disadvantage in cancer cells by reducing the cytotoxicity of antitumor agents, which is a cause of resistance. Consequently, the different pathways of DNA repair have been studied as potential targets for improving cancer treatments.

Some new antitumor drugs are emerging from the study of these targets, particularly those involved in the repair of single strand damage.[193] NER is the most flexible of all DNA repair mechanisms because of its ability to eliminate many DNA lesions. It recognizes single-strand breaks and bulky helix-distorting changes in the DNA. The common denominator of the different types of damage induced by the numerous chemicals to which NER-deficient cells are sensitive seems to be the generation of bulky base adducts that cause significant helical distortion in addition to a change in the DNA chemistry. The tight binding into the minor groove of DNA of the anticancer drug ecteinascidin 743 (trabectedin, ET-743, Yondelis®) that was discussed in Section 4 of Chapter 6 provides one example. This drug induces a distortion of the helix that would normally trigger the NER process, but it traps the repair machinery as it attempts to remove the ET-743/DNA adducts, causing the endonuclease components to create lethal single-strand breaks in the DNA rather than repairing it.[194,195] Accordingly, this drug showed decreased activity in NER-deficient cell lines compared to NER-proficient cell lines.[196]

Nemorubicin, a member of the anthracyclin family of anticancer drugs (see Chapter 4, Section 3), also exerts its activity primarily by NER inhibition.



Trabectedin



Nemorubicin

REFERENCES

1 Graves DE, Velea LM. *Curr Org Chem* 2000;**4**:915.
2 Sobell HM. Premeltons: the emergence of dynamic coherent structures in DNA. http://members.localnet.com/∼sobell.
3 Berman HM, Young PR. *Annu Rev Biophys Bioeng* 1981;**10**:87.
4 Gago F. *Methods* 1998;**14**:277.
5 Kapur A, Beck JL, Sheil MM. *Rapid Commun Mass Spectrom* 1999;**13**:2489.
6 Braña MF, Cacho M, Gradillas A, de Pascual-Teresa B, Ramos A. *Curr Pharm Des* 2001;**7**:1745.
7 Garbett NC, Graves DE. *Curr Med Chem Anticancer Agents* 2004;**4**:149.
8 Canals A, Purciolas M, Aymamí J, Coll M. *Acta Crystallogr D Biol Crystallogr* 2005;**61**:1009.
9 Froelich-Ammon SJ, Patchan MW, Osheroff N, Thompson R. *J Biol Chem* 1995;**270**:14988.
10 Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, Frei E. *Int J Cancer* 2003;**107**:885.
11 Poljakova J, Frei E, Gomez J, Aimova D, Eckschlager T, Hrabeta J, et al. *Cancer Lett* 2007;**252**:270.
12 Stiborová M, Poljaková J, Martınková E, Borek-Dohalská L, Eckschlager T, Kizek R, et al. *Interdiscipl Toxicol* 2011;**4**:98.
13 Auclair C, Paoletti C. *J Med Chem* 1981;**24**:289.
14 Stiborová M, Sejbal J, Borek-Dohalská L, Aimová D, Poljaková J, Forsterová K, et al. *Cancer Res* 2004;**64**:8374.
15 Auclair C. *Arch Biochem Biophys* 1987;**259**:1.
16 Rouëssé J, Spielmann M, Turpin F, Le Chevalier T, Azab M, Mondésir JM. *Eur J Cancer* 1993;**29A**:856.
17 Awada A, Giacchetti S, Gerard B, Eftekhary P, Lucas C, De Valeriola D, et al. *Ann Oncol* 2002;**13**:1925.
18 Pivot X, Awada A, Gedouin D, Kerger J, Rolland F, Cupissol D, et al. *Ann Oncol* 2003;**14**:373.
19 Poddevin B, Riou JF, Lavelle F, Pommier Y. *Mol Pharmacol* 1993;**44**:767.
20 Abigerges D, Armand JP, Chabot GG, Bruno R, Bissery MC, Bayssas M, et al. *Anticancer Drugs* 1996;**7**:166.
21 Miller CM, McCarthy FO. *RSC Adv* 2012;**2**:8883.
22 Gallego J, Ortiz AR, de Pascual-Teresa B, Gago F. *J Comput Aided Mol Des* 1997;**11**:114.
23 Takusagawa F, Wen L, Chu W, Li Q, Carlson RG, Takusagawa KT, et al. *Biochemistry* 1996;**35**:13240.
24 Kamitori S, Takusagawa F. *J Am Chem Soc* 1994;**116**:415.
25 For a review of the binding of actinomycin D to unstructured, single-stranded DNA, see Yoo H, Rill RL. *J Mol Recogn* 2001;**14**:145.
26 Bailey SA, Graves DE, Rill R. *Biochemistry* 1994;**33**:11493.
27 Sobell HM. *Proc Natl Acad Sci U S A* 1985;**82**:5328.

28 Ewesuedo RB, Iyer L, Das S, Koenig A, Mani S, Vogelzang NJ, et al. *J Clin Oncol* 2001;**19**:2084.
29 Ishida K, Asao T. *Nucl Acids Symp Ser* 1999;**42**:129.
30 Yoshida M, Kabe Y, Wada T, Asai A, Handa H. *Mol Pharmacol* 2008;**73**:987.
31 Rosell R, Carles J, Abad A, Ribelles N, Barnadas A, Benavides A, et al. *Invest New Drugs* 1992;**10**:171.
32 Allen SL, Budman DR, Fusco D, Kolitz J, Kreis W, Schulman P, et al. *Proc Am Assoc Cancer Res* 1994;**35**:325.
33 Braña MF, Ramos A. *Curr Med Chem Anticancer Agents* 2001;**1**:237.
34 De Isabella P, Zunino F, Capranico G. *Nucleic Acids Res* 1995;**23**:223.
35 Vaquero A, Portugal J. *Eur J Biochem* 1998;**251**:435.
36 Asai G, Yamamoto N, Toi M, Shin E, Nishiyama K, Sekine T, et al. *Cancer Chemother Pharmacol* 2002;**49**:468.
37 For a review of chartreusin, elsamicin A, and related anticancer antibiotics, see Portugal J. *Curr Med Chem Anticancer Agents* 2003;**3**:411.
38 Spielmann HP, Wemmer DE, Jacobsen JP. *Biochemistry* 1995;**34**:8542.
39 Lambert B, Rocques BP, Le Pecq J-B. *Nucleic Acids Res* 1988;**16**:1063.
40 Gao Q, Williams LD, Egli M, Rabinovich D, Le Chen S, Qugley G, et al. *Proc Natl Acad Sci U S A* 1991;**88**:2422.
41 Okamaoto M, Ohsato T, Nakada K, Isobe K, Spelbrink JN, Hayashi J, et al. *Curr Genet* 2003;**43**:364.
42 Braña MF, Castellano JM, Morán M, Pérez de Vega MJ, Romerdahl CR, Qian X-D, et al. *Anticancer Drug Des* 1993;**8**:257.
43 Villalona-Calero MA, Eder JP, Toppmeyer DL, Allen LF, Fram R, Velagapudi R, et al. *J Clin Oncol* 2001;**19**:857.
44 Bailly C, Carrasco C, Joubert A, Bal C, Wattez N, Hildebrand MP, et al. *Biochemistry* 2003;**42**:4136.
45 Wadler S, Tenteromano L, Cazenave L, Sparano JA, Greenwald ES, Rozenblit A, et al. *Cancer Chemother Pharmacol* 1994;**34**:266.
46 Gradishar WJ, Vogelzang NJ, Kilton LJ, Leibach SJ, Rademaker AW, French S, et al. *Invest New Drugs* 1995;**13**:171.
47 Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, et al. *Cancer Res* 2005;**65**:9047.
48 Cuesta-Seijo JA, Sheldrick GM. *Acta Crystallogr D Biol Crystallogr* 2005;**61**:442.
49 Leng F, Chaires JB, Waring MJ. *Nucleic Acids Res* 2003;**31**:6191.
50 Pfoh R, Cuesta-Seijo JA, Sheldrick GM. *Acta Crystallogr F* 2009;**65**:660.
51 Sordet O, Khan QA, Kohn KW, Pommier Y. *Curr Med Chem Anticancer Agents* 2003;**3**:271.
52 Denny WA. *Exp Opin Emerg Drugs* 2004;**9**:105.
53 For a monograph, see Pommier Y, editor. *DNA topoisomerases and cancer*. New York: Humana Press; 2012.
54 Kaufmann WK. *Proc Soc Exp Biol Med* 1998;**217**:327.
55 Hengstler JG, Heimerdinger CK, Schiffer IB, Gebhard S, Sagemüller J, Tanner B, et al. *EXCLI J* 2002;**1**:8.
56 Sordet O, Abby Goldman A, Pommier Y. *Mol Cancer Ther* 2006;**5**:3139.
57 Burden DA, Osheroff N. *Biochem Biophys Acta* 1998;**1400**:139.
58 For representative reviews of catalytic topoisomerase inhibitors, see. (a) Holden JA. *Curr Med Chem Anticancer Agents* 2001;**1**:1; (b) Larsen AK, Escargueil AE, Skladanowski A. *Pharmacol Ther* 2003;**99**:167.
59 Andoh T, Ishida R. *Biochim Biophys Acta* 1998;**1400**:155.
60 Hartman G, Pommier Y. In: Chabner BA, Longo DL, editors. *Cancer chemotherapy and biotherapy*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2010 [chapter 19].
61 Denny WA. *Expert Opin Invest Drugs* 1997;**6**:1845.
62 Denny WA, Baguley BC. *Curr Topics Med Chem* 2003;**3**:339.
63 For general reviews, see. (a) Bailly C. *Curr Med Chem* 2000;**7**:39; (b) Pommier Y. *Nat Rev Cancer* 2006;**6**:789; (c) Pommier Y. *Chem Rev* 2009;**109**:2894.
64 Burden DA, Osheroff N. *Biochim Biophys Acta* 1998;**1400**:139.

65 Pommier Y. *ACS Chem Biol* 2013;**8**:82.
66 For a monograph, see Adams VR, Burke TG, editors. *Camptothecins in cancer therapy*. Totowa, NJ: Humana Press; 2005.
67 For reviews of the role of camptothecin analogs in the treatment of cancer see: (a) Venditto VJ, Simanek EE. *Mol Pharm* 2010;**7**:307; (b) Giannini G. In: Hanessian S, editor. *Natural products in medicinal chemistry*. Weinheim, Germany: Wiley-VCH; 2014 [chapter 5]
68 Stewart DJ. *Oncologist* 2004;**9**:43.
69 Bencharit S, Morton CL, Howard-Williams EL, Danks MK, Potter PM, Redinbo MR. *Nat Struct Biol* 2002;**9**:337.
70 Nakanishi Y, Takayama K, Takano K, Inoue K, Osaki S, Wataya H, et al. *Am J Clin Oncol* 1999;**22**:399.
71 Toffoli G, Cecchin E, Corona G, Boiocchi M. *Curr Med Chem Anticancer Agents* 2003;**3**:225.
72 Moukarskaya J, Verschraegen C. *Hematol Oncol Clin North Am* 2012;**26**:507.
73 https://clinicaltrials.gov/show/NCT01832298.
74 Burke TG, Mi Z. *Anal Biochem* 1993;**212**:285.
75 Burke TG. *Ann N Y Acad Sci* 1996;**803**:29.
76 Pizzolato JF, Saltz LB. *Lancet* 2003;**361**:2235.
77 Tangirala RS, Antony S, Agama K, Pommier Y, Anderson BD, Bevins R, et al. *Bioorg Med Chem* 2006;**14**:6202.
78 Bailly C. *Crit Rev Oncol Hematol* 2003;**45**:91.
79 Príncipe P, Troconiz IF, Segura C, Garrido MJ, Cendros JM, Peraire C, et al. *J Clin Oncol* 2004;**22**:2046.
80 Trocóniz IF, Cendrós JM, Soto E, Pruñonosa J, Pérez-Mayoral A, Peraire C, et al. *Cancer Chemother Pharmacol* 2012;**70**:239.
81 Scott L, Soepenberg O, Verweij J, de Jonge MJ, Th Planting AS, McGovern D, et al. *Ann Oncol* 2007;**18**:569.
82 Kroep JR, Gelderblom H. *Exp Opin Invest Drugs* 2009;**18**:69.
83 Takagi K, Dexheimer TS, Redon C, Sordet O, Agama K, Lavielle G, et al. *Mol Cancer Ther* 2007;**6**:3229.
84 Bom D, Curran DP, Kruszewski S, Zimmer SG, Strode JT, Kohlhagen G, et al. *J Med Chem* 2000;**43**:3970.
85 http://www.medbravo.org/study?study=NCT01202370.
86 Munster PN, Daud AI. *Expert Opin Invest Drugs* 2011;**20**:1565.
87 Ulukan H, Swaan PW. *Drugs* 2002;**62**:2039.
88 García-Carbonero R, Supko JG. *Clin Cancer Res* 2002;**8**:641.
89 Clark JW. *Expert Opin Invest Drugs* 2006;**15**:71.
90 Takimoto CH, Thomas R. *Ann N Y Acad Sci* 2000;**922**:224.
91 Kehrer DFS, Bos AM, Verweij J, Groen HJ, Loos WJ, Sparreboom A, et al. *J Clin Oncol* 2002;**20**:1222.
92 Ajani J, Takimoto C, Becerra C, Silva A, Baez L, Cohn A, et al. *Invest New Drugs* 2005;**23**:479.
93 Abou-Alfa GK, Letourneau R, Harker G, Modiano M, Hurwitz H, Tchekmedyian NS, et al. *J Clin Oncol* 2006;**24**:4441.
94 Chatterjee A, Digumarti R, Katneni K, Upreti VV, Mamidi RN, Mullangi R, et al. *J Clin Pharmacol* 2005;**45**:453.
95 Crul M. *Curr Opin Invest Drugs* 2003;**4**:1455.
96 Pecorelli S, Ray-Coquard I, Tredan O, Colombo N, Parma G, Tisi G, et al. *Ann Oncol* 2010;**21**:759.
97 For a review of namitecan, see Beretta GL, Zuco V, De Cesare M, Perego P, Zaffaroni N. *Curr Med Chem* 2012;**19**:3488.
98 Wadkins RM, Potter PM, Vladu B, Marty J, Mangold G, Weitman S, et al. *Cancer Res* 1999;**59**:3424.
99 Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin AB, Stewart L. *Proc Natl Acad Sci U S A* 2002;**99**:15387.
100 Pommier Y, Marchand C. *Nat Rev Drug Discov* 2012;**11**:25.
101 Wadkins RM, Bearss D, Manikumar G, Wani MC, Wall ME, Von Hoff DD. *Curr Med Chem Anticancer Agents* 2004;**4**:327.

102 Staker BL, Feese MD, Cushman M, Pommier Y, Zembower D, Stewart L, et al. *J Med Chem* 2005;**48**:2336.
103 Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin AB, Stewart L. *Proc Natl Acad Sci U S A* 2002;**99**:15387.
104 Meng L, Liao Z, Pommier Y. *Curr Topics Med Chem* 2003;**3**:305.
105 Teicher BA. *Biochem Pharmacol* 2008;**75**:1262.
106 Prudhomme M. *Curr Med Chem Anticancer Agents* 2004;**4**:509.
107 Gani OA, Engh RA. *Nat Prod Rep* 2010;**27**:489.
108 Ruegg UT, Burgess GM. *Trends Pharmacol Sci* 1989;**10**:218.
109 Goel S, Wadler S, Hoffman A, Volterra F, Baker C, Nazario E, et al. *Invest New Drugs* 2003;**21**:103.
110 Urasaki Y, Laco G, Takebayashi Y, Bailly C, Kohlhagen G, Pommier Y. *Cancer Res* 2001;**61**:504.
111 Bailly C, Dassonneville L, Colson P, Houssier C, Fukasawa K, Nishimura S, et al. *Cancer Res* 1999;**59**:2853.
112 (a) Yoshinari T, Ohkubo M, Fukasawa K, Egashira S, Hara Y, Matsumoto M, et al. *Cancer Res* 1999;**59**:4271; (b) Meng L-H, Liao Z-Y, Pommier Y. *Curr Top Med Chem* 2003;**3**:305.
113 (a) Ohkubo M, Nishimura T, Honma T, Nishimura I, Ito S, Yoshinari T, et al. *Bioorg Med Chem Lett* 1999;**9**:3307; (b) Saif MW, Diario RB. *Clin Colorectal Cancer* 2005;**5**:27.
114 Hurwitz HI, Cohen RB, McGovren JP, Hirawat S, Petros WP, Natsumeda Y, et al. *Cancer Chemother Pharmacol* 2007;**59**:139.
115 Antony S, Agama KK, Miao ZH, Takagi K, Wright MH, Robles AI, et al. *Cancer Res* 2007;**67**:10397.
116 Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, et al. *Nat Rev Cancer* 2008;**8**:957.
117 Kurtzberg LS, Roth S, Krumbholz R, Crawford J, Bormann C, Dunham S, et al. *Clin Cancer Res* 2011;**17**:2777.
118 Bailly C. *Curr Med Chem Anticancer Agents* 2004;**4**:363.
119 Baunbæk D, Trinkler N, Ferandin Y, Lozach O, Ploypradith P, Somsak Rucirawat S, et al. *Mar Drugs* 2008;**6**:514.
120 Quesada AR, García-Grávalos MD, Fernández-Puentes JL. *Br J Cancer* 1996;**74**:677.
121 Plisson F, Huang XC, Zhang H, Khalil Z, Capon RJ. *Chem Asian J* 2012;**7**:1616.
122 Ishibashi F, Tanabe S, Oda T, Iwao M. *J Nat Prod* 2002;**65**:500.
123 Facompré M, Tardy C, Bal-Mahieu C, Colson P, Pérez C, Manzanares I, et al. *Cancer Res* 2003;**63**:7392.
124 Marco E, Laine W, Tardy C, Lansieaux A, Iwao M, Ishibashi F, et al. *J Med Chem* 2005;**48**:3796.
125 Caldecott KW. *Nat Rev Genet* 2008;**9**:619.
126 Beretta GL, Cossa G, Gatti L, Zunino F, Perego P. *Curr Med Chem* 2010;**17**:1500.
127 Deweese JE, Osheroff N. *Nucleic Acids Res* 2009;**37**:738.
128 For reviews, see. (a) Demeunynck M, Charmantray F, Martelli A. *Curr Pharm Des* 2001;**7**:1703; (b) Belmont P, Bosson J, Godet T, Tiano M. *Anticancer Agents Med Chem* 2007;**7**:139
129 Ketron AC, Denny WA, Graves DE, Osheroff N. *Biochemistry* 2012;**51**:1730.
130 Sung WJ, Kim DH, Sohn SK, Kim JG, Baek JH, Jeon SB, et al. *Jpn J Clin Oncol* 2005;**35**:612.
131 Burnett A, Wetzler M, Lowenberg B. *J Clin Oncol* 2011;**29**:487.
132 Nelson EM, Tewey KM, Liu LF. *Proc Natl Acad Sci U S A* 1984;**81**:1361.
133 Demeunynck M. *Exp Opin Ther Pat* 2004;**14**:55.
134 Twelves C, Campone M, Coudert B, Van den Bent M, de Jonge M, Dittrich C, et al. *Ann Oncol* 2002;**13**:777.
135 Dittrich C, Coudert B, Paz-Ares L, Caponigro F, Salzberg M, Gamucci T, et al. *Eur J Cancer* 2003;**39**:330.
136 Verschraegen CF. *IDrugs* 2002;**5**:1000.
137 Saeki T, Eguchi K, Takashima S, Sugiura T, Hida T, Horikoshi N, et al. *Cancer Chemother Pharmacol* 2004;**54**:459.
138 Bastasch M, Panella TJ, Kretzschmer SL, Graham D, Mayo M, Williamson S. *Invest New Drugs* 2002;**20**:339.
139 Plastaras JP, Dedon PC, Marnett LJ. *Biochemistry* 2002;**41**:5033.
140 Datsch DC, Gleseler F. *DNA Repair* 2007;**6**:1618.

141 Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. *Pharmacol Rev* 2004;**56**:185.
142 Takenaka S, Takagi M. *Bull Chem Soc Jpn* 1999;**72**:327.
143 Smith CK, Davies GJ, Dodson EJ, Moore MH. *Biochemistry* 1995;**34**:415.
144 Wierzbowska A, Robak T, Pluta A, Wawrzyniak E, Cebula B, Holowiecki J, et al. *Eur J Haematol* 2008;**80**:115.
145 Krapcho AP, Maresch MJ, Hacker MP, Hazelhurst L, Menta E, Oliva A, et al. *Curr Med Chem* 1995;**2**:803.
146 Sissi C, Palumbo M. *Curr Top Med Chem* 2004;**4**:219.
147 Borchmann P, Reiser M. *IDrugs* 2003;**6**:486.
148 Borchmann P, Schnell R. *Expert Opin Invest Drugs* 2005;**14**:1055.
149 Beggiolin G, Crippa L, Menta E, Manzotti C, Cavalletti E, Pezzoni G, et al. *Tumori* 2001;**6**:407.
150 Gonsette RE. *J Neural Sci* 2004;**223**:81.
151 De Isabella P, Palumbo M, Sissi C. *Biochem Pharmacol* 1997;**53**:161.
152 For reviews of phodophyllotoxin, see. (a) Gordaliza M, Castro MA, Miguel del Corral JM, San Feliciano A. *Curr Pharm Design* 2000;**6**:1811; (b) Gordaliza M, Miguel del Corral JM, Castro MA, García-García PA, Gómez-Zurita MA. *Toxicon* 2003;**44**:441; (c) Liu Y-Q, Yang L, Tian X. *Curr Bioact Comp* 2007;**3**:37.
153 Hande KR, Eur J. *Cancer* 1998;**34**:1514.
154 Baldwin EL, Osheroff N. *Curr Med Chem Anticancer Agents* 2005;**5**:363.
155 Wu CC, Li T-K, Farh L, Lin L-Y, Lin T-S, Yu Y-J, et al. *Science* 2011;**333**:459.
156 Morris SK, Lindsley JE. *J Biol Chem* 1999;**274**:30690.
157 Gantchev TG, Hunting D. *J Mol Pharmacol* 1998;**53**:422.
158 Kagan VE, Yalowich JC, Borisenko GG, Tyurina YY, Tyurin VA, Thampatty P, et al. *Mol Pharmacol* 1999;**56**:494.
159 Zheng N, Felix CA, Pang S, Boston R, Moate P, Scavuzzo J, et al. *Clin Cancer Res* 2004;**10**:2977.
160 Byl JA, Cline SD, Utsugi T, Kobunai T, Yamada Y, Osheroff N. *Biochemistry* 2001;**40**:712.
161 Barret JM, Kruczynski A, Vispe S, Annereau JP, Brel V, Guminski Y, et al. *Cancer Res* 2008;**68**:9845.
162 Gentry AC, Pitts SL, Jablonsky MJ, Bailly C, Graves DE, Osheroff N. *Biochemistry* 2011;**50**:3240.
163 Barret JM, Kruczynski A, Vispé S, Annereau JP, Brel V, Guminski Y, et al. *Cancer Res* 2008;**68**:9845.
164 Kluza J, Mazinghien R, Irwin H, Hartley JA, Bailly C. *Anticancer Drugs* 2006;**17**:155.
165 Meng LH, Zhang JS, Ding J. *Biochem Pharmacol* 2001;**62**:733.
166 Hu C-X, Zuo ZL, Xiong B, Ma J-G, Geng M-Y, Lin LP, et al. *Mol Pharmacol* 2006;**70**:1593.
167 Niltiss JL, Pourquier P, Pommier Y. *Cancer Res* 1997;**57**:4564.
168 Kraut EH, Bendetti J, Balcerzak SP, Doroshow JH. *Invest New Drugs* 1992;**10**:347.
169 Andoh T. *Biochimie* 1998;**80**:235.
170 Hasinoff PB, Abram ME, Bernabe M, Khélifa T, Allan WP, Yalowich JC. *Mol Pharmacol* 2001;**59**:453.
171 Salzer WL, Devidas M, Carroll WL, Winick N, Pullen J, Hunger SP, et al. *Leukemia* 2010;**24**:355.
172 Vavrova A, Jansova H, Mackova E, Machacek M, Haskova P, Tichotova L, et al. *PLoS One* 2013;**8**:e76676.
173 Neidle S, Parkinson G. *Nature Rev Drug Discov* 2002;**1**:383.
174 Mergny J-L, Riou J-F, Mailliet P, Teulade-Fichou M-P, Gilson E. *Nucl Acid Res* 2002;**30**:839.
175 Cunningham AP, Love WK, Zhang RW, Andrews LG, Tollefsbol TO. *Curr Med Chem* 2006;**13**:2875.
176 For selected reviews of telomerase inhibitors, see. (a) Huo L, Tang JWS, Huang J, Huang P, Huang C, Kung H, et al. *Cell Mol Immun* 2006;**3**:1; (b) Liu J-P, Chen W, Schwarer AP, Li H. *Biochim Biophys Acta* 2010;**1805**:35; (c) Sprouse AA, Steding CE, Herbert B-S. *J Cell Mol Med* 2012;**16**:1.
177 Biffi G, Tannahill D, McCafferty J, Balasubramanian S. *Nature Chem* 2013;**5**:140.
178 De Cian A, Lacroix L, Douarre C, Temime-Smaali N, Trentesaux C, Riou J-F, et al. *Biochimie* 2008;**90**:131.
179 Kerwin SM. *Curr Pharm Des* 2000;**6**:441.
180 Riou JF, Guittat L, Mailliet P, Laoui A, Renou E, Petitgenet O, et al. *Proc Natl Acad Sci U S A* 2002;**99**:2672.
181 (a) Liu D, O'Connor MS, Qin J, Songyang Z. *J Biol Chem* 2004;**279**:51338; (b) De Lange T. *Gen Devel* 2005;**19**:2100.

182 Palm W, de Lange T. *Annu Rev Genet* 2008;**42**:301.
183 Martínez P, Blasco MA. *Nat Rev Cancer* 2011;**11**:161.
184 Rosu F, Gabelica V, Smargiasso N, Mazzucchelli G, Shin-Ya K, De Paw E. *J Nucleic Acids* 2010, article id 121259.
185 (a) Rodriguez R, Müller S, Yeoman JA, Trentesaux C, Riou J-F, Balasubramanian S. *J Am Chem Soc* 2008;**130**:15758; (b) Balasubramanian S, Neidle S. *Curr Opin Chem Biol* 2009;**13**:345.
186 Balasubramanian S, Hurley LH, Neidle S. *Nat Rev Drug Discov* 2011;**10**:261.
187 Li Q, Xiang JF, Zhang H, Tang YL. *Curr Pharm Des* 2012;**18**:1973.
188 Pascolo E, Wenz C, Lingner J, Hauel N, Priepke H, Kaufmann I, et al. *J Biol Chem* 2002;**277**:15566.
189 Bashash D, Ghaffari SH, Zaker F, Hezave K, Kazerani M, Ghavamzadeh A, et al. *Cancer Invest* 2012;**30**:57.
190 (a) Rankin AM, Faller DV, Spanjaard RA. *Anticancer Drugs* 2008;**19**:329; (b) For a review, see Buseman CM, Wright WE, Shay JW. *Mutat Res* 2012;**730**:90.
191 Herbert BS, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, et al. *Oncogene* 2005;**24**:5262.
192 Puri N, Girard J. *J Cancer Sci Ther* 2013;**5**:1000e127.
193 Berthet N, Boturyn D, Constant J-F. *Expert Opin Ther Patents* 1999;**9**:401.
194 Takebayashi K, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, et al. *Nat Med* 2001;**7**:961.
195 Zewail-Foote M, Ven-Shun L, Kohn H, Bearss D, Guzmán M, Hurley LH. *Chem Biol* 2001;**135**:1.
196 Damia G, Silvestri S, Carrassa L, Filiberti L, Faircloth GT, Liberi G, et al. *Int J Cancer* 2001;**92**:583.

CHAPTER

EPIGENETIC THERAPY OF CANCER

8

CONTENTS

1 INTRODUCTION

The development and progression of cancer involves both genetic and epigenetic changes leading to the alteration of gene expression and cell phenotype. The term "epigenetics" refers to heritable phenotypes resulting from changes in chromosomes without alterations in the primary DNA sequence.[1]

Chromatin is the complex of DNA and protein that makes up the chromosome. The human genome corresponds to 3 billion base pairs of the DNA double helix, two copies of which make up to 2 m of DNA chains that have to be stored within the tiny micron-sized nucleus of each cell. The smallest structural units in chromatin are nucleosomes ("10-nm fibers"), which are formed by approximately 200 DNA base pairs wrapped around a core of eight DNA-associated proteins called histones

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00008-9

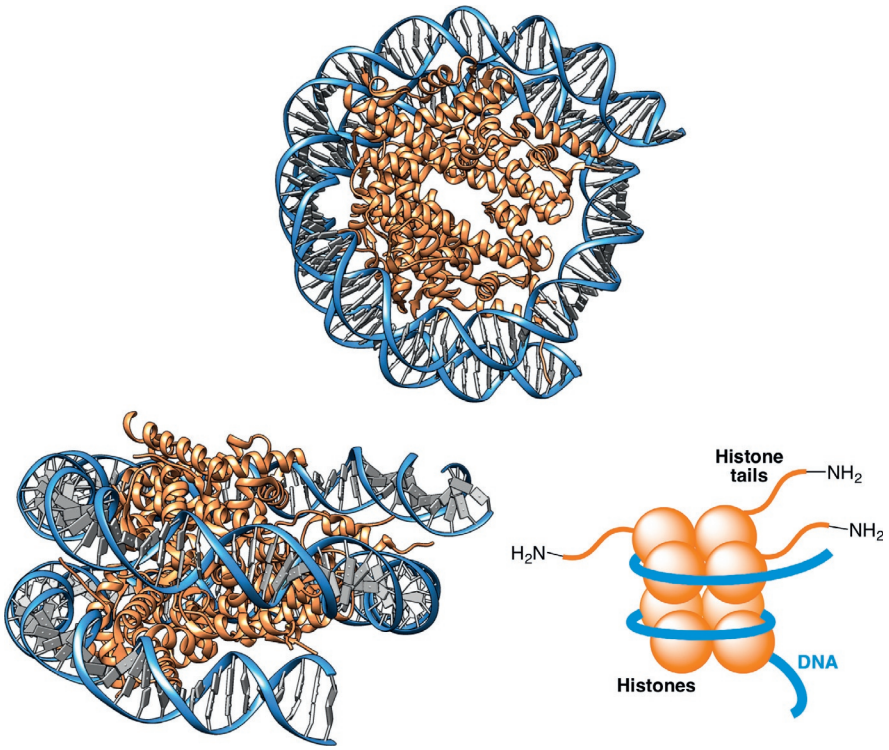


FIGURE 8.1

Structure of the nucleosome. Generated from Protein Data Bank reference 3AV1 and displayed with Chimera 1.8.1.

(Figure 8.1). Alterations in the structure and modification status of chromatin represent powerful regulatory mechanisms for gene expression and genome stability.

Regarding its accessibility for nuclear proteins, chromatin can exist in either an open or a closed configuration, and the regulation of these changes involves epigenetic mechanisms such as DNA methylation and post-translational histone modifications.

Promoter hypermethylation and histone deacetylation are epigenome alterations that are common in tumorigenesis. Promoters of tumor suppressor genes frequently increase DNA methylation by DNA methyltransferases (DNMTs) and/or histone deacetylation by DNA histone deacetylases (HDACs), leading to gene silencing. For these reasons, these events have become attractive anticancer targets.[2]

Epigenetic studies are also important to select cancer treatments because variations in clinical responses to different anticancer drugs are frequently related to epigenetic changes.[3] A growing body of evidence indicates that resistance to cancer drugs involves a reversible "drug-tolerant" state and that HDACs play a key role in its development.[4] For instance, a phase II study with dacarbazine in colorectal cancers for which standard therapies with oxaliplatin, irinotecan, fluoropyrimidines, and

cetuximab or panitumumab had failed concluded that objective clinical responses were confined to those tumors harboring epigenetic inactivation of the DNA repair enzyme O^6-methylguanine–DNA–methyltransferase (MGMT).[5] The deficiency of this enzyme, due to hypermethylation of the corresponding gene leading to its silencing, occurs in approximately 40% of colorectal cancers.

A summary of the drugs discussed in this chapter is given in Table 8.1.

Table 8.1 Anticancer drugs acting through epigenetic mechanisms

Mechanism	Type	Drugs
Inhibition of DNA methyltransferases (DNMTs)	Nucleosides	5-Azacitidine (Vidaza®) Decitabine (Dacogen®) Fluoro-2′-deoxycytidine DHAC
	Non-nucleosides	Epigallocatechin-3-gallate (EGCG) IM25 RG-108
	Antisense oligonucleotides	MG-98
Inhibition of histone deacetylases (HDAC)	Short-chain fatty acids	Valproic acid (Depakote®)
	Hydroxamic acids	Trichostatin A (TSA) Vorinostat (SAHA, Zolinza®) Pyroxamide Belinostat (PDX-101, Beleodaq®) Panobinostat (LBH-589) Dacinostat (NVP-LAQ-824) Abexinostat (PCI-24781) Pracinostat (SB-939) Resminostat (4SC-201, RAS410) Givinostat (ITF-2357) Quisinostat (JNJ-26481585) Rocilinostat (ACY-1215)
	Cyclic tetrapeptides	Romidepsin (FK-228, FR-901228, Istodax®) Trapoxins Apicicin CHAP-31
	Benzamides	Entinostat (MS-275) Tacedinaline (CI-994) Mocetinostat (MGCD-0103)
	Thiols	Psammaplin A
Inhibition of histone deacetylase 4 (HDAC4)		Tasquinimod
Inhibition of sirtuins (SIRTs)		Sirtinol Salermide EX-527

Continued

Table 8.1 Anticancer drugs acting through epigenetic mechanisms—cont'd

Mechanism	Type	Drugs
Inhibition of bromodomain		(+)-JQ1 GSK525762A (I-BET762) TEN-010
Inhibition of histone methyltransferases (HMTs)		Mithramycin A (aureolic acid, plicamycin, Mithracin®) BIX 01294
Inhibition of lysine-specific demethylases (LSDs or KDMs)		ORY-1001

2 INHIBITORS OF DNA METHYLATION: REACTIVATION OF SILENCED GENES

DNA methylation in eukaryotes occurs by the covalent modification of DNA bases, preferently cytosine residues, by DNMT enzymes. In the human genome, CpG dinucleotides are concentrated in the so-called "CpG islands," in which are embedded the majority of human gene promoters. Methylation of such DNA regions correlates with long-term silencing of gene expression because the associated structural changes in DNA block the binding of transcriptional factors or recruit transcription repressors called methyl-binding proteins (MBDs) that subsequently recruit histone deacetylases (HDACs) and histone methyltransferases (HMTs), as shown in Figure 8.2.

The methylation pattern is normally maintained throughout life, but in older individuals, deviations from this pattern start to appear, which can lead to genomic instability. Because the silencing of tumor suppressor genes is considered one of the early key events in the development of cancer, one specific goal of epigenetic chemotherapy is to prevent the hypermethylation in DNA.[6]

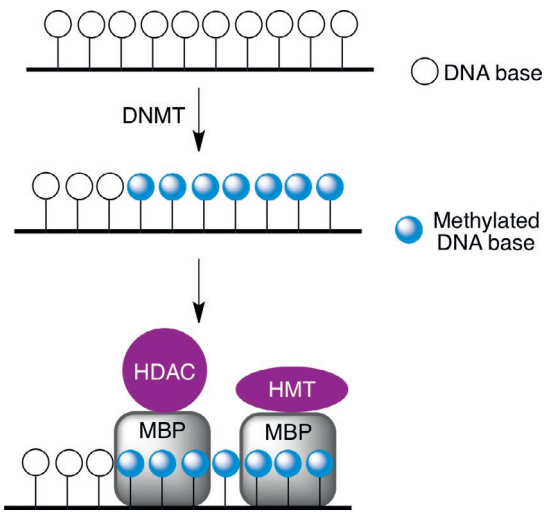


FIGURE 8.2

The role of DNA methylation in long-term silencing of gene expression.

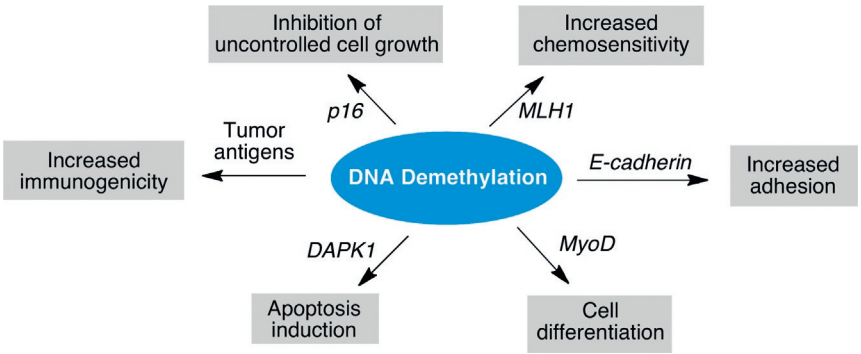


FIGURE 8.3

Effects of DNA demethylation on genes encoding different proteins. *DAPK1*, death-associated protein kinase 1 (mediates γ-interferon-induced programmed cell death); *p16*, cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1; *MLH1*, DNA mismatch repair protein Mlh1; *E-cadherin*, calcium-dependent adhesion transmembrane protein; *MyoD*, a myogenic regulatory factor.

DNA demethylating agents have the advantage of low toxicity because they are aimed at the activation of genes involved in apoptosis pathways and cell cycle regulation and do not cause immediate cell death as do most other chemotherapeutic drugs.[7] However, their effects are transient, and the aberrant patterns can be re-established upon removal of the drugs. The use of demethylating agents can potentiate the expression of oncogenes, but it has been observed that the overall response to decreased methylation is the abrogation of tumorigenicity (Figure 8.3).

The DNA methylation reaction, which uses as a methyl donor the *S*-adenosylmethionine cofactor, transforms the cytosine residues **8.1** into their 5-methyl derivatives **8.2** (Figure 8.4).

The role of DNMTs in this reaction is twofold. On the one hand, a carboxylic group from the enzyme acts as an acid catalyst by protonation of N-3, and on the other hand, a thiol group in a cysteine residue located in the catalytic pocket attacks the activated $C_6=C_5–C_4=N_3$ system of the starting material **8.1** to yield intermediate **8.3**. This compound contains an enamine system that is nucleophilic at the C5 position, and the enzyme enhances this nucleophilicity by abstraction of the N(3)–H proton allowing displacement of the highly electrophilic methyl group attached to the sulfonium unit in *S*-adenosylmethionine, to give **8.4** and *S*-adenosylhomocysteine. Finally, the free enzyme is released from structure **8.4** by a β-elimination reaction giving the product **8.2** (Figure 8.5).

2.1 NUCLEOSIDE INHIBITORS OF DNA METHYLTRANSFERASES

Some nucleoside analogs that have a modified cytosine ring attached to a ribose or deoxyribose moieties behave as DNMT inhibitors. Therefore, their main mechanism of anti-neoplastic activity is based on the induction of DNA hypomethylation, leading to renewed transcription of previously silenced tumor suppressor genes.[8] The most interesting compounds of this class are 5-azacitidine (Vidaza®), its epimer fazarabine, decitabine (Dacogen®), 5-fluoro-2′-deoxycytidine, 5,6-dihydro-5-azacitidine, and zebularine. Due to the poor oral absorption of nucleosides, these compounds have to be administered by injection.[9]

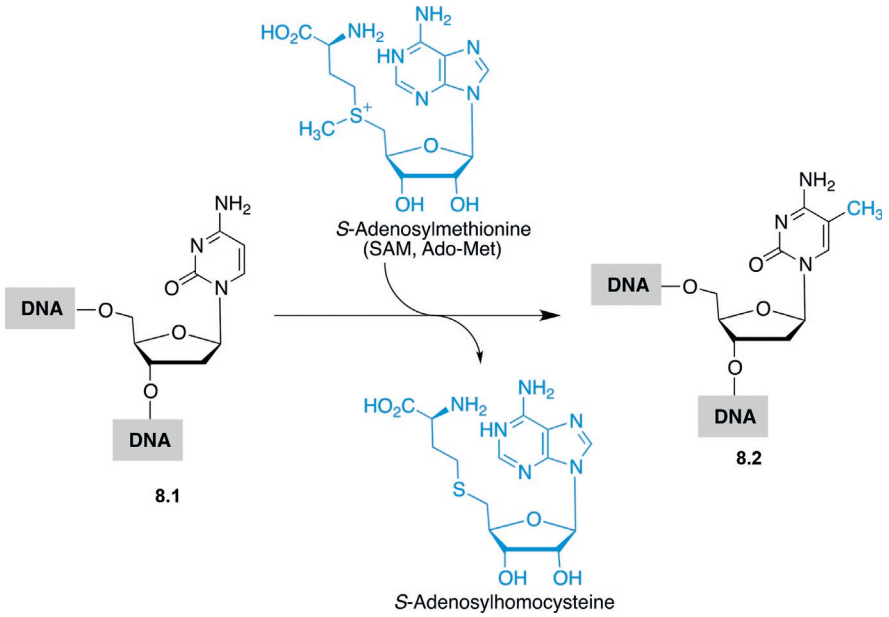
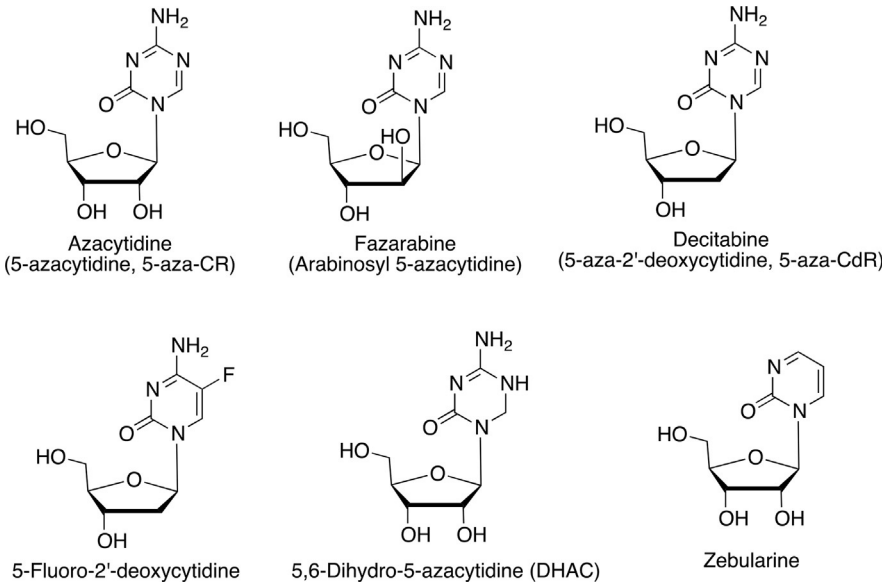


FIGURE 8.4

Methylation of cytosine by DNA methyltransferases.



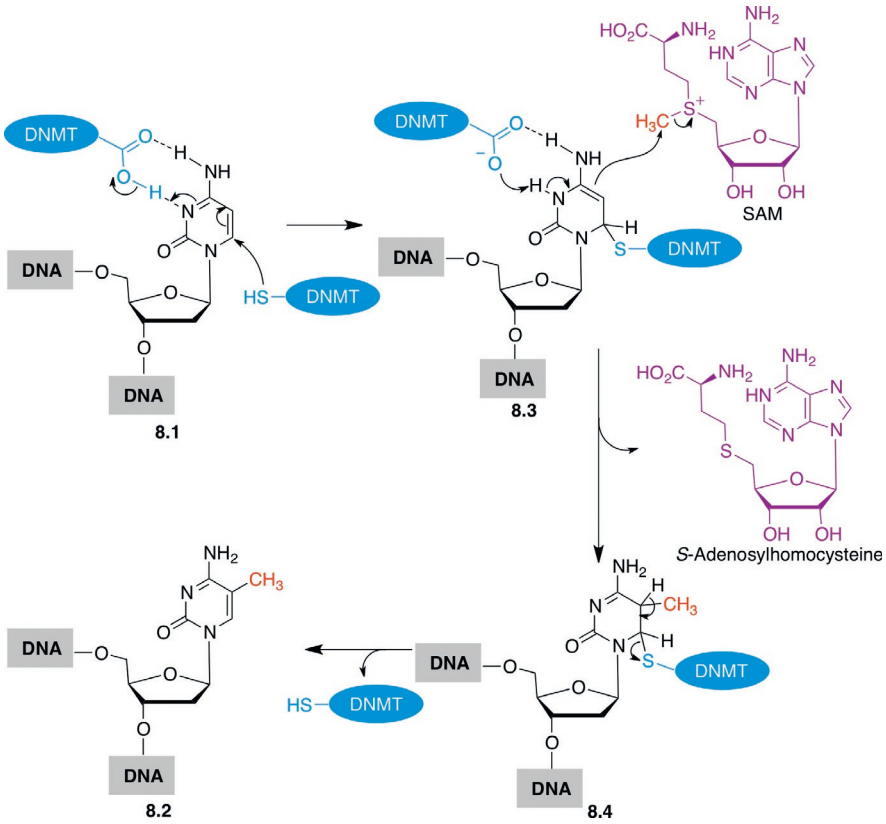


FIGURE 8.5

Mechanism of cytosine methylation by DNA methyltransferases.

Nucleoside inhibitors are converted by kinases and ribonucleotide reductases into deoxynucleotides that can be incorporated into DNA and complexed by the DNMT enzyme similarly to its natural substrates, allowing subsequent inhibition. Formation of covalent complexes with DNMTs results in enzyme depletion and, finally, a reversal of the methylation pattern.[10] In the case of decitabine, attack of the mercapto group in the active site to **8.5** gives adduct **8.6**, and its methylation at the N-5 position takes place normally to give **8.7**, but the lack of a hydrogen atom at N-5 after methylation prevents the elimination reaction necessary to restore the enzyme, which is thus inactivated (Figure 8.6).

5-Azacitidine[11] and its 2′-deoxy analog decitabine demonstrated the expected correlation between loss of methylation in specific gene regions and activation of the associated genes. Phase II clinical studies of 5-azacitidine as an antitumor drug took place in 1972, but its ability to inhibit DNA methylation was established in 1980. Beginning in 1993, a number of studies proved its efficacy in the treatment of myelodysplastic syndrome (MDS), leading to its approval by the U.S. Food and Drug Administration (FDA) in 2004 for this indication.[12] Decitabine is also a long-known anticancer drug that was tested in the clinic in the 1970s using the maximum tolerated doses, with myelosuppression as

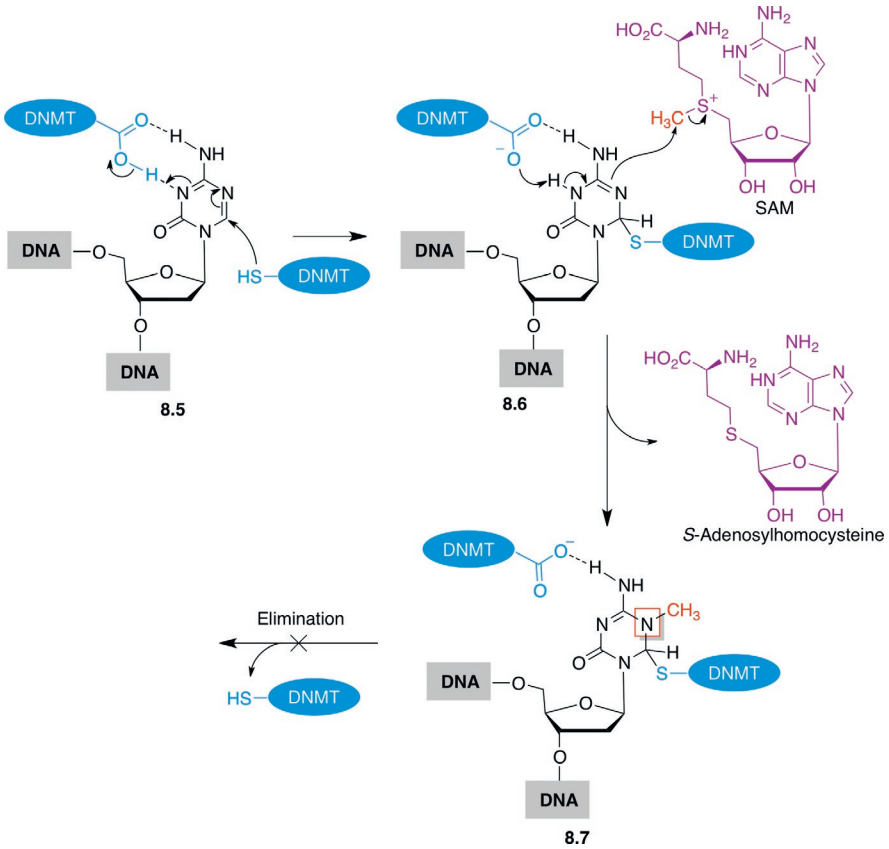


FIGURE 8.6

Mechanism of DNMT inhibition by decitabine.

its main side effect. It was not until 2004 that it was shown to have higher efficacy at much lower doses, with a correlation with DNA demethylation.[13] In 2003, it received orphan drug status for treatment of MDS, being approved for this indication by the FDA in 2006. Decitabine was also studied in patients with chronic myelogenous leukemia resistant to imatinib,[14] and it received an orphan drug designation for the treatment of acute myeloid leukemia (AML) and the European Medicines Agency (EMA) approval for this indication in 2012.

Currently, there is a revived interest in decitabine for cancers in which epigenetic silencing of critical regulatory genes has occurred. Treatment with decitabine reverses the methylation of the PDZ-LIM domain-containing protein 2 (PDLIM2), an essential terminator of NF-κB activation that is repressed in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (the tumorigenicity of these cells is suppressed by restoring the expression of this promoter).[15] Decitabine also induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human breast cancer MDA-231 cells.[16] In summary, decitabine is the DNMT inhibitor currently most favored for clinical

applications despite its high genotoxicity, which indicates that epigenetic therapies could benefit from further optimization of treatment schedules and from clinical development of alternative drugs.[17]

5-Fluoro-2′-deoxycytidine is another DNA demethylating agent that is undergoing clinical studies.[18] Its mechanism of action involves incorporation into DNA as nucleotide **8.8**, followed by methylation at C-5 mediated by nucleophilic attack of a cysteine residue in the active pocket of DNMT to give intermediate **8.9**, which is methylated by SAM to **8.10**. The absence of a proton at C-5 prevents the elimination reaction necessary for liberating the free enzyme (Figure 8.7).

5-Azacitidine, decitabine, and 5-fluoro-2′-deoxycytidine suffer from low *in vitro* and *in vivo* stabilities, which may hinder their effective delivery to the tumor site. *In vitro* stability can be improved by suppression of the $N_5=C_6$ imine function in order to prevent the addition of nucleophiles, by suppression of either the double bond or the nitrogen atom. These analogs, such as 5,6-dihydro-5-azacitidine (DHAC) and zebularine, are a less toxic alternative to decitabine and sometimes are associated with tetrahydrouridine, an inhibitor of cytosine deaminase that extends their half-life.[19] DHAC reached

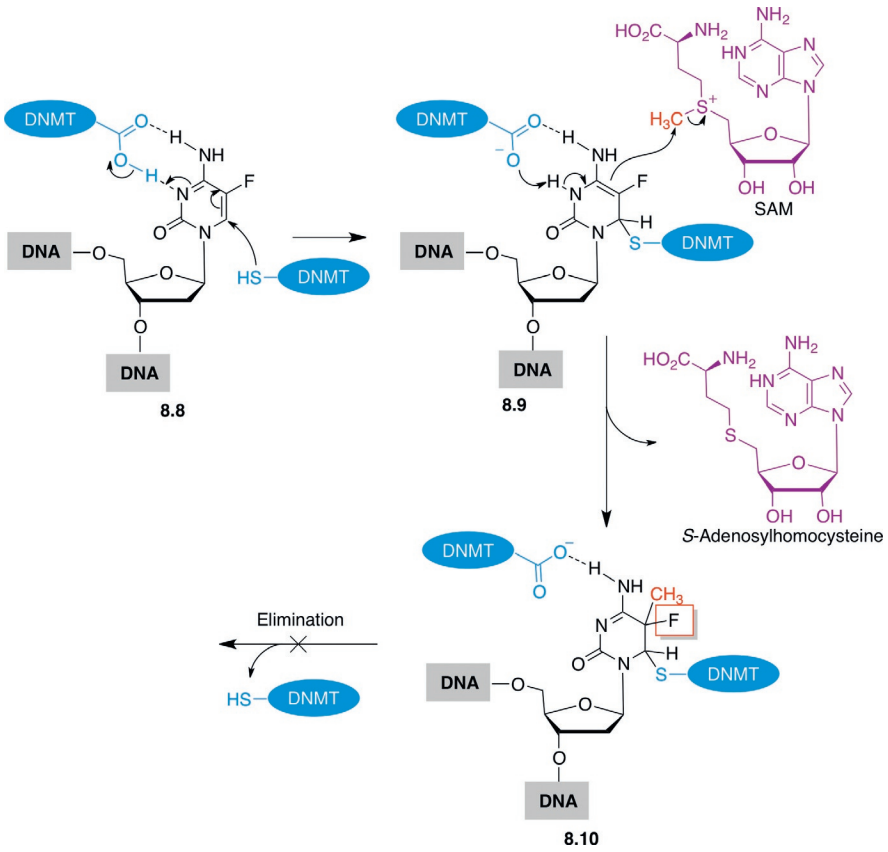


FIGURE 8.7

Mechanism of DNMT inhibition by 5-fluoro-2′-deoxycytidine.

phase II clinical studies for ovarian cancer and lymphomas, but it did not show sufficient efficacy. It has also been evaluated for other indications, such as pleural malignant mesothelioma.[20]

Zebularine was originally developed as a potent cytidine deaminase inhibitor because it lacks an amino group at position 4 of the pyrimidine. Its demethylating activity was not recognized until 2003.[21] After incorporation into DNA as nucleotide **8.11**, it forms a stable covalent adduct with DNMT (Figure 8.8), as proven by X-ray diffraction studies. In this case, the formation of intermediate **8.12** is not followed by methylation, a behavior that correlates with the low electron density of the C-5 position due to the absence of the 4-amino group, in agreement with molecular orbital calculations.[22]

Zebularine preferentially depletes DNA methyltransferase 1 (DNMT1) and induces expression of anticancer-related antigen genes in cancer cells.[23] Its main drawbacks are its low bioavailability and the need for high doses, which can be explained by its activity as an inhibitor of cytidine deaminase. This inhibition is due to analogy between the transition state of the reaction catalyzed by this enzyme (**8.13**) and zebularine hydrate **8.14**,[24] which is a dehydro analog of the previously mentioned inhibitor of cytidine deaminase tetrahydrouridine (Figure 8.9). This secondary effect causes part of the dose of

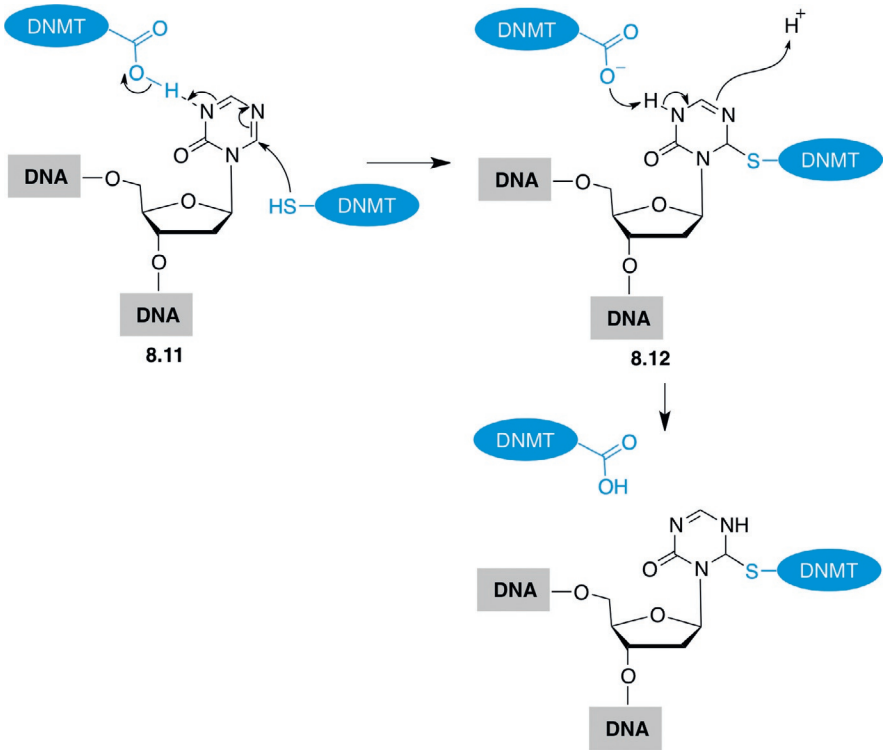


FIGURE 8.8

Mechanism of DNMT inhibition by zebularine.

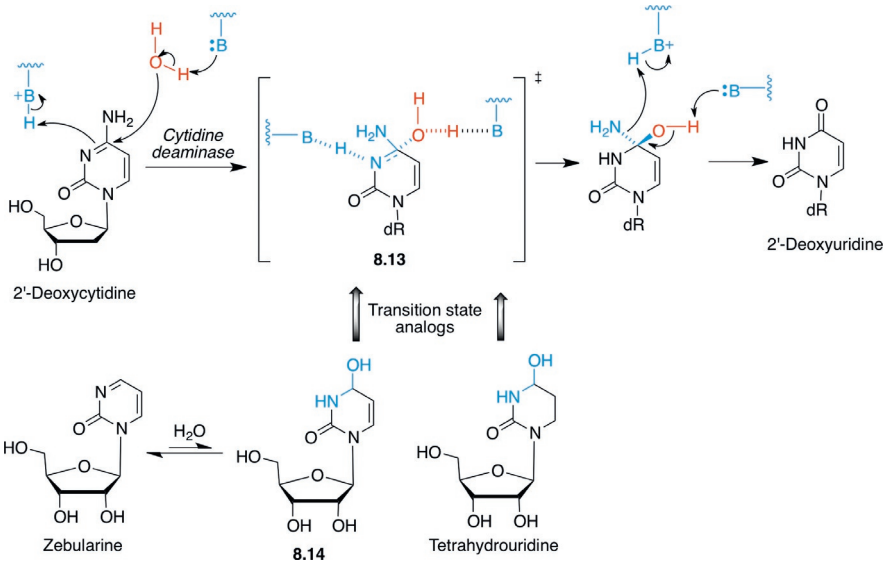


FIGURE 8.9

Inhibition of cytidine deaminase by zebularine hydrate due to its analogy with the reaction transition state.

zebularine to be sequestered by this enzyme and not available for DNMT. In addition, inhibition of cytidine deaminase results in increased levels of deoxycytidine, which competes with zebularine for incorporation into DNA, a necessary step for DNMT inhibition. Despite the promising preclinical results of zebularine, this drug has not entered clinical trials, and emphasis has been placed on identifying prodrugs that circumvent its bioavailability and metabolic limitations as well as on finding less toxic analogs.

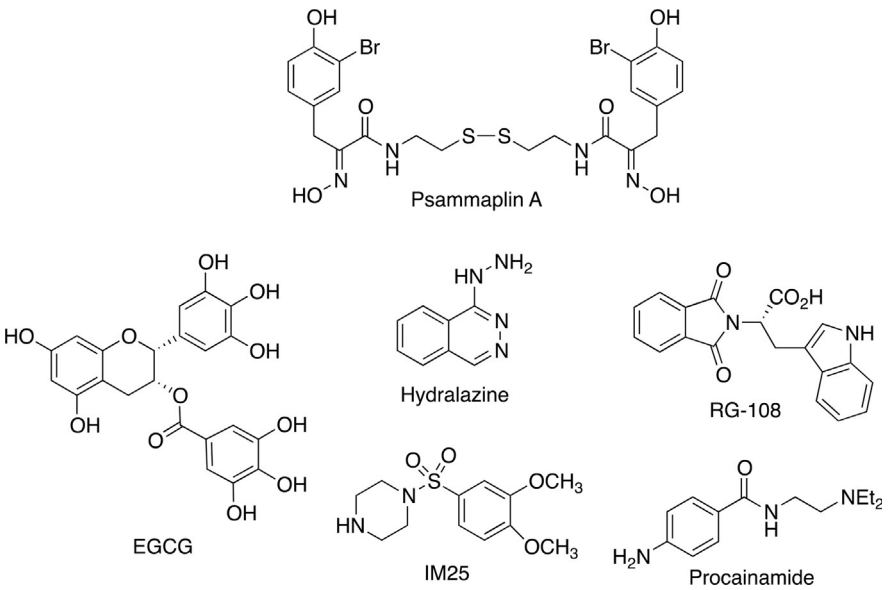
2.2 NON-NUCLEOSIDE INHIBITORS OF DNA METHYLTRANSFERASE

These inhibitors have the advantage of binding directly to the catalytic region of the enzyme without needing to be first incorporated into DNA, but their effects on cellular viability have not been analyzed systematically. One of these compounds is psammaplin A, a symmetric, dimeric hydroxyiminotyrosine-based natural product, isolated from various *Verongid* sponges and characterized in 1987, whose physiologic instability precluded its direct clinical development.[25] This compound was initially characterized as a dual inhibitor of DNMT and HDAC, the two main epigenetic modifiers of tumor suppressor gene activity, but recent studies have failed to show significant DNMT inhibitory activity in the natural product[26] and in a number of its analogs.[27]

Another natural DNMT inhibitor isolated from green tea is the polyphenol (–)-epigallocatechin-3-gallate (EGCG).[28] Various clinical studies have revealed that treatment with this compound inhibits tumor incidence in different organs, and several workers have demonstrated that it has potential in cancer prevention because it reduces the activity of DNMTs, proteases, and dihydrofolate reductase. Other

studies have shown that the effect of EGCG on DNA methylation might be more indirect than previously thought and that its cytotoxic effects can probably be attributed to oxidative stress induction.[29] In fact, EGCG inhibits DNMTs in cell-free assays, but it did not show a detectable effect on cellular DNA methylation.

Some unnatural DNMT inhibitors that have undergone preclinical study are also known. Hydralazine (apresoline) is a vasodilator used to treat severe hypertension and has also been used successfully as a treatment for myelodysplastic syndrome; the tryptophan derivative RG-108[30] and IM25, as potent as decitabine in terms of demethylation but with much lower cytotoxicity, are other DNMT inhibitors.[31] Furthermore, procainamide, an anti-arrhythmic drug with a good safety profile, inhibits DNMT1 by reducing the affinity with its two substrates: hemimethylated DNA (in which methylcytosine is in only one of the two DNA strands) and *S*-adenosylmethionine. Procainamide causes growth arrest and reactivation of tumor suppressor genes in cancer cells, but it requires high concentrations to be effective in suppressing cancer cell growth.



The extensive conservation of the catalytic domains of all DNMTs has allowed the use of X-ray structures of bacterial methylases as a basis for ligand design.[32] This model was validated through the design and evaluation of the new nucleoside analog N^4-fluoroacetyl-5-azacitidine[33] and of non-nucleoside compounds such as RG-108, currently in preclinical studies.[34] The binding of the previously mentioned natural inhibitor EGCG to this model is shown in Figure 8.10. Recently, molecular dynamics of the crystal structure of human DNMT1 and docking studies have allowed the establishment of a pharmacophore model.[35]

MG-98 is an antisense oligonucleotide of human DNMT1 that prevents the translation of the DNMT1 gene into the corresponding mRNA. It has reached phase II clinical studies.[36]

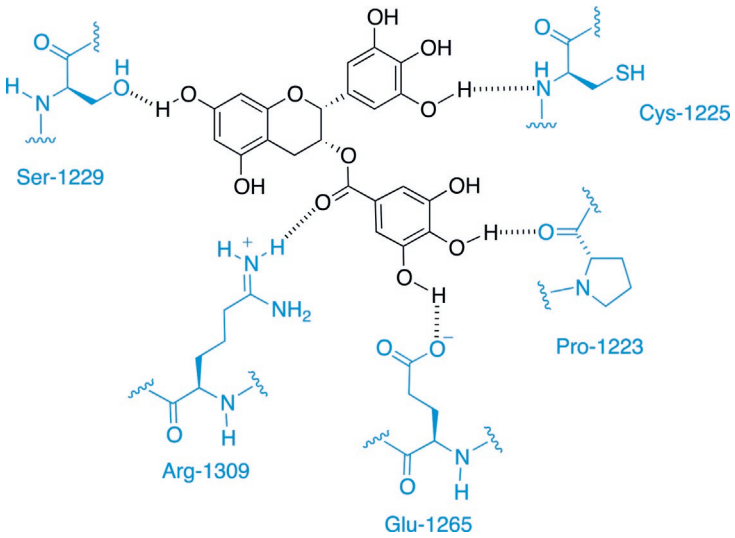


FIGURE 8.10

Interactions between DNMT and its natural inhibitor EGCG.

3 INHIBITORS OF HISTONE AND OTHER PROTEIN DEACETYLASES

Although histones do not interact with polymerase enzymes directly, their modification can affect the way DNA wraps around them and thereby influence which genes are expressed. The amino-terminal tails of histone proteins protrude out of the nucleosome and are subject to epigenetic transformations, including methylation of lysine and arginine residues; acetylation; ubiquitylation of lysines; and phosphorylation of serine and threonines. All of them play a regulatory role in gene expression, but whereas lysine acetylation is usually correlated with transcription deactivation, lysine methylation is associated with transcription activation or repression, depending on the residue and degree of methylation.

The basic ε-amino groups of lysine units are protonated under physiological conditions and interact electrostatically with the negatively charged phosphate backbone of DNA. Acetylation of these amino groups in the histone tails eliminates their basicity and consequently their positive charge. This weakens the electrostatic interactions of histones with DNA, opening up access to DNA for transcription factors and polymerases, and therefore enhancing transcription (Figure 8.11). The level of histone acetylation is regulated by histone acetylases and HDACs, which respectively add and remove the acetyl groups from lysine. Because HDAC activity is associated with increased growth and proliferation of cancer cells, there is a growing interest in the development of HDAC inhibitors as anticancer agents.[37]

Almost all known HDAC inhibitors induce the expression of the *p21WAF1/CIP1* gene, which leads to inhibition of cyclin D–CDK4 complex formation, resulting in cell cycle arrest and cell differentiation. HDAC inhibitors also lead to apoptotic and anti-angiogenic effects (Figure 8.12).

The HDAC family of proteins can be divided into zinc-dependent (class I and II) and zinc-independent, NAD-dependent (class III) enzymes, with the latter having being only recently implicated

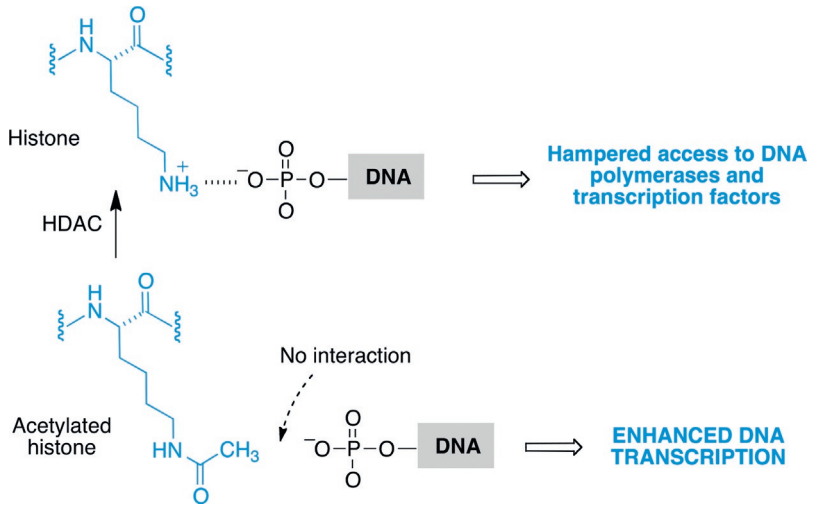


FIGURE 8.11

Mechanism by which histone acetylation enhances DNA transcription.

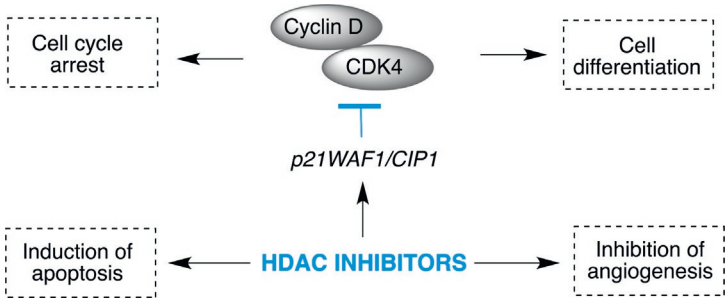


FIGURE 8.12

Summary of the biological effects of HDAC inhibitors.

in proliferation control. The proposed mechanism for the hydrolysis of the *N*-acetyl lysine residues in a zinc-dependent HDAC belonging to class I (HDAC1) is based on crystallographic studies of a bacterial deacetylase and is summarized in Figure 8.13.[38] It involves polarization of the carbonyl oxygen of the acetyl group and activation of a molecule of water by a charge-relay system formed by aspartic and histidine residues (**8.15**) in a process analogous to the one taking place in the activation of the serine hydroxyl in serine proteases by glutamic acid in zinc proteases. Nucleophilic attach of water onto the carbonyl leads to the formation of a tetrahedral intermediate, stabilized by two zinc–oxygen interactions, similarly to zinc proteases, and possibly by a hydrogen bond with the Tyr-303 hydroxyl (**8.16**). In the final step, the tetrahedral intermediate evolves by cleavage of the C–N bond with concomitant protonation from another His–Asp charge-relay system (**8.17**).

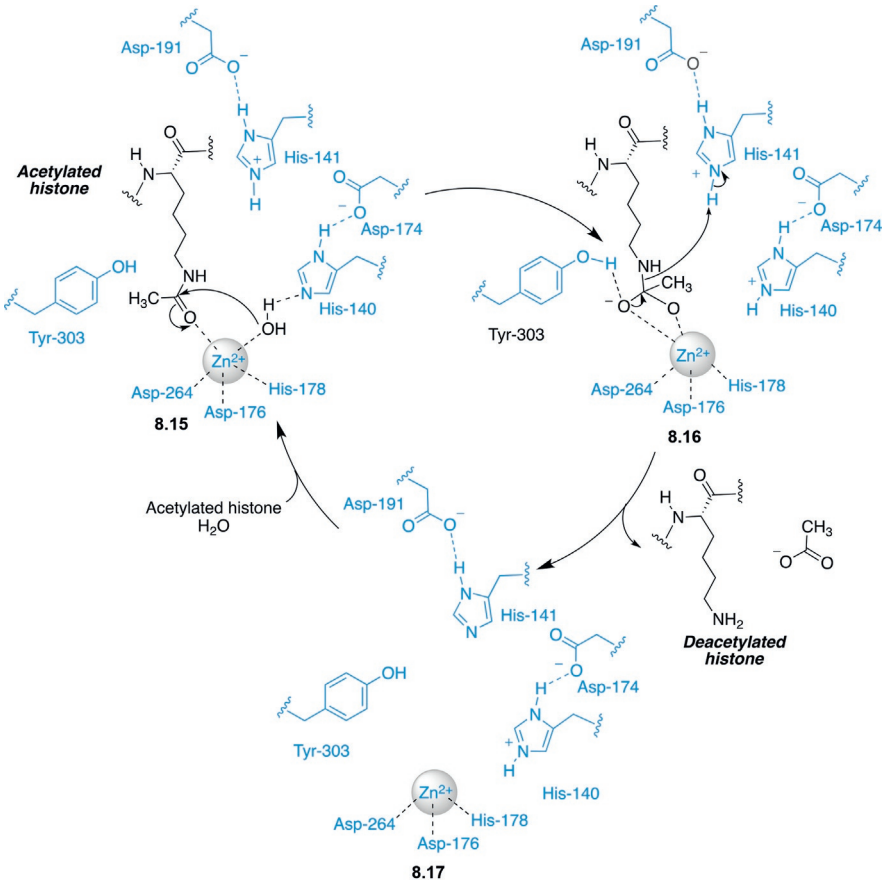


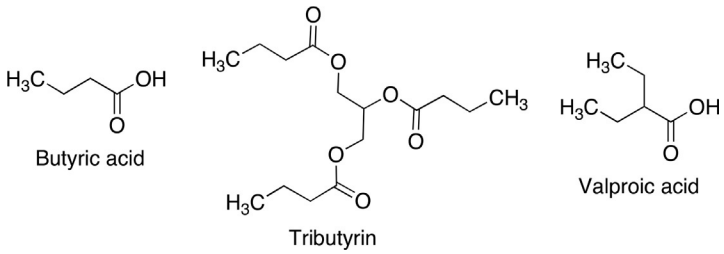
FIGURE 8.13

Proposed mechanism for the reaction catalyzed by Zn^{2+}-dependent HDAC.

HDAC inhibitors can be classified into five main categories: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, benzamides, and thiols.

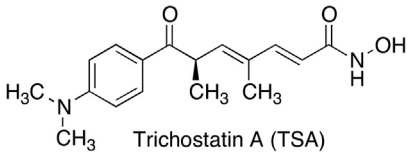
3.1 SHORT-CHAIN FATTY ACIDS

These compounds normally have low potencies, but they have become a useful tool in the study of HDAC inhibitors. Butyric and valproic acids were the first known HDAC inhibitors. Tribityrin is a prodrug of burytic acid with favorable pharmacokinetic and efficacy profiles that has entered clinical studies for solid tumors,[39] whereas the anticonvulsant valproic acid (Depakote®) is a dual inhibitor of HDAC1 and HDAC2 that is in trials for cervical cancer and ovarian cancer and, in combination with all-*trans* retinoic acid, for the treatment of AML in elderly patients.[40]



3.2 HYDROXAMIC ACIDS

Some potent inhibitors of HDAC belong to this class of compounds that, as discussed in the context of matrix metalloprotease inhibitors (see Chapter 11, Section 2.2), are potent Zn^{2+} chelators. Trichostatin A (TSA) is an antifungal antibiotic isolated from *Streptomyces* that was first shown to be a potent inducer of cell differentiation and cell cycle arrest and later reported to have anti-HDAC activity. It selectively inhibits the classes 1–7 and 9 of mammalian HDAC families of enzymes at the nanometer level. Despite its proven antitumor activity, it demonstrated too many undesired effects to be advanced into the clinic.[41]



The oral drug vorinostat, also known as SAHA (from *s*uberoyl*a*nilide *h*ydroxamic *a*cid) and commercialized as Zolinza®, was the first member of this class to enter human clinical trials[42] and the first HDAC inhibitor to reach the market. It was approved by the FDA in 2006 for cutaneous T-cell lymphoma (CTCL) in patients who have progressive, persistent or recurrent disease or following failure of two systemic therapies, and it was also approved for multiple myeloma (MM) with slight advantage over Velcade®. This compound was discovered in the course of studies on why dimethyl sulfoxide (DMSO) causes growth arrest and terminal differentiation of murine erythroleukemia cells. After other polar, small-molecule solvent species were examined, it was decided to link two amide groups that could lead to strong binding if there were two or more receptor sites for the amide species near each other. The first compound of this type was hexamethylene bisacetamide (HMBA). These amides, as well as the sulfoxide group in DMSO, can be assumed to bind a receptor site through hydrogen bonds or through metal ion chelation. In either case, it seemed likely that a hydroxamic acid would be a better binder than an amide group. Thus, a series of bishydroxamic acids was synthesized, including the compound with six methylenes—suberic bishydroxamic acid (SBHA)—which, as hoped, was more potent than the analogous bisamide. Based on the reasoning that if the hydroxamic acid group was binding to a metal ion it seemed unlikely that the other end of the dimeric species would do the same, it was decided to put a hydrophobic group at the nonhydroxamic acid end. The new synthesized molecule was SAHA, which was sixfold more potent than SBHA in causing cell growth arrest and cell death (Figure 8.14).

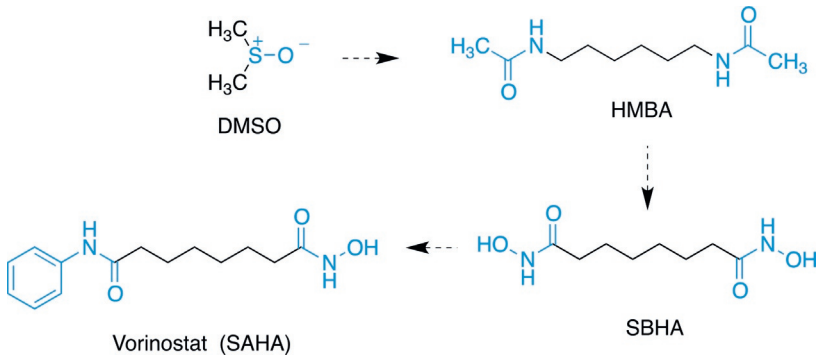
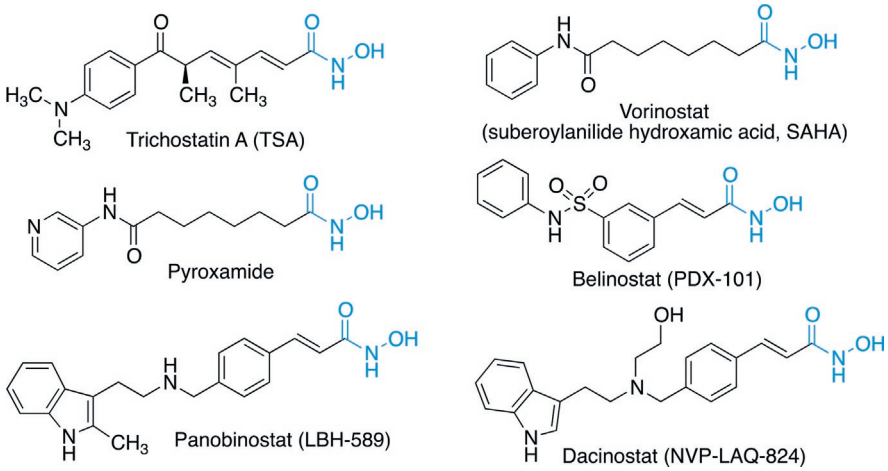


FIGURE 8.14

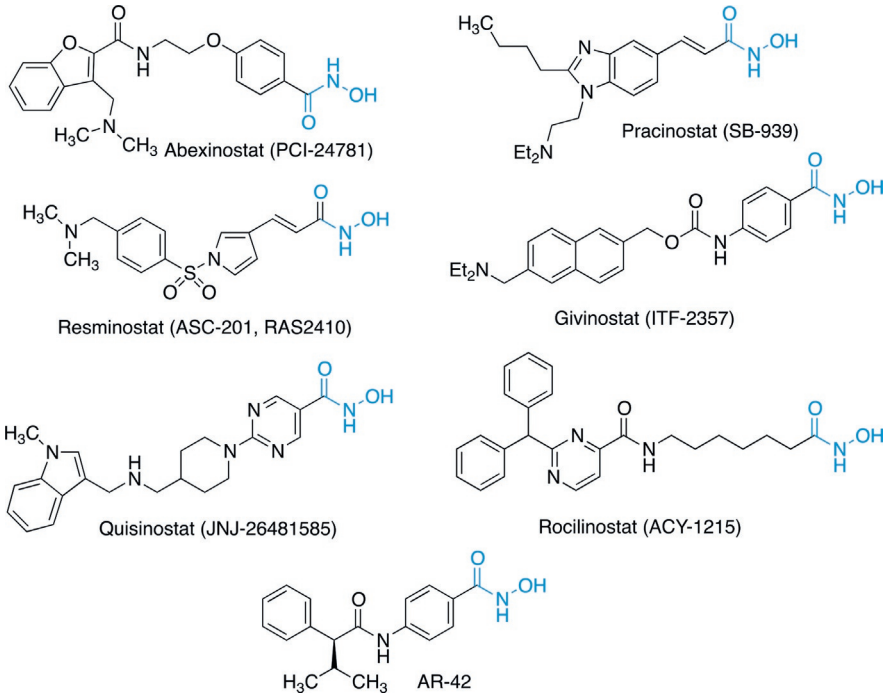
Stages in the design of vorinostat from DMSO.

The similarity of the structure of SAHA to that of trichostatin A suggested that SAHA acted by HDAC inhibition. Finally, when a complex of SAHA with a histone deacetylase-like protein (HDLP) was examined by X-ray crystallography, it was shown that the hydroxamic acid group chelated a zinc atom at the bottom of a cavity and the phenyl group was lying on the hydrophobic surface of the enzyme.[43]

Pyroxamide is a bioisoster of vorinostat that is also under clinical assays in patients with advanced malignancies.[44] Several hydroxamic derivatives of cinnamic acid, such as belinostat (PDX-101, Beleodaq®), panobinostat (LBH-589, Farydak®),[45] and dacinostat (NVP-LAQ-824),[46] are also under clinical evaluation for hematological and solid tumors. Belinostat entered a phase II trial for relapsed ovarian cancer and showed good results for T cell lymphoma. It was approved by the FDA in 2014 for the treatment of relapsed or refractory peripheral T-cell lymphoma. Panobinostat is a pan-DAC inhibitor got accelerated aprroval by the FDA on 2/23/2015 for use in patients with MM who have received at least two previous treatments. In multiple combinations entered phase I–III studies for cutaneous T cell lymphoma, myelodysplastic syndromes, myelofibrosis, and other hematologic malignancies.[47]



Abexinostat (PCI-24781) is in phase II clinical trials for B-cell lymphoma, and it is also being studied for different types of cancer, including MM.[48] Pracinostat (SB-939) is a new orally active hydroxamate-based HDAC inhibitor with an improved pharmacokinetic profile compared to that of SAHA. It has entered phase II clinical tests in combination with 5-azacitidine in high-risk myelodysplastic syndrome, and it is also a prospective new drug for treatment of malaria.[49] It is also a pan-DAC inhibitor. Resminostat (4SC-201, RAS410) is an oral pan-HDAC inhibitor with a favorable safety profile that induces growth arrest and apoptosis in solid and hematological tumors. In addition, it sensitizes tumors to other anticancer therapies and is in clinical development with encouraging results for hepatocellular carcinoma (as a single agent or in combination with sorafenib),[50] Hodgkin's lymphoma, and colorectal carcinoma. Givinostat (ITF-2357) is in numerous phase II clinical trials for different cancers, including refractory leukemias and myelomas.[51] Other hydroxamic acid HDAC inhibitors, such as quisinostat (JNJ-26481585),[52] the orally bioavailable, specific inhibitor of HDAC6 rocilinostat (ACY-1215),[53] and AR-42 have entered phase I clinical trials. AR-42 is also noteworthy because it rescues structural and functional brain deficits in a mouse model of Kabuki syndrome, a rare congenital disorder accompanied by congenital anomalies and a moderate intellectual disability that is caused by haploinsufficiency for either of two genes that promote the opening of chromatin.[54]



The interaction of the hydroxamic acid family with the human HDAC active site is exemplified in Figure 8.15 for the case of vorinostat.[55] The hydroxamic acid group coordinates the zinc cation in the enzyme active site through the hydroxamate and carbonyl oxygens, and it also establishes hydrogen bonds with two histidines belonging to the charge-relay systems and with the Tyr-308 hydroxyl group.

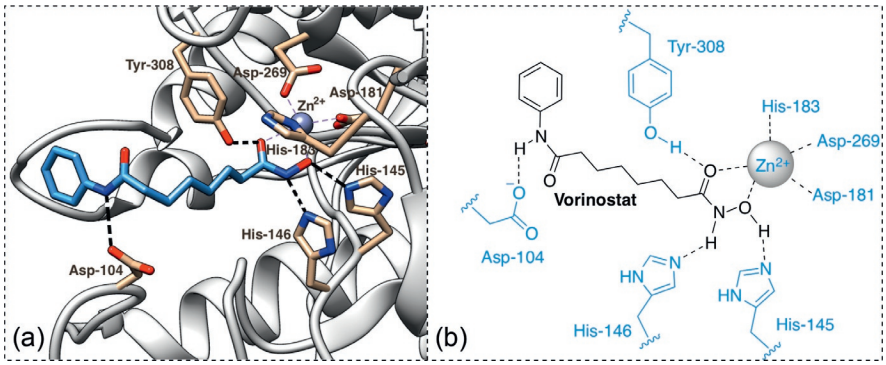


FIGURE 8.15

Interaction of vorinostat with the human HDAC2 active site. The three-dimensional structure was generated from Protein Data Bank reference 4LXZ, and displayed with Chimera 1.8.1.

An additional hydrogen bond is formed between an Asp residue and the vorinostat amide NH group. The hydroxamic acid hydroxyl group replaces a Zn^{2+}-bound water molecule of the active structure, and additional Van der Waals contacts (not shown) are established between hydrophobic enzyme amino acid residues and the inhibitor lipophilic chain. A similar mode of interaction has been described for trichostatin A.[56]

3.3 CYCLIC TETRAPEPTIDES

Some cyclic tetrapeptides are potent inhibitors of HDACs. The best-known compound of this group is romidepsin (FK-228, FR-901228, Istodax®), a depsipeptide isolated from a *Chromobacterium* that was approved by the FDA in 2009 for cutaneous T-cell lymphoma,[57] although it was rejected in 2012 by the EMA. It also entered clinical studies for the treatment of chronic lymphocytic leukemia and AML.[58] Romidepsin is a prodrug that is activated inside the cells by glutathione. The four-carbon chain between the free sulfhydryl and the cyclic depsipeptide core of this reduced active drug (RedFK) forms a covalent disulfide bond with the only cysteine residue present in the HDAC pocket[59] (Figure 8.16).

Trapoxins A and B are hydrophobic cyclotetrapeptides isolated from the fungus *Helicoma ambiens* that contain pipecolinic acid and proline residues, respectively. They also have two phenylalanines and an unusual amino acid bearing a side chain that contains an epoxide group. These compounds are potent enzyme inactivators, but they are too toxic for clinical use. In view of their structures, it would be reasonable to think that they irreversibly inhibit HDACs through a covalent bond that involves its epoxy group.[60] However, the α-epoxyketone moiety is not essential for activity, as can be deduced from the structure of apicidin, a fungal metabolite with antiprotozoal activity that also inhibits HDACs through induction of the *p21WAF1/Cip1* gene.[61] Apicidin is under preclinical assays as an anticancer agent.[62] CHAP-31, a trapoxin B analog in which the epoxy group has been replaced with a hydroxamate function,[63] is also under preclinical assays.[64]

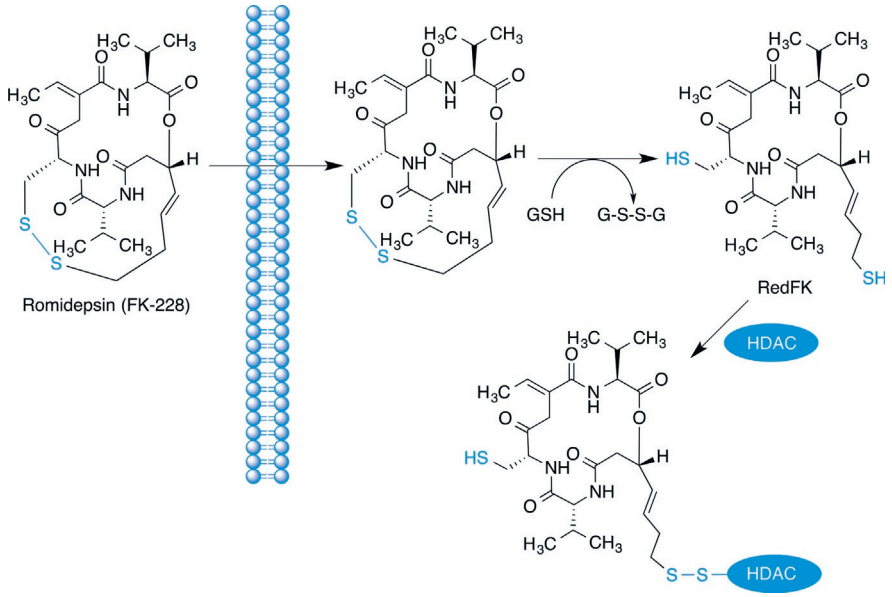
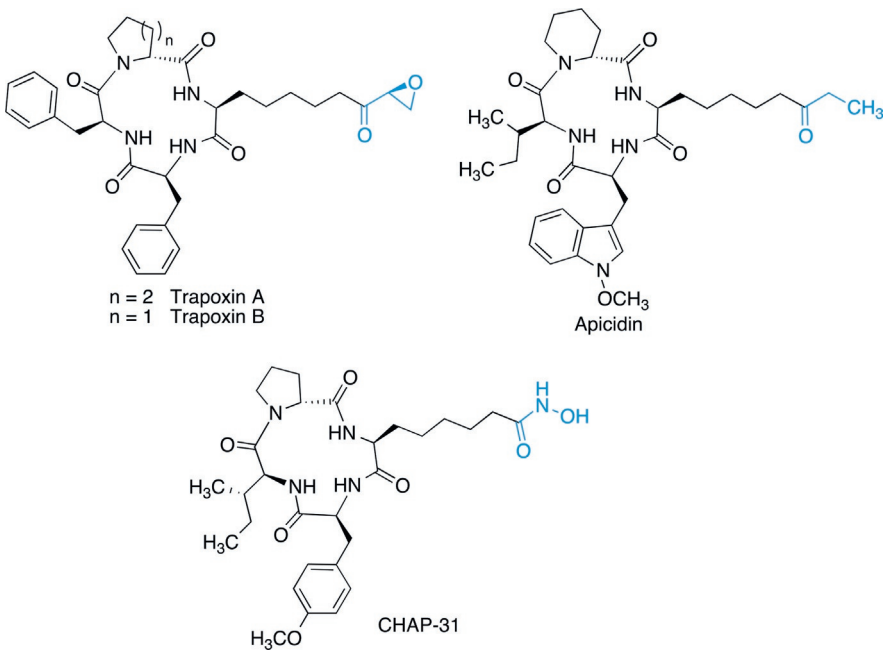


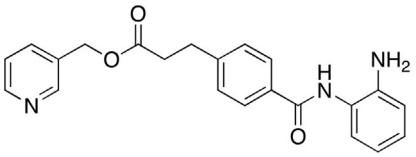
FIGURE 8.16

Romidepsin bioactivation.

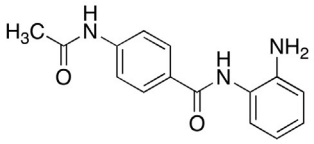


3.4 BENZAMIDES

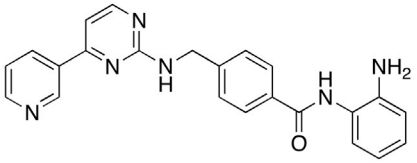
The synthetic benzamides entinostat (MS-275),[65] tacedinaline (CI-994),[66] and mocetinostat (MGCD-0103)[67] are being clinically tested in a variety of tumors, alone or in combination with other drugs. Entinostat is in phase II trials for Hodgkin's lymphoma, lung cancer, and breast cancer.[68] A phase II clinical trial in relapsed/refractory follicular lymphoma has been completed for mocetinostat. It has also been tested in multiple phase I and II trials, either as a single agent or in combination with 5-azacitidine (Vidaza®) or gemcitabine (Gemzar®). Mocetinostat received orphan drug designation from the FDA and EMA for the treatment of Hodgkin's lymphoma and AML. In 2014, the FDA also granted orphan drug designation for this compound for the treatment of diffuse large B-cell lymphoma and also for bladder cancer patients with specific genetic alterations. The presence of an *ortho* amino group into the *N*-phenylbenzamide substituent is essential for activity, and therefore it can be assumed to play a key role in the binding to the active site. In the case of entinostat, binding to zinc has been demonstrated.



Entinostat (MS-275)



Tacedinaline (CI-994, *p*-*N*-acetyldinaline)



Mocetinostat (MGCD-0103)

3.5 THIOLS

Since the initial report about its potent HDAC inhibition activity, the previously mentioned marine natural product psammaplin A has been the model for new HDAC inhibitors and for structure–activity relationship (SAR) studies. Psammaplin A is a prodrug that requires reduction of its disulfide functionality to the corresponding thiol, which acts as a zinc-binding group within the active site of the HDAC protein (Figure 8.17).[26] Its oxime unit is important for high potency and selectivity, but it may be replaced by other groups. Psammaplin A also inhibits topoisomerase II and aminopeptidase N, with *in vitro* angiogenesis suppression.[69]

Highly potent heterocyclic *N*-2-(thioethyl)picolinamide HDAC inhibitors, such as **8.18**, have been discovered by using computational modeling based on the psammaplin A pharmacophore.[70] However, probably because a thiol group is not an ideal functional feature due to potential off-target effects and low metabolic stability *in vivo*, thioester derivatives have been studied as possible prodrugs. Surprisingly, the thioester **8.19** is a potent HDAC inhibitor, despite the fact that previous SAR studies suggested that modification of the thiol functionality should detrimentally affect HDAC potency.[71]

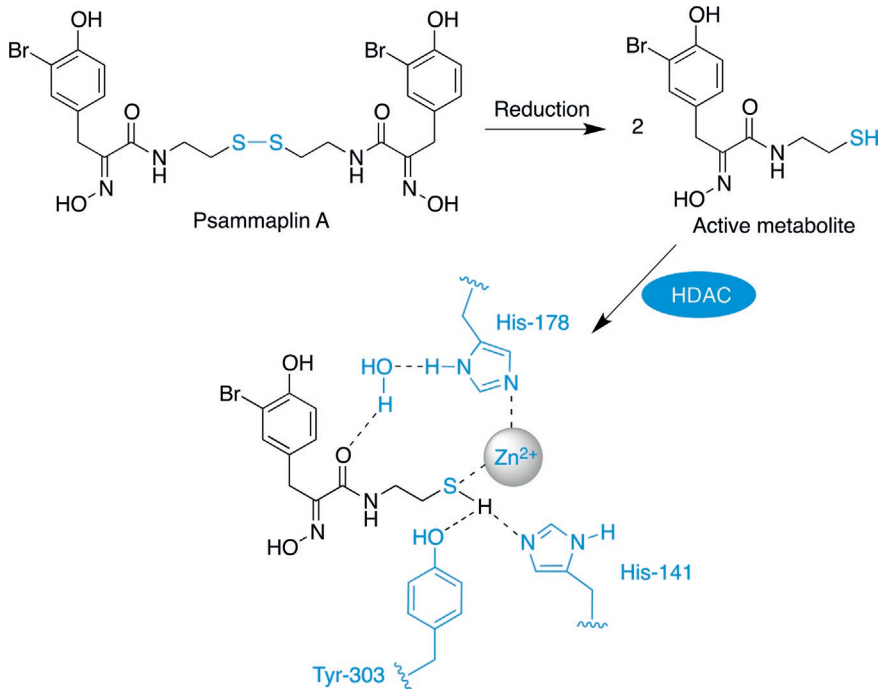
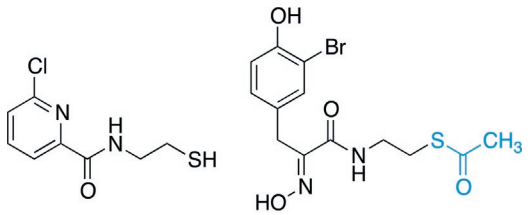


FIGURE 8.17

Bioactivation of psammaplin A and interaction of the active thiol with HDAC1.

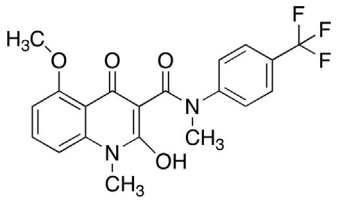


8.18 **8.19**

3.6 INHIBITORS OF HDAC4

The histone deacetylase HDAC4 could be a useful target for new anticancer therapies because its inhibition induces *p21WAF1/Cip1* gene expression and arrests cancer cell growth.[72] Tasquinimod is an allosteric modulator of this enzyme that inhibits HDAC4 client transcription factors bound at promoter/enhancers where epigenetic reprogramming is required for cancer cell survival and angiogenic response. It is an orally active antiangiogenic drug that is currently in phase III clinical trials for the

treatment of castration-resistant prostate cancer, and it is effective as a monotherapeutic agent against human prostate, breast, bladder, and colon tumor xenografts.[73] The efficacy of tasquinimod can be further enhanced in combination with the targeted thapsigargin prodrug G-202, which selectively kills tumor endothelial cells in the tumor microenvironment (see Chapter 13, Section 2.1).

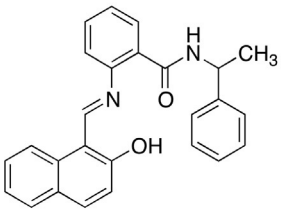


Tasquinimod

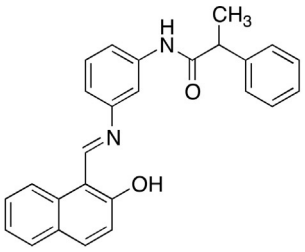
3.7 INHIBITORS OF SIRTUINS

Sirtuins (SIRTs; from *S*ilent *I*nformation *R*egulators) belong to the class III HDACs and play key roles in the maintenance of metabolic homeostasis. They have primarily protective functions against many age-related diseases, including neurodegeneration, cardiovascular disease, and cancer. In relation to cancer, SIRT1 regulates several tumor suppressor genes, such as *p53*, whereas SIRT2 regulates a G_2/M mitotic checkpoint and tubulin stability. SIRT1 is upregulated in several cancers, including lymphomas, leukemia, soft tissue sarcomas, prostate cancer, and lung and colon carcinomas. Overexpression of SIRT2 can significantly prolong the mitotic (M) phase to prevent the induction of chromosomal instability, particularly in response to microtubule inhibitor-mediated mitotic stress. Consistently, tumors with high levels of SIRT2 are refractory to chemotherapy, especially to microtubule poisons.[74]

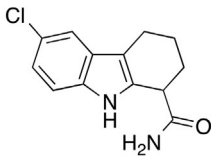
Sirtinol, salermide, and EX-527 are examples of SIRT inhibitors that have been preclinically evaluated. Sirtinol (ALX-270-308) is a SIRT1 inhibitor whose anticancer potential is related to an impaired activation of the Ras–MAPK pathway.[75] Salermide is a strong *in vitro* inhibitor of SIRT1 and SIRT2 that produces cell death in a wide range of human cancer cell lines, primarily by reactivation of proapoptotic genes epigenetically repressed exclusively in cancer cells by SIRT1.[76] EX527 is a SIRT1 inhibitor that, similarly to the HDAC inhibitor trichostatin A, increases the acetylation at Lys-382 of p53 after different types of DNA damage in several cell lines, confirming that p53 acetylation is regulated by both SIRT1 and HDACs.[77] It has been suggested that SIRT inhibitors require combined targeting of both SIRT1 and SIRT2 to induce p53 acetylation and cell death.[78]



Sirtinol



Salermide



EX-527

3.8 BROMODOMAIN INHIBITORS

Bromodomains are modules contained within epigenetic proteins that recognize acetyl groups on histones and play critical roles in influencing gene transcription by functioning as readers of epigenetic marks. They recognize special marks on DNA–protein complexes and attract gene-activating proteins to those spots. Family members of the bromodomain and extra-terminal (BET) protein, most notably bromodomain-containing protein 4 (BRD4), are a new and highly promising class of epigenetic targets that, once bound to chromatin, can influence gene expression and are directly implicated in the activation of oncogenes such as *MYC*, one of the most common overexpressed genes in many cancers. The DNA-binding protein Myc is involved in up to 70% of cancers, but it is generally considered undruggable because the active parts of its structure are not accessible to small-molecule drugs. A way for lowering Myc levels in hematopoietic cancers emerged from the discovery that the incurable nature of MLL-AF9 AML depends on the presence of BRD4. This discovery was possible by screening libraries of approximately 1000 short hairpin RNA molecules (shRNAs) designed to knockout 234 genes coding for the key proteins involved in epigenetic-driven gene expression. Since then, BET bromodomain inhibition has demonstrated its potential in several cancers, including multiple myeloma, AML, non-small cell lung cancer, and neuroblastoma. BET bromodomain inhibition has been shown to decrease Myc levels, leading to cancer cell death.

Development of small-molecule inhibitors of bromodomain binding to histones and other acetyl lysine-containing proteins is in its early stages, but it has already yielded some promising compounds,[79] most of which are derived from fused thienodiazepine or benzodiazepine frameworks.[80] The first of these compounds is (+)-JQ1, which inhibits the BRD4 isoform, was developed to treat the BRD4-driven rare nuclear protein in testis (NUT) midline carcinoma (NMC), but when it was used on human MLL-AF9 AML cells, Myc levels rapidly plunged and these cells rapidly stopped multiplying, being differentiated into macrophages.[81] BRD4 functioning is vital not only for fast-growing leukemias but also for many, if not most, dangerous lymphomas and myelomas. Thus, cell lines from most human multiple myeloma patients show high sensitivity to JQ1.[82] X-ray diffraction studies have shown that (+)-JQ1 occupies the same bromodomain as the *N*-acetyl lysine residues, thereby deactivating transcription. The binding of JQ1 to BET displaces chromatin, and the gain in compacted chromatin attenuates DNA damage response signaling, which enhances the lethality of anticancer drugs such as bortezomib.[83]

JQ1 has also been investigated for other applications. Thus, this compound also inhibits a testis-specific bromodomain (BRDT), essential for chromatin remodeling during spermatogenesis, although early evidence suggests that BRDT does not promote Myc synthesis. Occupancy of the BRDT acetyl lysine pocket by JQ1 blocks the production of sperm in the testes and generates a complete and reversible contraceptive effect.[84] OTX015 is a close structural analog of JQ1 that is in phase I clinical studies for hematological malignancies.

GSK-525762A, also known as I-BET762, is another potent benzodiazepine inhibitor that disrupts the function of the BET family of bromodomains (BRD2, BRD3, and BRD4). It was discovered following a lead optimization approach and is currently under evaluation in a phase I/II clinical trial for NMC and other cancers.[85] NMC is a highly aggressive cancer with very limited treatment options that is caused by the fusion of two genes, one that encodes the reader protein BRD4 and another gene called *NUT*. This fusion encodes a mutant protein, NUT–BRD4, which seems to act as a reader, spurring errant gene expression and forcing cells to lose their identity and become cancerous.

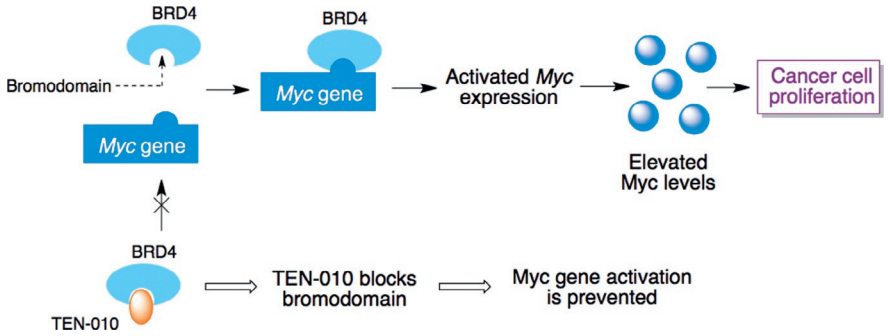
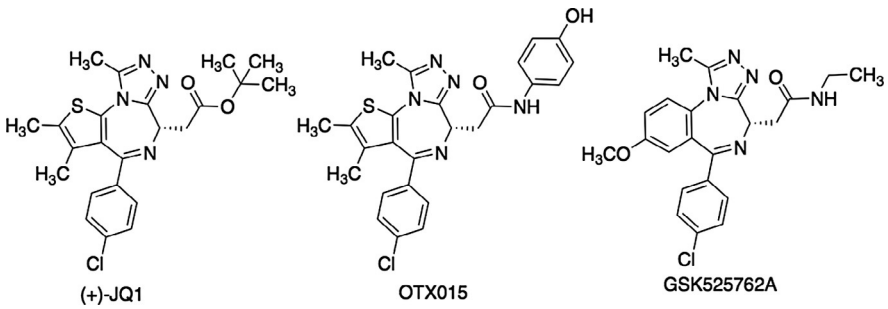


FIGURE 8.18

Prevention fo Myc gene activation by the bromodomain blocker TEN-010

TEN-010 (NCT-01987362), whose structure has not been disclosed at the time of writing, is another highly selective, potent BET bromodomain inhibitor that occupies the bromodomains of BRD4 and prevents binding to the *Myc* gene. As a result, BRD4 no longer activates *Myc* gene expression, resulting in decreased proliferation and cancer cell death (Figure 8.18). It has entered phase I clinical trials in patients with NMC.



X-ray crystal structures of JQ1 and GSK525762A in complex with BRD4 showed nearly identical interactions, with the methyltriazole moiety occupying the acetyl lysine binding site of the protein (KAc). The two adjacent nitrogen atoms of the 1,2,4-triazole ring mimic the carboxy group of acetyl lysine and give a hydrogen bond with the NH_2 group of asparagine 140 and a water-mediated hydrogen bond with the hydroxy group of tyrosine 97. The methyl substituent of the triazole binds to a small hydrophobic pocket—the same one that is occupied by the methyl fragment of the acetyl group in acetyl lysine (Figure 8.19).

Another potential application of bromodomain inhibitors is the treatment of heart disease because BET proteins regulate the growth of heart muscle cells and activate a broad set of genes involved in heart failure. Thus, treatment with JQ1 inhibited this abnormal pattern of gene activity and protected against heart wall thickening, the formation of scar tissue, and pump failure in a mouse model of cardiac disease. RVX208 is a bromodomain inhibitor that has been specifically designed for the treatment of cardiovascular disease and is in phase II clinical studies.

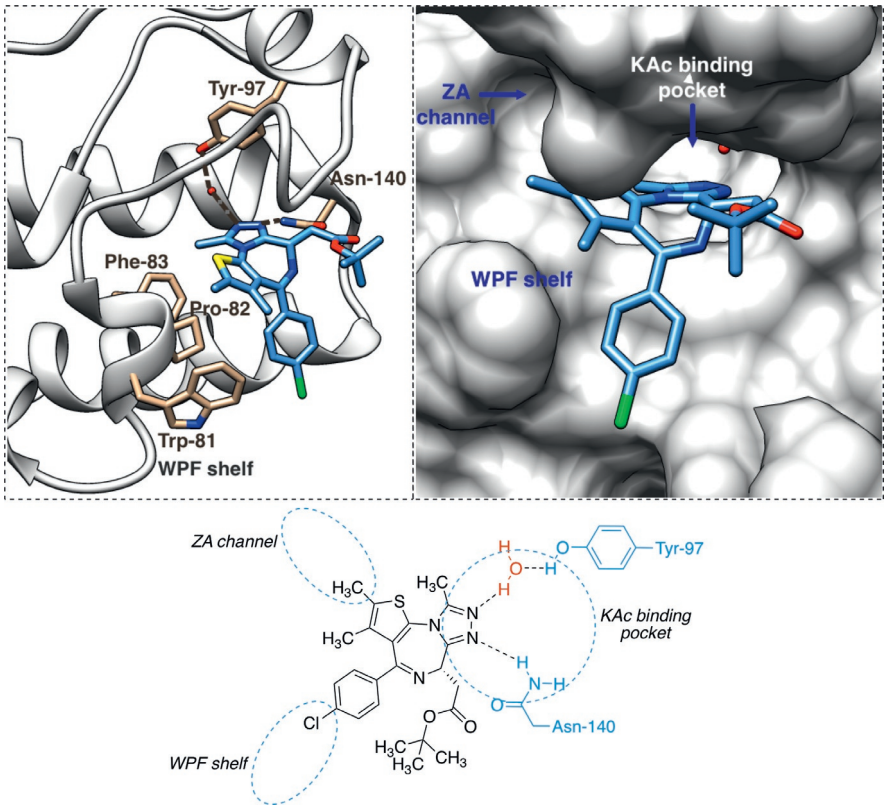
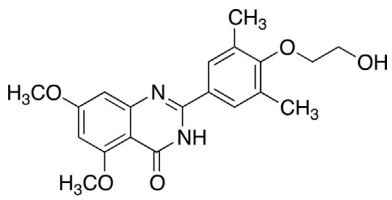


FIGURE 8.19

Interactions of (+)-JQ-1 with the BRD4 active site, including the KAc binding pocket, the ZA channel, and the WPF shelf, formed by the Trp-81, Pro-82, and Phe-83 residues. The three-dimensional structures were generated from Protein data Bank reference 3MXF and displayed with Chimera 1.81.



4 REGULATORS OF HISTONE METHYLATION

In a manner similar to acetylation, the methylation of histone lysine residues can be regulated at individual genes through the recruitment of lysine methyltransferases (KMT) and demethylases. Several of these pathways are relevant to cancer.[86] The addition of methyl groups to histones by histone

methyltransferases can either activate or further repress transcription, depending on the amino acid being methylated and the presence of other methyl or acetyl groups in the vicinity.[87]

4.1 INHIBITORS OF HISTONE METHYLTRANSFERASES

Several proteins are able to catalyze the addition of methyl groups to lysine or arginine residues of histones using *S*-adenosylmethionine (SAM) as the methyl donor (Figure 8.20).

Specific lysines in H3 and H4 histones can be mono-, di-, or trimethylated, whereas arginines can be monomethylated, asymmetrically dimethylated, or symmetrically dimethylated (Figure 8.21).

Although histone methylation does not alter the positive charge of the amino groups, several reader proteins specifically recognize this transformation and recruit additional enzymes whose activity may alter the local chromatin environment, thus affecting transcription. Consequently, activating or inactivating mutations, as well as overexpression of specific methyltransferases, can result in disease development.

The fact that histone lysine methylation is a much slower process than histone lysine acetylation (a half-life of 0.3–4 days compared to 2–40 min for histone acetylation) has led to the suggestion that methylation could impose memory on gene transcription and could be a potential example of heritable epigenetic control.

Among histone lysine *N*-methyltransferases, overexpression of EZH2, which mediates histone H3K27 trimethylation,[88] has been found in various cancers, and its inhibition is associated with gene silencing. This enzyme is coordinately expressed and functions upstream of the histone methyl transferase MMSET, which mediates H3K36 dimethylation. The discovery of 3-deazaneplanocin A (DZNep) as an inhibitor of EZH2 opened the possibility to pharmacologic inhibition of histone methylation[89] and the identification of HZH2–MMSET axis as an attractive therapeutic target in cancer.[90] BIX 01294, another inhibitor of MMSET, is a promising preclinical candidate for the treatment of some patients with multiple myeloma because this enzyme is overexpressed in myelomas with the translocation t(4,14).[91] Mithramycin A (aureolic acid, plicamycin, Mithracin®) is a natural antibiotic that, through binding to GC-rich regions in DNA, prevents the approach of HMTs, causing the DNA to coil up and be inaccessible for transcription. Mithramycin A is also a strong activator of the tumor suppressor p53 protein in human hepatoma cells,[92] being used for the treatment of patients with Paget's disease of bone as well as for several other forms of cancer.

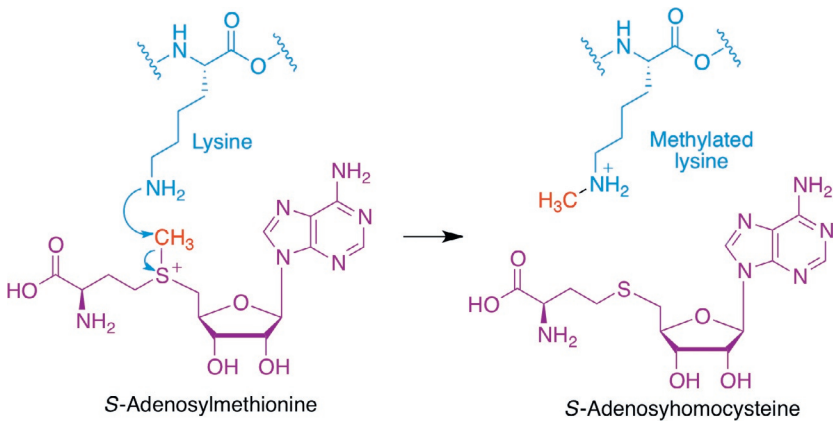


FIGURE 8.20

Mechanism of lysine methylation.

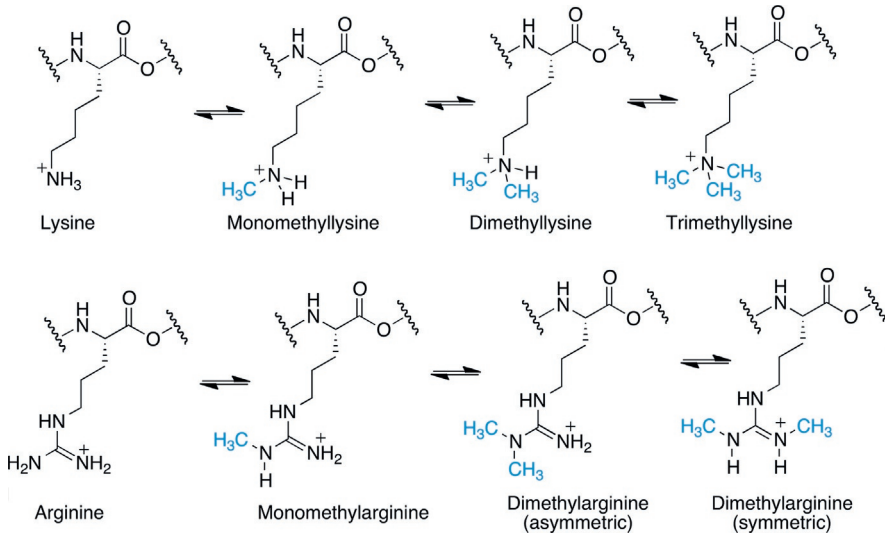
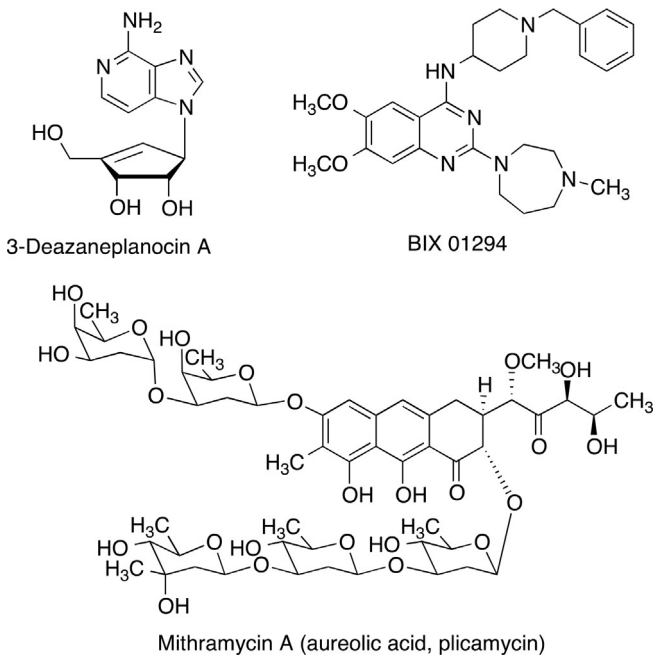


FIGURE 8.21

Stages in the methylation of lysine and arginine.



4.2 LYSINE-SPECIFIC DEMETHYLASES (LSDs OR KDMs) AND THEIR INHIBITORS

Some years ago, histone methylation was believed to be a stable modification that was only erased upon histone exchange or during DNA replication, but this idea was rejected following the demonstration that lysine-specific demethylase 1 (LSD1) catalyzes the demethylation of H3K4me1 and H3K4me2. Today, histone lysine demethylases are regarded as a specific group of eraser proteins that are implicated in the epigenetic control of cellular differentiation and in the development and maintenance of cancer, with the strongest biological evidence obtained for the LSD1, JARID1B, FBXL10, and JMJD2 families.

Lysine-specific demethylases LSD1[93] and LSD2, also known as KDMs, have an amine oxidase-like (AOL) domain and a chromatin factor-associated SWIRM domain. The SWIRM domain is thought to participate in protein–protein interactions rather than in protein–DNA interactions, which may explain the ability of LSDs to recognize different substrates. The AOL domain displays two subdomains, one that binds to the cofactor FAD and another that binds to the substrate, whose interface forms the catalytic center. Lysine demethylation catalyzed by these enzymes is likely to occur through the hydride transfer mechanism showed in Figure 8.22.[94]

The catalytic domains of the LSD proteins share sequence homology with monoaminooxidases MAO-A and MAO-B, which are responsible for the oxidative deamination of dopamine and serotonin, respectively. Accordingly, a few inhibitors of these enzymes, most notably tranylcypromine (a mechanism-based inhibitor that forms a covalent adduct with the FAD cofactor in the AOL domain), also inhibit LSD1, presumably via the same mechanism (Figure 8.23).[95]

To avoid the adverse effects of nonselective amine oxidase inhibitors, several derivatives of tranylcypromine with enhanced potency and target selectivity for LSD1 have been obtained through modification of the phenyl and the amino groups, and some of these derivatives have proved to be potent and selective LSD1 inhibitors.[96]

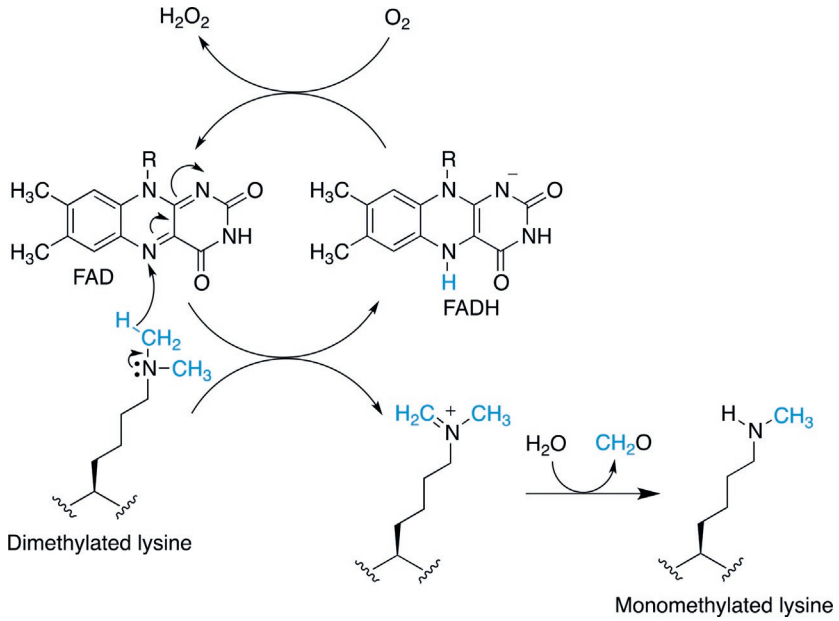


FIGURE 8.22

Mechanism of lysine demethylation by LSDs (KDMs).

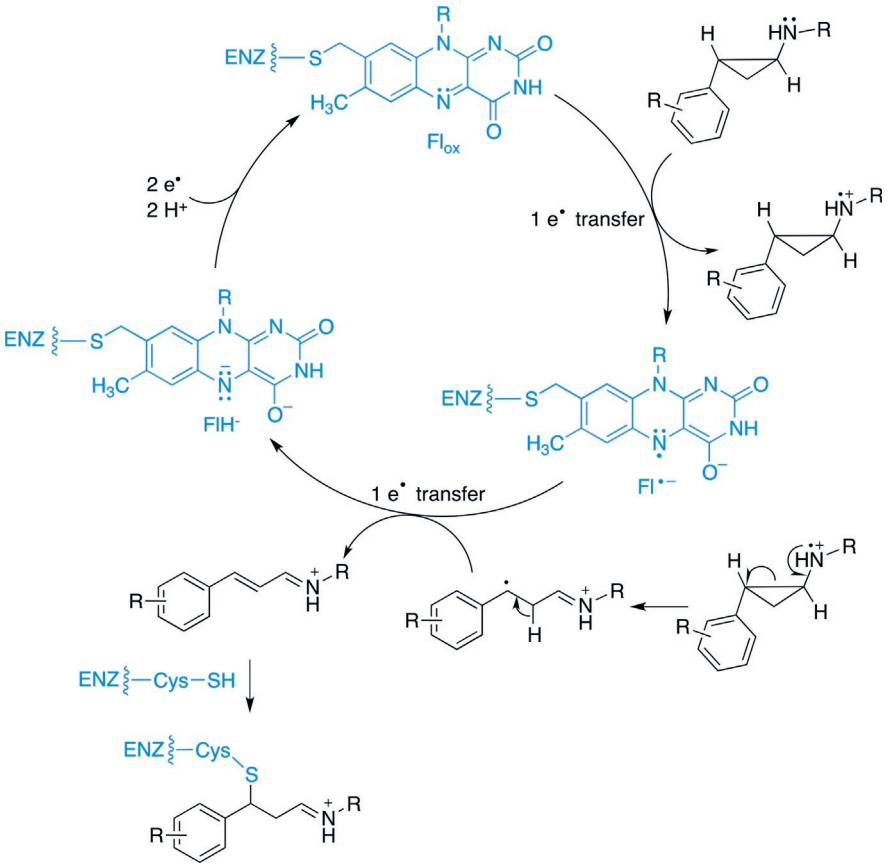
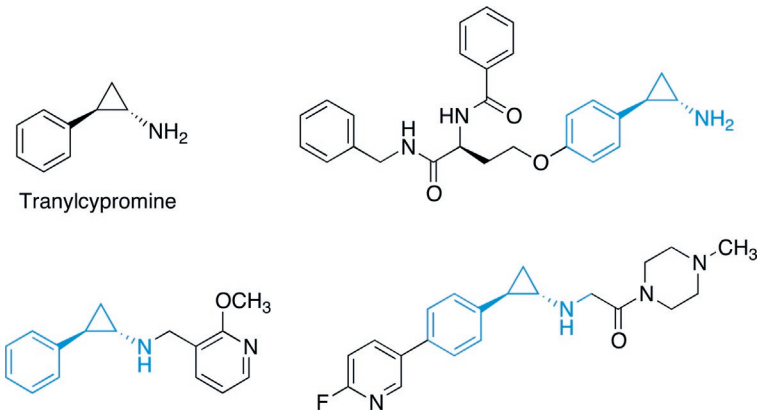


FIGURE 8.23

The mechanism of action of tranylcypromine.



Phase I/IIA clinical trials have been initiated in patients with relapsed or refractory acute leukemia with ORY-1001, a novel highly selective and orally active LSD1 inhibitor previously designed by EMA as an orphan drug, and whose structure has not been determined.[97] Apart from the tranylcypromine derivatives, no truly promising drug candidates that selectively target LSD1 have been published, although within the pharmaceutical industry, there is considerable interest in their development.[98]

Another family of LSD enzymes contains the catalytic Jumonji C (JMJC) domain, which binds to the cofactors Fe(II) and 2-oxoglutarate. This complex reacts with oxygen to form a highly active oxoferryl [Fe(IV) = O] intermediate that hydroxylates one *N*-methyl group of the methylated lysine substrate, and the resulting unstable hemiaminal releases the *N*-hydroxymethyl group from nitrogen in the form of formaldehyde (Figure 8.24). This mechanism allows the demethylation of mono-, di-, and trimethylated lysines.[99]

Because potential tumor suppressors and oncogenes are both present within the Jumonji protein family, subclass-specific inhibitors are necessary to determine how these enzymes act and if they are beneficial for patients. Among the inhibitors of JMJC demethylases that have been reported, few have sufficient potency

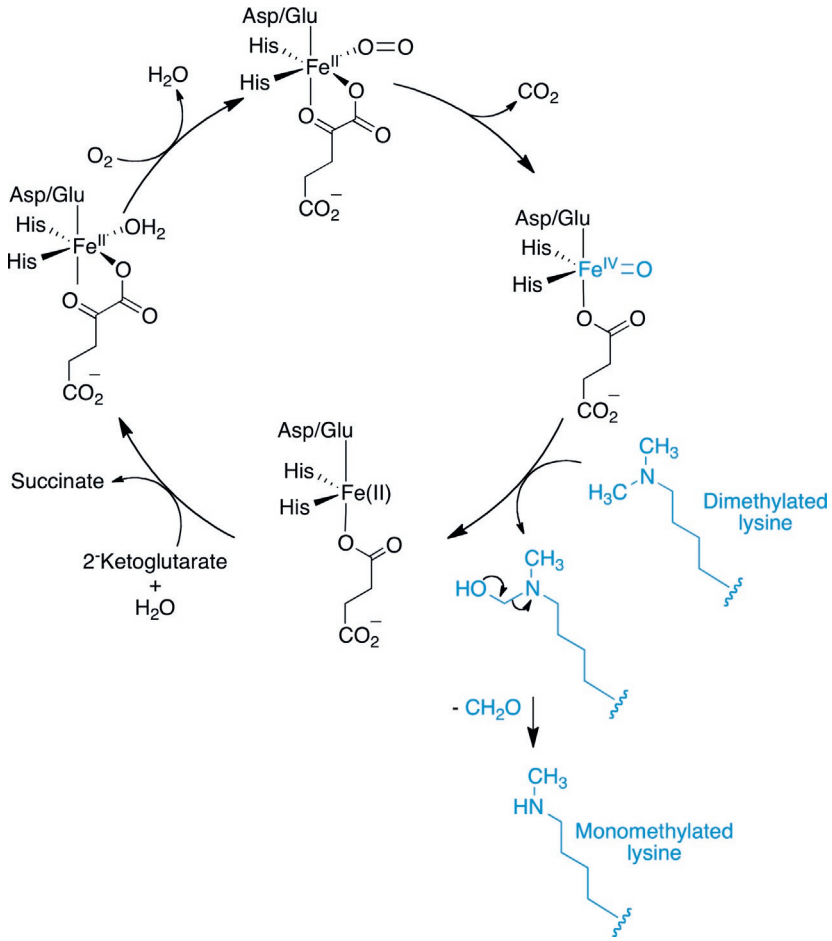
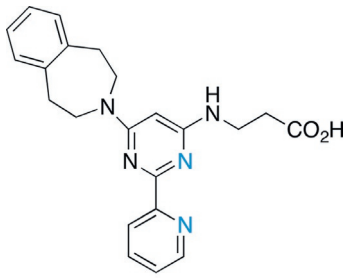


FIGURE 8.24

Mechanism of lysine demethylation by LSD enzymes containing the catalytic Jumonji C (JMJC) domain.

and selectivity to be considered lead structures for drug development. One of the most encouraging is GSK-J1, an inhibitor of the JMJD3 subfamily that binds competitively to the 2-oxoglutarate cofactor and is able to chelate the active site Fe(II) by the pyridine and pyrimidine nitrogens.[100]



GSK-J1

REFERENCES

1 Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. *Genes Dev* 2009;**23**:781.
2 For selected reviews, see. (a) Yoo CB, Jones PA. *Nature Rev Drug Discov* 2006;**5**:37; (b) Mair B, Kubicek S, Nijman SMB. *Trends Pharmacol Sci* 2014;**35**:136.
3 For a review, see Helin K, Dhanak D. *Nature* 2013;**502**:480.
4 Hou J, Wu J, Dombkowski A, Zhang K, Holowatyj A, Boerner JL, et al. *Am J Transl Res* 2012;**4**:247.
5 Amatu A, Sartore-Bianchi A, Moutinho C, Belotti A, Bencardino K, Chirico G, et al. *Clin Cancer Res* 2013;**19**:1.
6 Yoo CB, Cheng JC, Jones PA. *Biochem Soc Trans* 2004;**32**:910.
7 Lyko F, Brown R. *J Natl Cancer Inst* 2005;**97**:1498.
8 For reviews of DNA methyltransferase inhibitors, see. (a) Goffin J, Eisenhauer E. *Ann Oncol* 2002;**13**:1699; (b) Ren J, Singh BN, Huang Q, Li Z, Gao Y, Mishra P, et al. *Cell Signal* 2011;**23**:1082.
9 Kaminskas E, Farrell AT, Wang Y-C, Sridhara R, Padzur R. *Oncologist* 2005;**10**:176.
10 Gowher H, Jeltsch A. *Cancer Biol Ther* 2004;**3**:1062.
11 Issa J-P, Kantarjian H. *Nature Rev Drug Discov* 2005;**4**:S6.
12 Kaminskas E, Farrell AT, Abraham S, Baird A, Hsieh L-S, Lee S-L, et al. *Clin Cancer Res* 2005;**11**:3604.
13 Kuykendall JR. *Ann Pharmacother* 2005;**39**:1700.
14 Issa JPJ, Gharibyan V, Cortes J, Jelinek J, Morris G, Verstovsek S, et al. *J Clin Oncol* 2005;**23**:3948.
15 Qu Z, Fu J, Yan P, Hu J, Cheng SY, Xiao G. *J Biol Chem* 2010;**285**:11786.
16 Xu J, Zhou JY, Tainsky MA, Wu GS. *Cancer Res* 2007;**67**:1203.
17 Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. *Cancer Res* 2006;**66**:2794.
18 https://clinicalstudies.info.nih.gov/detail/A_2006-C-0221.html.
19 Matoušová M, Votruba I, Otmar M, Tloušťvá E, Günterová J, Mertlíková-Kaiserová H. *Epigenetics* 2011;**6**:769.
20 Dhingra HM, Murphy WK, Winn RJ, Raber NM, Hong WK. *Invest New Drugs* 2004;**9**:69.
21 Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. *J Natl Cancer Inst* 2003;**95**:399.
22 Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. *J Mol Biol* 2002;**321**:591.
23 Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, et al. *Cancer Cell* 2004;**6**:151.
24 Jeong LS, Buenger G, McCormack JJ, Cooney DA, Hao Z, Márquez VE. *J Med Chem* 1998;**41**:2572.
25 Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH. *Mol Cancer Ther* 2005;**4**:333.
26 Garcia J, Franci G, Pereira R, Benedetti R, Nebbioso A, Rodríguez-Barrios F. *Bioorg Med Chem* 2011;**19**:3637.

27 Baud MG, Leiser T, Haus P, Samlal S, Wong AC, Wood RJ, et al. *J Med Chem* 2012;**55**:1731.
28 Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, et al. *Cancer Res* 2003;**63**:7563.
29 Elbling L, Weiss RM, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R, et al. *FASEB J* 2005;**19**:807.
30 Brueckner B, García-Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. *Cancer Res* 2005;**65**:6305.
31 Lin Y-S, Shaw YIH, Wang SC, Hsu Ch-Ch, Teng I-W, Tseng M-J, et al. *J Biomed Sci* 2011;**18**:3.
32 For a review, see Medina-Franco JL, Caulfield T. *Drug Discov Today* 2011;**16**:418.
33 Siedlecki P, García Boy R, Comagic S, Schirrmacher R, Wiessler M, Zielenkiewicz P, et al. *Biochem Biophys Res Commun* 2003;**306**:558.
34 Savickiene J, Treigyte G, Jazdauskaite A, Borutinskaite VV, Navakauskiene R. *Cell Biol Int* 2012;**36**:1067.
35 Yoo J, Kim J, Robertson KD, Medina-Franco JL. *Adv Prot Chem Struct Biol* 2012;**87**:219.
36 https://clinicaltrials.gov/ct/show/NCT00324220.
37 For representative reviews, see. (a) Marks PA, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. *Nature Rev Cancer* 2001;**1**:194; (b) Johnstone RW. *Nature Rev Drug Discov* 2002;**1**:287; (c) Monneret C. *Eur J Med Chem* 2005;**40**:1; (d) Moradei O, Maroun CR, Paquin I, Vaisburg A. *Curr Med Chem Anticancer Agents* 2005;**5**:529; (e) Carey N, La Thangue NB. *Curr Opin Pharmacol* 2006;**6**:369; (f) Falkenberg KJ, Johnstone RW. *Nature Rev Drug Discov* 2014;**13**:673.
38 Flinnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, et al. *Nature* 1999;**401**:188.
39 Khanwani SL, Edelman MJ, Tait N. *Proc Am Soc Clin Oncol* 2001;**20**:86a.
40 Raffoux E, Chaibi P, Dombret H, Degos L. *Haematologica* 2005;**90**:986.
41 Rodríguez-Paredes M, Esteller M. *Nature Med* 2011;**17**:330.
42 Duvic M, Talpur R, Ni X, Zhang C, Hazarika P, Kelly C, et al. *Blood* 2007;**109**:31.
43 Marks PA, Breslow R. *Nature Biotechnol* 2007;**25**:84.
44 https://www.clinicaltrials.gov/ct/show/NCT00042900;
jsessionid=47CCC05C87B745FACD2295C56507E1A6?order=48.
45 Giles F, Fischer T, Cortes J, García-Manero G, Beck J, Ravandi F, et al. *Clin Cancer Res* 2006;**12**:4628.
46 De Bono JS, Kristeleit R, Tolcher A, Fong P, Pacey S, Karavasilis V, et al. *Clin Cancer Res* 2008;**14**:6663.
47 Maiso P, Carvajal-Vergara X, Ocio EM, López-Pérez R, Mateo G, Gutiérrez N, et al. *Cancer Res* 2006;**66**:5781.
48 http://1.usa.gov/YZGz99.
49 Sumanadasa SD, Goodman CD, Lucke AJ, Skinner-Adams T, Sahama I, Haque A, et al. *Antimicrob Agents Chemother* 2012;**56**:3849.
50 Wu L-M, Yang Z, Zhou L, Zhang F, Xie H-Y, Feng X-W, et al. *PLoS One* 2010;**5**:e14460.
51 Finazzi G, Vannucchi AM, Martinelli V, Ruggeri M, Nobile F, Specchia G, et al. *Br J Haematol* 2013;**161**:688.
52 Tong W-G, Wei Y, Stevenson W, Kuang S-Q, Fang Z, Zhang M, et al. *Leuk Res* 2010;**34**:221.
53 http://myeloma.org/pdfs/ASH2012_Raje_3929.pdf.
54 Bjornsson HT, Benjamin JS, Zhang L, Weissman J, Gerber EE, Chen Y-C, et al. *Sci Transl Med* 2014;**6**:256ra135.
55 Lauffer BE, Mintzer R, Fong R, Mukund S, Tam C, Zilberleyb I, et al. *J Biol Chem* 2013;**288**:26926.
56 Undevia SD, Kindler HL, Janisch L, Olson SC, Schilsky RL, Vogelzang NJ, et al. *Ann Oncol* 2004;**15**:1705.
57 (a) Whittaker SJ, Demierre MF, Kim EJ, Rook AH, Lerner A, Duvic M, et al. *J Clin Oncol* 2010;**28**:4485; (b) Coiffier B, Pro B, Prince HM, Foss F, Sokol L, Greenwood M, et al. *J Clin Oncol* 2012;**30**:631.
58 Byrd JC, Marcucci G, Parthun MR, Xiao JJ, Klisovic RB, Moran M, et al. *Blood* 2005;**105**:959.
59 Furamai R, Matsuyama A, Kobashi N, Lee K-H, Nishiyama M, Nakajima H, et al. *Cancer Res* 2002;**62**:4916.
60 Furamai R, Komatsu Y, Nishino N, Khochbin S, Yoshida M, Horinouchi S. *Proc Natl Acad Sci U S A* 2001;**98**:87.
61 Kwon SH, Ahn SH, Kim YK, Bae G-U, Yoon JW, Hong S, et al. *J Biol Chem* 2002;**277**:2073.

62 Buoncervello M, Borghi P, Romagnoli G, Spadaro F, Belardelli F, Toschi E, et al. *Neoplasia* 2012;**14**:855.
63 Komatsu Y, Tomizaki K-Y, Tsukamoto M, Kato T, Nishino N, Sato S, et al. *Cancer Res* 2001;**61**:4459.
64 Murakami K, Matsubara H, Hoshino I, Akutsu Y, Miyazawa Y, Matsushita K, et al. *Oncology* 2010;**78**:62.
65 https://www.clinicaltrials.gov/ct/show/NCT00101179.
66 Hubeek I, Comijn EM, Van der Wilt CL, Merriman RL, Padron JM, Kaspers GJ, et al. *Oncol Rep* 2008;**19**:1517.
67 https://www.clinicaltrials.gov/ct/show/NCT00374296;jsessionid=92563A45C8B57CDD90D171943745C815?order=8.
68 Witta SE, Jotte RM, Konduri K, Neubauer MA, Spira AI, Ruxer RL, et al. *J Clin Oncol* 2012;**30**:2248.
69 Shima JS, Leeb H-S, Shinc J, Kwonm HJ. *Cancer Lett* 2004;**203**:163.
70 Baud MGJ, Haus P, Leiser T, Meyer-Almes F-J, Fuchter MJ. *ChemMedChem* 2013;**8**:149.
71 Baud MGJ, Leiser T, Petrucci V, Gunaratnam M, Neidle S, Meyer-Almes F-J, et al. *Beilstein J Org Chem* 2013;**9**:81.
72 Mottet D, Pirotte S, Lamour V, Hagedorn M, Javerzat S, Bikfalvi A, et al. *Oncogene* 2009;**28**:243.
73 Isaacs JT, Antony L, Dalrymple SL, Brennen WN, Gerber S, Hammers H, et al. *Cancer Res* 2013;**73**:1386.
74 For a review, see Sebastián C, Satterstrom FK, Halgis MC, Mostosiavsky R. *J Biol Chem* 2012;**287**:4244.
75 Ota H, Tokunaga E, Chang K, Hikasa M, Iijima K, Eto M, et al. *Oncogene* 2006;**25**:176.
76 Lara E, Mai A, Calvanese V, Altucci L, López-Nieva P, Martínez-Chantar ML, et al. *Oncogene* 2009;**28**:781.
77 Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS, et al. *Mol Cell Biol* 2006;**26**:28.
78 Peck B, Chen Ch-Y, Ho KK, Di Fruscia P, Myatt SS, Coombes RCh, et al. *Mol Cancer Ther* 2010;**9**:844.
79 For representative reviews, see; (a) Filippakopoulos P, Knapp S. *Nature Rev Drug Discov* 2014;**13**:337; (b) Gallenkamp D, Gelato KA, Haendler B, Weinmann H. *ChemMedChem* 2014;**9**:438; (c) Sánchez R, Meslamani J, Zhou MM. *Biochim Biophys Acta Gene Regul Mech* 2014;**1839**:676
80 For a review of diazepine derivatives as bromodomain inhibitors, see Smith SG, Sánchez R, Zhou MM. *Chem Biol* 2014;**21**:573.
81 Filippakopoulos P, Qi PJ, Picaud S, Shen Y, Smith WB, Fedorov O, et al. *Nature* 2010;**468**:1067.
82 Zuber J, Vakoc C. *Nature* 2011;**478**:524.
83 Floyd SR, Pacold ME, Huang Q, Clarke SM, Lam FC, Cannell IG, et al. *Nature* 2013;**498**:246.
84 Matzuk MM, McKeown MR, Filippakopoulos P, Li Q, Ma L, Agno JE, et al. *Cell* 2012;**150**:673.
85 Mirguet O, Gosmini R, Toum J, Clément CA, Barnathan M, Brusq JM, et al. *J Med Chem* 2013;**56**:7501.
86 Varier RA, Timmers M. *Biochem Biophys Acta Rev Cancer* 2011;**1815**:75.
87 Greer EL, Yang S. *Nature Rev Genet* 2012;**13**:343.
88 Enroth S, Rada-Iglesias A, Andersson R, Wallerman O, Wanders A, Pahlman L, et al. *BMC Cancer* 2011;**11**:450.
89 (a) Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. *Mol Cancer Ther* 2009;**8**:1579; (b) Kikuchi J, Takashina T, Kinoshita I, Kikuchi E, Shimizu Y, Sakakibara-Konishi J, et al. *Lung Cancer* 2012;**78**:138.
90 Asangani IA, Ateeq B, Cao Q, Dodson L, Pandhi M, Kunju LP, et al. *Mol Cell* 2013;**49**:180.
91 Munshi NC, Anderson KC. *Clin Cancer Res* 2013;**19**:1881.
92 Koutsodontis G, Kardassis D. *Oncogene* 2004;**23**:9190.
93 Højfeldt JW, Agger K, Helin K. *Nature Rev Drug Discov* 2013;**12**:917.
94 Yu T, Higashi M, Cembran A, Gao J, Truhlar DG. *J Phys Chem B* 2013;**117**:8422.
95 Vintém APB, Price NT, Silverman RC, Ramsay RR. *Bioorg Med Chem* 2005;**13**:3487.
96 Mimasu S, Umezawa N, Sato S, Higuchi T, Umehara T, Yokoyama S. *Biochemistry* 2010;**49**:6494.
97 Maes T, Tirapu T, Mascaró C, Ortega A, Estiarte A, Valls N, et al. *J Clin Oncol* 2013;**31**(Suppl.):e13543.
98 Lohse B, Kristensen JL, Kristensen LH, Agger K, Helin K, Gajhede M, et al. *Bioorg Med Chem* 2011;**19**:3625.
99 Højfeldt JW, Agger K, Helin K. *Nature Rev Drug Discov* 2013;**12**:917.
100 Kruidenier L, Chung Ch, Cheng Z, Liddle J, Che K, Joberty G, et al. *Nature* 2012;**488**:404.

CHAPTER

ANTICANCER DRUGS TARGETING TUBULIN AND MICROTUBULES

9

CONTENTS

1 INTRODUCTION

Microtubules are filamentous intracellular structures that are responsible for several crucial aspects of cell morphology. They form the cytoskeleton in eukaryotic cells and are also responsible for various kinds of their movements because they are part of the cilia and flagella. They are rigid and relatively straight and serve as tracks to deliver vesicles and organelles to distal cellular regions. The integrity of microtubule structures is essential for cells to go through various cell cycle checkpoints because without it, programmed cell death or apoptosis is triggered. Finally, according to a controversial theory, consciousness depends on orchestrated coherent quantum processes in groups of microtubules within brain neurons.[1]

Microtubules are hollow structures formed by 13 parallel protofilaments that grow and shorten by the reversible, noncovalent addition of tubulin dimers at their ends. Tubulin is a protein that contains two subunits called α and β in a head-to-tail arrangement. Microtubules and free tubulin dimers are

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00009-0

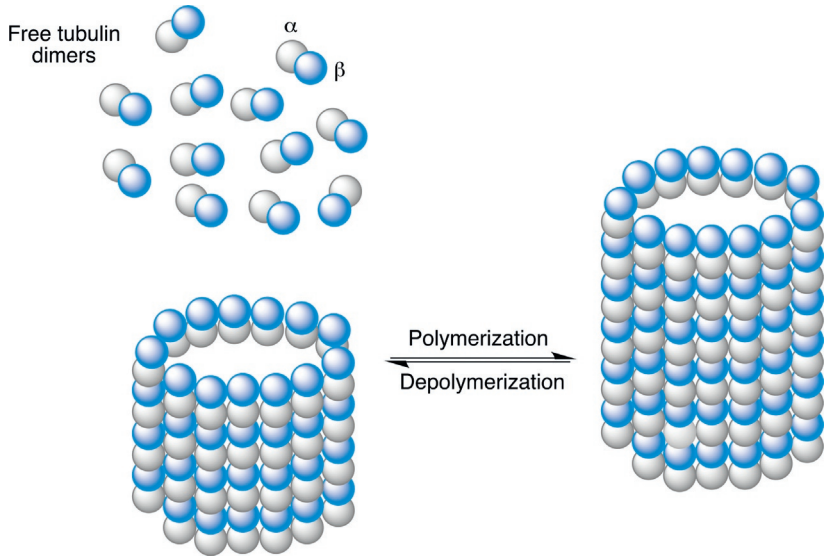


FIGURE 9.1

Dynamic equilibrium between microtubules and tubulin dimers.

involved in a highly dynamic equilibrium, which is very sensitive to external factors (Figure 9.1). The α,β-tubulin dimers bind to GTP before assembling onto the (+) end of the microtubule, and when the dimer is incorporated, the molecule of GTP corresponding to the β subunit eventually hydrolyzes to GDP. Dimers bound to GTP tend to remain in the microtubule, whereas those bound to GDP tend to fall apart. For this reason, GTP hydrolysis is essential for the dynamic instability of the microtubule and hence for the polymerization–depolymerization equilibrium.

A very important structure generated from microtubules is the mitotic spindle, which is used by eukaryotic cells to segregate their chromosomes correctly during cell division and to allow the transfer of the chromosomes of the original cell to the daughter cells. During cell division, microtubules in the cytoplasmic network depolymerize, and the tubulin thus liberated is again polymerized to give the mitotic spindle.

Several important antitumor drugs exert their action by disrupting these equilibria. These drugs are known as microtubule-targeting agents. They act either by binding to tubulin and inhibiting polymerization or by binding to the microtubules and inhibiting depolymerization by stabilizing them.[2,3] This leads to inhibition of the formation of the mitotic spindle; therefore, these compounds behave as antimitotic agents.

Microtubules are the main target of cytotoxic natural products, and most of the drugs discussed in this chapter were discovered in large-scale screens of natural materials. These compounds are highly successful in cancer treatment;[4] indeed, it has been argued that microtubules represent the best cancer target known so far. This conclusion seems to be supported by the fact that from an evolutionary standpoint, the microtubule seems to be a preferred target for natural cytotoxic agents because a large number of plants and marine organisms produce structurally diverse compounds able to potently bind nearly identical sites on microtubules.

Drugs acting on microtubules bind to several sites of tubulin and at different positions on the microtubules, but they all suppress microtubule dynamics, thereby blocking mitosis at the metaphase/anaphase transition and inducing cell death. The spindle microtubules are much more dynamic than the cytoskeletal ones, and they exchange their tubulin units with the soluble pools with half-times of approximately 15 sec, which explains why drugs that interfere with microtubule dynamics are so effective against dividing cells.

Based on their behavior at high concentrations, antitumor drugs acting on microtubules have been traditionally classified into two groups: drugs that inhibit microtubule polymerization (microtubule-destabilizing agents) and drugs that stimulate microtubule polymerization (microtubule-stabilizing agents). Although this time-sanctioned classification is adopted for organizing this chapter, it is probably overly simplistic because it has been shown that at low concentrations, both types of drugs act similarly by stabilizing spindle microtubule dynamics.[5]

Microtubule inhibitors such as taxanes and the *Vinca* alkaloids are important anticancer drugs used in the treatment of breast, ovarian, and lung cancer.[6] However, the response of cells to microtubule inhibitors is highly variable,[7] potentially compromising clinical efficacy. How these drugs cause cell death remains unclear, but induction of mitotic arrest appears to be a key aspect of the mechanism.[8] By perturbing the mitotic spindle, these drugs activate the spindle assembly checkpoint, which delays mitotic exit by inhibiting the ubiquitin ligase activity of the anaphase-promoting complex/cyclosome (see Chapter 11, Section 2.1.3).

An interesting feature of drugs acting on microtubules is the synergistic effects that can often be found among them, potentially allowing their combination, avoiding high doses of any individual drug.

2 DRUGS THAT INHIBIT MICROTUBULE POLYMERIZATION

There are three main binding sites of drugs to tubulin, which are designed according to their best known ligands as the *Vinca*, colchicine, and taxol sites (Figure 9.2). For some of them, further research has uncovered the existence of different subsites corresponding to different ligand structural families.

2.1 COMPOUNDS BINDING AT THE *VINCA* SITE

2.1.1 Vinca *Alkaloids and Their Synthetic Analogs*

Vincristine and vinblastine are complex molecules produced by the leaves of the rosy periwinkle plant *Catharanthus roseus* (*Vinca rosea*), whose potent cytotoxicity was discovered in 1958. They were introduced in cancer chemotherapy in the late 1960s and remain in widespread clinical usage. Despite their very similar structures and common mechanism of action, they have widely different toxicological properties and antitumor spectra. Thus, vinblastine is currently used in the treatment of Hodgkin's disease and metastatic testicular tumors, for which it is combined with bleomycin and cisplatin, whereas vincristine is used in the treatment of leukemia and lymphomas.

Several semisynthetic analogs of these alkaloids[9] are also in clinical use. The most notable of these are vindesine, used mainly to treat melanoma and lung carcinomas and—associated with other drugs—to treat uterine cancers, and the nor-derivative vinorelbine, used for non-small cell lung cancer, metastatic breast cancer, and ovarian cancer and first approved in 1995 for the first of these indications. The fluorinated analog vinflunine showed a better antitumor activity than vinorelbine and vinblastine in preclinical studies and entered clinical development.[10]

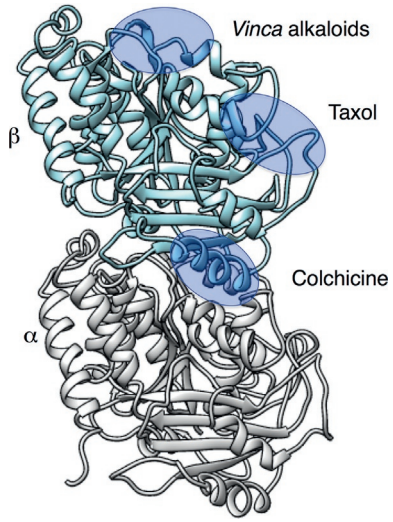
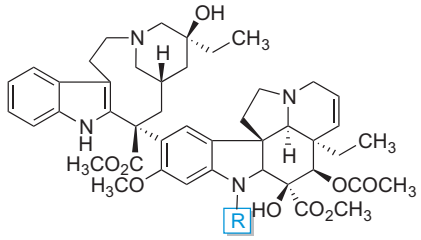
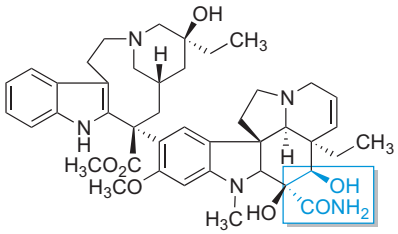


FIGURE 9.2

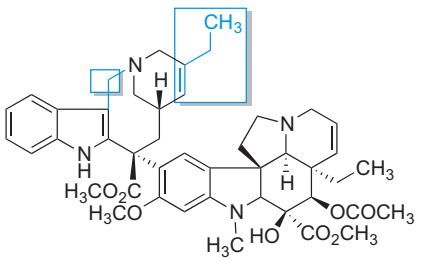
Main sites of action of antimicrotubule anticancer drugs. The tubulin dimer was generated from Protein Data Bank reference 1TUB and displayed with Chimera 1.8.



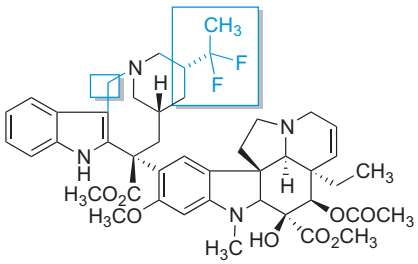
R = CHO Vincristine
R = CH_3 Vinblastine



Vindesine



Vinorelbine



Vinflunine

The *Vinca* alkaloids specifically block cells in mitosis with metaphase arrest, and hence they are antimitotic drugs. Their biological activity is explained by their specific binding to the β subunit of tubulin dimers in a region called the *Vinca* domain. Binding is fast and reversible, and it induces a

conformational change in tubulin, increasing its affinity for itself and leading to the formation of paracrystalline aggregates. This decreases the pool of free tubulin dimers available for microtubule assembly, resulting in a shift of the equilibrium toward microtubule shrinkage and disassembly. These phenomena result in microtubule depolymerization and destruction of the mitotic spindles, as verified in HeLa cells at high concentrations (10–100 nM) (Figure 9.3). As a consequence, dividing cells are blocked in mitosis with condensed chromosomes.

The previously described mechanism led to the *Vinca* alkaloids being thought for many years to act solely as microtubule-depolymerizing agents. However, recent observations have shown that at concentrations that are low but clinically relevant (0.8 nM in HeLa cells), the spindle microtubules are not depolymerized but mitosis is still blocked and cells die by apoptosis. This suggests that the block is due to suppression of microtubule dynamics rather than to microtubule depolymerization.

One of the drawbacks of *Vinca* alkaloids and their analogs is their neurotoxicity, which is probably related to the fact that microtubules are a key component of neurons. Another problem associated with the use of *Vinca* alkaloids is the easy development of resistance, normally mediated by the overexpression of the Pgp 170 transport protein (see Chapter 14, Section 2).

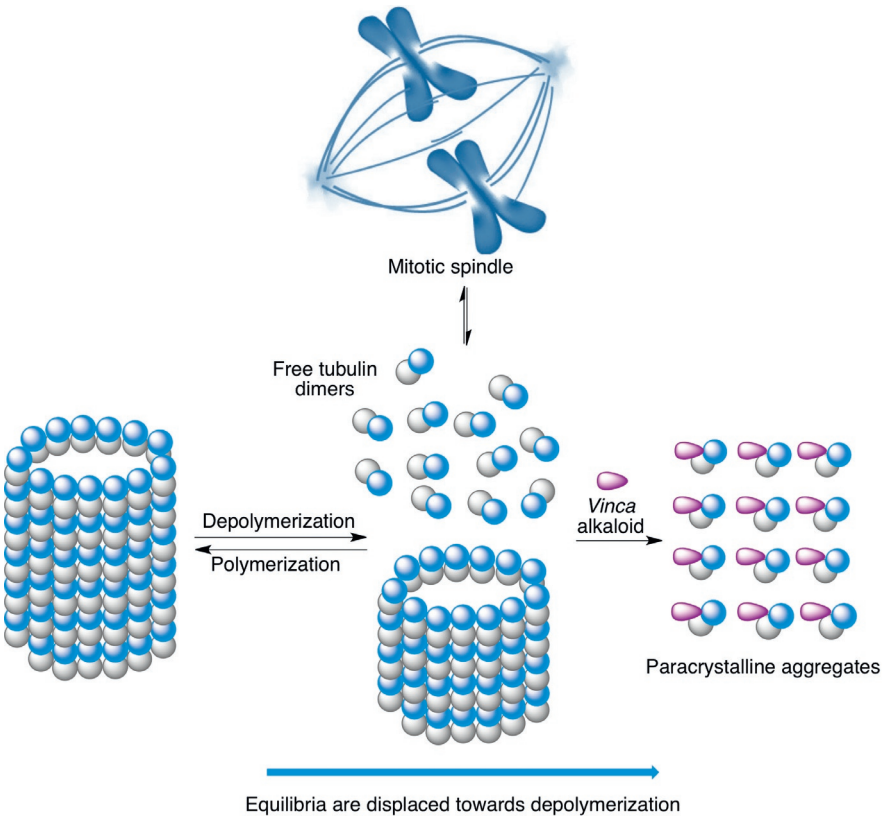


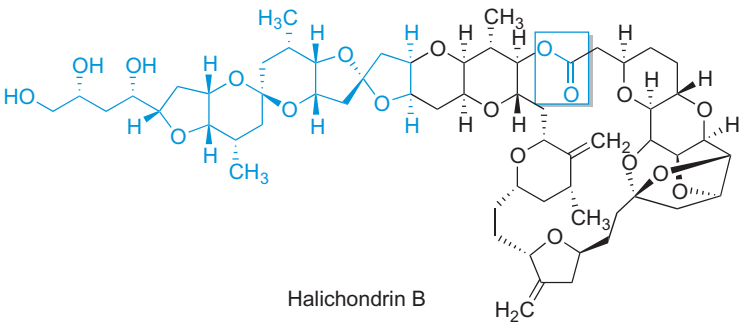
FIGURE 9.3

Depolymerization of microtubules following binding of *Vinca* alkaloids.

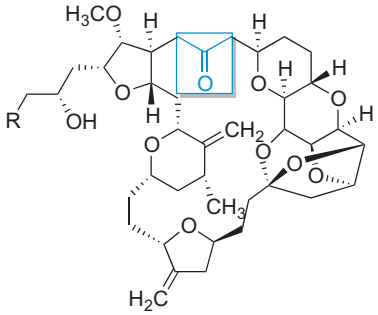
2.1.2 Marine Natural Products Binding at the Vinca Domain and Their Analogs

Marine organisms are a rich source of antitumor compounds that have probably evolved as defense mechanisms in the highly competitive marine environment, and many of these compounds are in advanced preclinical or clinical stages of development.[11] Those acting on microtubules by binding at the *Vinca* domain include the halichondrins, the dolastatins, the hemiasterlins, the cryptophicins, and the spongistatins.

Halichondrin B is a complex polyether macrolide first isolated from the marine sponge *Halichondria okadai*. It has an extraordinarily high potency as an antitumor agent and a high therapeutic index. Although its scarcity in natural sources has hampered efforts to develop halichondrin B as a new anticancer drug, the existence of a route allowing its total synthesis[12] has paved the way for the preparation of structurally simpler, fully synthetic analogs that retain the remarkable potency of the parent compound, especially the closely related eribulin (E-7389, ER-086526) and ER-076349.[13] In addition to the deletion of a large region of the molecule, in these compounds the readily biodegradable lactone group has been replaced with a ketone. Eribulin mesylate (Halaven®), the most active of these synthetic compounds, was approved by the U.S. Food and Drug Administration (FDA) in 2010 and by the European Medicines Agency in 2011 for the treatment of late-stage metastatic breast cancer in patients who have previously received at least two conventional chemotherapeutic regimens.[14] It is also being investigated for use in a variety of other solid tumors, including non-small cell lung cancer, prostate cancer, and sarcoma,[15] and it is in phase II clinical studies in patients with soft tissue sarcoma.[16] The primary antimitotic mechanism of action of eribulin is suppression of microtubule growth.[17]



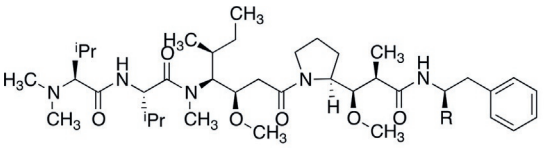
Halichondrin B



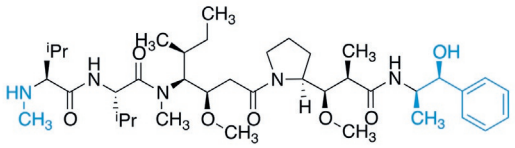
R = NH_2 Eribulin (E-7389, ER-086526, Halaven®)
R = OH ER-076349

Dolastatin 10, a linear peptide,[18] was isolated in 1987 from an Indian Ocean mollusk, the sea hare *Dolabella auricularia*. Although this compound progressed to phase II trials as a single agent, it did not demonstrate significant antitumor activity against prostate cancer[19] nor metastatic melanoma.[20] Soblidotin (auristatin PE, TZT-1027) is another dolastatin 10 analog that has undergone clinical trials.[21]

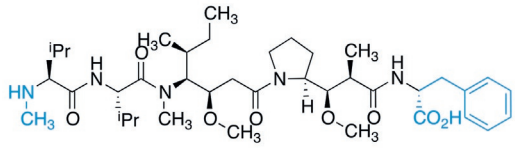
Vedotin (monomethylauristatin E) is a secondary metabolite from a *Symploca* cyanophyte. Due to its high potency, it has been used as the cytotoxic payload of a large number of drug–antibody conjugates, which are discussed in Section 4.6 of Chapter 13.



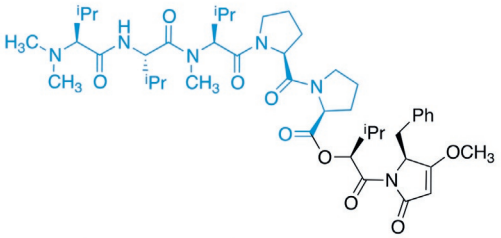
Dolastatin 10 R = 
Soblidotin R = H



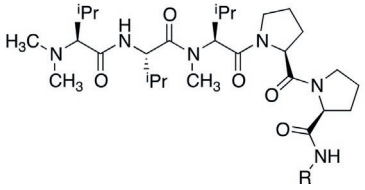
Vedotin (monomethylauristatin E)



Monomethylauristatin F

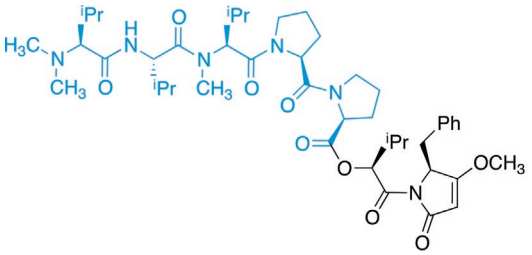


Dolastatin 15

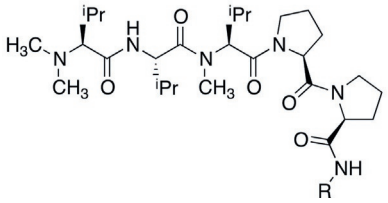


R = $PhCH_2$ Cemadotin (LU-103793)
R = $C(CH_3)_3$ Synthadotin

Dolastatin 15 is a related seven-subunit depsipeptide, also obtained from *D. auricularia*. Many synthetic analogs of this natural product have been prepared, among which cemadotin (LU-103793)[22] and synthadotin (ILX651) have entered clinical trials. The latter compound has the advantage of being orally active and seems to be promising for the treatment of non-small lung cell cancer and refractory prostate cancer.[23]



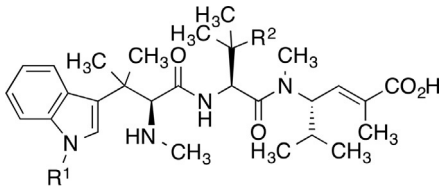
Dolastatin 15



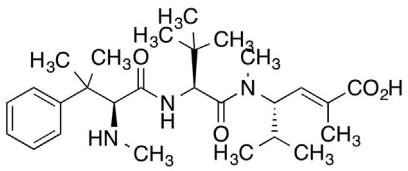
R = $PhCH_2$ Cemadotin (LU-103793)
R = $C(CH_3)_3$ Synthadotin

The dolastatins bind to a "peptide site" very close to the *Vinca* domain.[24] In connection with this finding, it has been shown that the *Vinca* domain in tubulin may be composed of a series of overlapping domains rather than being a single entity because different levels and types of competition were found when selected tubulin interactive agents were used to investigate the binding characteristics of a tritium-labeled dolastatin probe.[25]

Hemiasterlins are a family of natural tripeptides originally isolated from the South African sponge *Hemiasterella minor*. These agents show cytotoxicity in the nanomolar range and inhibit tubulin assembly, probably by binding at the "peptide binding site" shared with the dolastatins and cryptophycins.[26] In comparison with other marine peptides, hemiasterlins have the advantage of a simpler structure, and this has allowed the preparation of many synthetic analogs, one of which, taltobulin (HTI-286),[27] has the advantage of being a poor substrate for P-glycoprotein drug transporters and is undergoing clinical trials.[28]

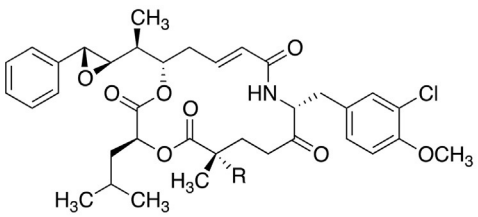


$R^1 = R^2 = Me$ Hemiasterlin
$R^1 = H, R^2 = Me$ Hemiasterlin A
$R^1 = R^2 = H$ Hemiasterlin B
$R^1 = Me, R^2 = H$ Hemiasterlin C

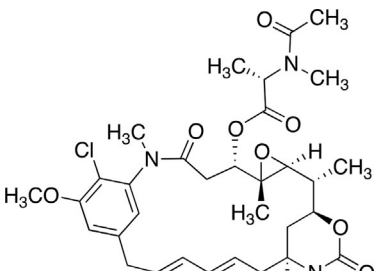


Taltobulin (HTI-286)

Cryptophycin-1 is a depsipeptide isolated from the cyanobacterium *Nostoc* species that was initially described as an antifungal agent[29] and was later shown to have antimitotic and cytotoxic activity. Subsequently, many cryptophycins have been isolated and prepared by synthesis, the most important one being cryptophicin 52 (LY-3555703), which entered clinical trials for the treatment of solid tumors.[30] The cryptophycins are among the most potent antimitotic agents described, and their binding is very strong and poorly reversible, making them relatively exempt from efflux by the Pgp-170-mediated multidrug resistance (MDR) mechanism.[31] The somewhat related natural product maytansine 1 is another inhibitor of tubulin polymerization, approximately 1000-fold more potent than vincristine. Although its toxicity led to abandoning clinical trials, it was approved by the FDA in 2013 as the conjugate trastuzumab emtansine or ado-trastuzumab emtansine (Kadcyla®) (see Chapter 13, Section 4.6).

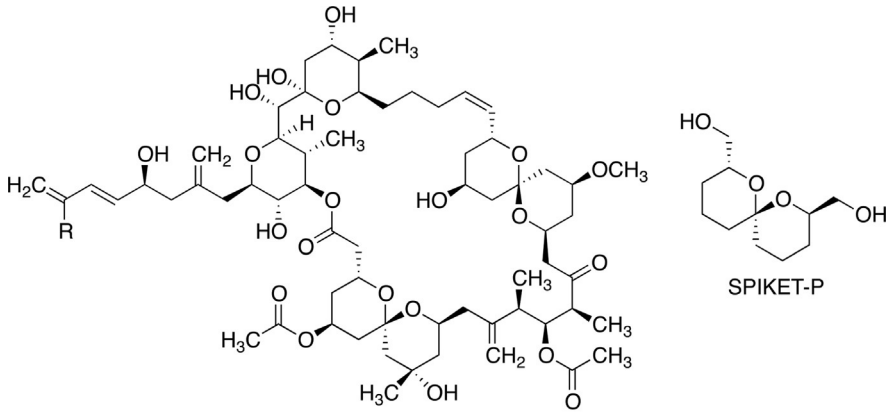


Cryptopyicin 1 R = H
Cryptopyicin 52 R = CH_3
(LY-355703)



Maytansine 1

The spongistatins[32] are macrocyclic lactones containing six pyran rings, four of which are incorporated into two spiroketal moieties, which were isolated from sponges of the *Hytrios* genus. The spongistatins elicit extraordinarily potent (10^{-11} M) cytotoxic responses, especially in solid tumors, and they are being examined in phase I clinical trials.[33] Spongistatin 1 is a noncompetitive inhibitor of the binding of [^{3}H]vinblastine and [^{3}H]dolastatin to tubulin, in contrast to competitive patterns obtained with vincristine versus [^{3}H]vinblastine and with a stereoisomer of dolastatin 10 versus [^{3}H]dolastatin 10. Because dolastatin 10 is itself a noncompetitive inhibitor of *Vinca* alkaloid binding to tubulin, this implies the existence of at least three distinct binding sites in the *Vinca* domain.[34] Molecular modeling studies of the binding of the spongistatins led to the discovery of a hydrophobic pocket containing an unusual cluster of 10 aromatic amino acids, which allowed the rational design of the SPIKET compounds, containing a single spiroketal system. SPIKET-P inhibited the division of human breast cancer cells at low-nanomolar concentrations.[35]



R = Cl Spongistatin 1
R = H Spongistatin 2

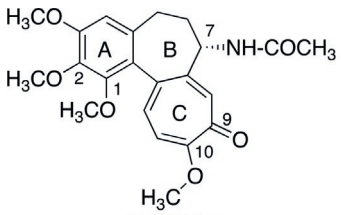
2.2 COMPOUNDS BINDING AT THE COLCHICINE SITE

The colchicine site[36] is named from the well-known tropolone alkaloid isolated from the autumn crocus *Colchicum autumnale*, a plant widely employed in traditional medicine and still used in the treatment of gout. It was the first compound to be identified as a tubulin binder, but it has not been employed in cancer chemotherapy as such. Several compounds binding in the colchicine site, including the combrestatins 2-methoxyestradiol and ABT-751, are under clinical investigation[37] and have the advantage of showing few issues with MDR, the main cause for anticancer chemotherapy failure (see Chapter 14, Section 2). Furthermore, they seem to have little sensitivity to mutations in tubulin and overexpression of the βIII-tubulin isoform, which is a significant mechanism of resistance to other tubulin targeting agents (e.g., paclitaxel and vinorelbine).

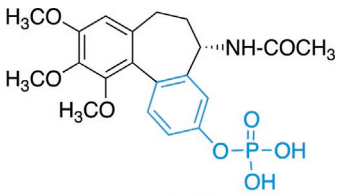
In addition to their antimitotic properties, some of these compounds are receiving much attention because of their vascular actions, which are due to the crucial role of microtubules in the regulation of endothelial cell biology (see Section 5).

Colchicine played a fundamental role in studies of mitosis, but it has not found significant use in cancer treatment because of the toxicity shown in clinical trials. Similarly to *Vinca* alkaloids, colchicine depolymerizes microtubules at high concentrations and stabilizes microtubule dynamics at low concentrations. It first binds to soluble tubulin, leading to a complex that copolymerizes into the ends of the microtubules and suppresses their dynamics because it binds more tightly to its tubulin neighbors than free tubulin. The structural features required for this binding were elucidated by extensive structure–activity relationship (SAR) studies that showed the importance of the 9-keto function and the methoxy groups at C-1, C-2, and C-10. The 7-acetamido function is not required for binding to tubulin and may be replaced by other substituents, although the stereochemistry of this center is critical for antimitotic activity probably because of the effect of this substituent in the overall conformation of the colchicine molecule. Ring B appears to be responsible for the irreversible nature of colchicine binding to tubulin, although it may also contribute to its toxic effects. Finally, the tropolone ring C may be replaced by a suitably substituted benzene ring with retention of the antimitotic activity. Thus, compound ZD6126, a water-soluble phosphate prodrug, was examined for the

treatment of metastatic colorectal cancer, although the clinical study was halted during phase II due to the observation of cardiotoxicity.[38]



Colchicine



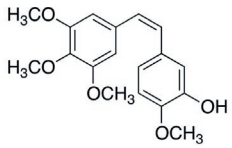
ZD6126

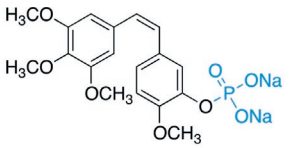
The combretastatins are natural stilbenoid phenols isolated from the African willow tree *Combretum caffrum*.[39,40] They bear structural similarities with colchicine because both possess a trimethoxyphenyl ring and the aromatic tropone ring of colchicine can be considered as related to the isovanillinyl group in the combretastatins. The most active member of the family is combretastatin A-4, which is a very effective antimitotic agent due to its rapid binding to tubulin at the colchicine site. Structure–activity studies showed that the *cis* configuration of its stilbene moiety is a critical structural feature for its activity. Its low water solubility stimulated the preparation of a number of prodrugs,[41] the most studied of which is 4-*O*-phosphate fosbretabulin (CA4P, Zybrestat®). This compound has been studied in phase II for anaplastic thyroid cancer, non-small cell lung cancer, and relapsed ovarian cancer, among others.[42] Combretastatin A-1 diphosphate (CA-1P, Oxi4503) is under preclinical studies for the treatment of patients with relapsed and refractory acute myelogenous leukemia and myelodisplastic syndrome. Ombrabulin (AVE8062) is another orally bioavailable amino acid prodrug of a combretastatin analog with improved water solubility that disrupts the blood vessel formation in tumors and has undergone a number of clinical studies.[43]

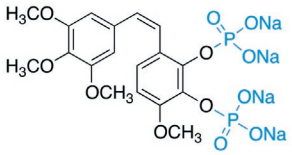
The *cis*-olefin structure of combretastatins is associated with *in vivo* stability problems due to its *cis–trans* isomerization. The following are the main approaches that have been explored to overcome this problem:

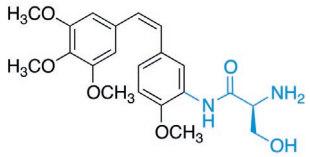
1. Displacement of one of the aromatic rings to give a 1,1-diarylethylene derivative. These compounds are known as isocombretastatins, exemplified by CC5097, a dual inhibitor of tubulin polymerization and phosphodiesterase-4.
2. Replacement of the two-carbon *cis*-olefin fragment by a one-carbon bridge. This led to the preparation of the phenstatins, which are benzophenone derivatives with highly potent antimitotic activity and a water solubility higher than that of the equivalent combretastatins. BNC-105P is a modified phenstatin in which one of the phenyl rings has been replaced by a benzofuran. It is a prodrug that is transformed into the active form (BNC-105) by phosphatases in plasma and endothelial cells. A phase II clinical study of the use of BNC105P as second-line therapy for advanced malignant pleural mesothelioma has been carried out.[44]
3. Replacement of the stilbene unit by two phenyl rings linked by sulfate/sulfonamide units or heterocycles (pyrazole, imidazole, triazole, and 2-oxazinone, among others).[37] These compounds are still under preclinical study and are not discussed further.

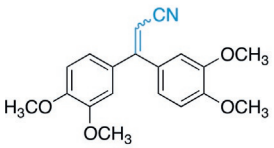
The vascular effects of combretastatin A-4 and its derivatives are discussed in Section 5.

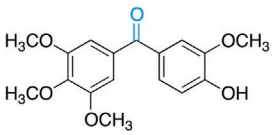

Combretastatin A-4 (CA-4)

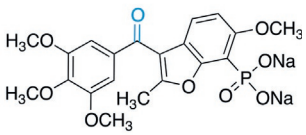

Fosbretabulin (CA4P, Zybrestat)


Oxi4503

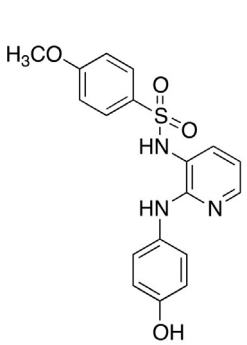

Ombrabulin (AVE8062)

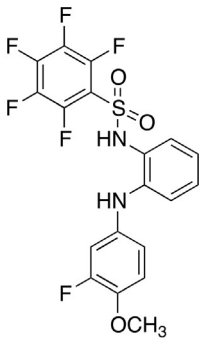

CC5097


Phenstatin


BCN-105P

The methoxybenzenesulfonamide derivative E-7010 (ABT-751)[45] is an oral tubulin binder that has been clinically investigated in solid tumors and refractory hematological malignancies,[46] and T-138067 is a related sulfonamide that has been studied in phase II clinical trials.


E-7010 (ABT-751)


T-138067

Whereas E-7010 is a standard colchicine domain ligand, T-138067 acts by a different mechanism despite their similarity because it binds covalently to β-tubulin by S_N-Ar attack of its Cys-239 residue to the pentafluorophenyl ring of the drug, thereby preventing the polymerization of the tubulin dimers to microtubules (Figure 9.4).[47]

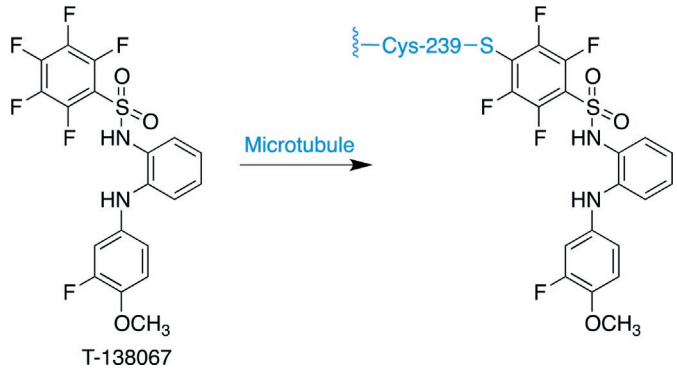
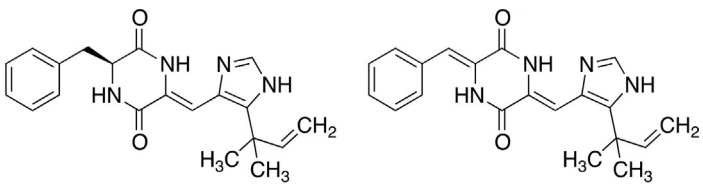


FIGURE 9.4

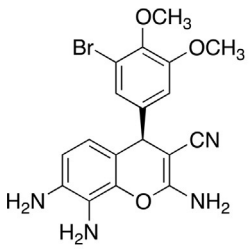
Covalent binding of T-138067 to tubulin.

The natural diketopiperazine (−)-phenylahistin (halimide), isolated from *Aspergillus ustus* in 1997, was shown to inhibit the cell cycle in the G_2/M phase by inhibiting tubulin polymerization. Plinabulin (NPI-2358), a simplified analog of the natural product, entered phase II clinical trials. A large number of analogs of this lead compound have been prepared with a view to improve its potency and aqueous solubility.[48] Plinabulin derivatives probably do not interact with the colchicine binding site. Instead, they interact with the boundary region between the α- and β-tubulins around the colchicine site.

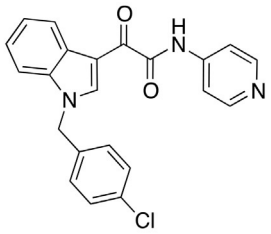


Several additional heterocyclic compounds have been identified as ligands of the colchicine binding site. Crolibulin (EPC2407) is a member of a family of 4-aryl-4*H*-chromenes that inhibits tubulin polymerization and induces apoptosis. This compound is undergoing a number of clinical studies, including a phase II clinical trial for the treatment of anaplastic thyroid cancer. Indibulin (D-24851, ZIO-301), an indole derivative, is an antimitotic drug active against various human tumor cell lines that has interesting features, including oral activity and the absence of neurotoxicity (the dose-limiting toxicity for most tubulin-binding drugs), and it is under clinical trials.[49] The 4-anilinoquinazoline derivative verubulin (MPC-6827, Azixa®) inhibited tubulin polymerization and was active in several cancer mouse models, and these promising findings spurred its clinical evaluation.[50] The structurally related

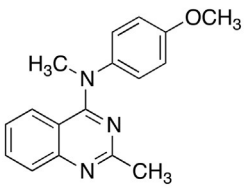
CYT-997 is an orally active 4-aminopyrimidine derivative that binds to the colchicine site and acts as a vascular disrupting agent. This compound is in phase II clinical trials in combination with carboplatin for the treatment of relapsed glioblastoma multiforme.[51] Denibulin (MN-029) is a benzimidazole carbamate that reversibly inhibits microtubule assembly and had antivascular effects in tumors, leading to the induction of necrosis. A phase I clinical study of this compound in patients with advanced solid tumors showed a good tolerance and a decrease in vascular parameters of the tumor.[52] CI-980 [(*S*)-(−)-NSC 613862] is a 1,2-dihydropyrido[3,4-*b*]pyrazine derivative that potently inhibits tubulin polymerization by binding to the colchicine binding site and has undergone phase II clinical trials in previously untreated extensive small cell lung cancer.[53]



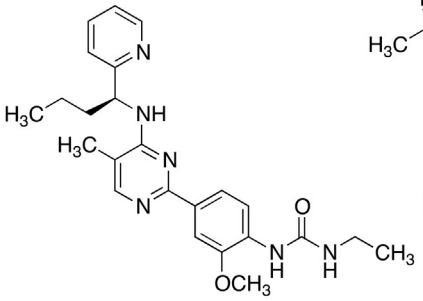
Crolibulin (EPC-2407)



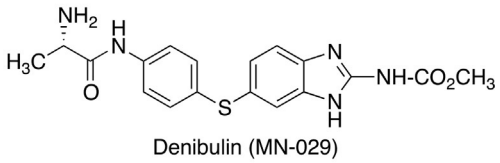
Indibulin (D-24851, ZIO-301)



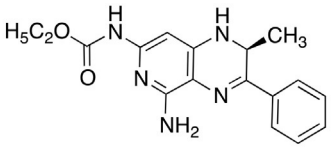
Verubulin (MPC-6827, Azixa)



CYT-997

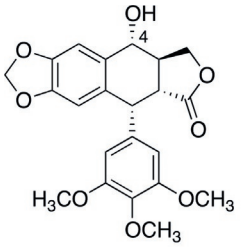


Denibulin (MN-029)

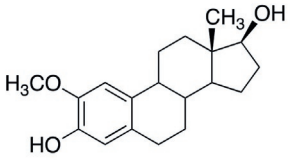


CI-980

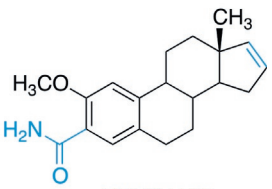
Finally, some terpene-type ligands of the colchicine binding site are discussed. The lignane derivative podophyllotoxin, which was mentioned in Section 6.3.1 of Chapter 7 as the lead compound in the development of a family of topoisomerase II inhibitors, is also a ligand of the colchicine site.[54] Its tubulin binding is greatly reduced by epimerization at C-4 (epipodophyllotoxin) and completely abolished by the presence of sugar molecules, as found in etoposide. Another compound that binds tubulin at the colchicine domain and is undergoing clinical trials is 2-methoxyestradiol (2-ME), which is being studied for the treatment of solid tumors.[55] This compound is metabolized by phase II conjugative metabolism at C-3 and C-17 and also by oxidation of its C-17 hydroxy to a ketone (2-methoxyestrone). The conjugated forms of 2-ME are inactive, and C-17 oxidation results in 10- to 100-fold loss in activity *in vitro*; these observations have prompted the design of ENMD-1198, which binds to the colchicine binding site in tubulin and displays both antiangiogenic and vascular-disrupting properties;[56] it has entered phase I clinical trials.



Podophyllotoxin



2-Methoxyestradiol



ENMD1198

3 MICROTUBULE-STABILIZING AGENTS: COMPOUNDS BINDING AT THE TAXANE SITE

The primary ligand for this site in tubulin is the natural terpene taxol, although several structurally dissimilar natural products (epothilones, eleutherobin, discodermolide, and others) were found to share the same mechanism of action. Based on extensive SAR studies and molecular modeling, a plausible common pharmacophore for those microtubule-stabilizing agents has been proposed.

3.1 TAXANES

Paclitaxel (taxol) is the most important natural product in cancer chemotherapy and one of the most successful cancer drugs ever produced, being widely employed in the treatment of breast, ovarian, and lung carcinomas. It was isolated from the Pacific yew *Taxus brevifolia*, and its anticancer activity was discovered in the 1960s during a large-scale plant-screening program sponsored by the National Cancer Institute. Enormous supply problems were encountered initially because the location of taxol in the bark required sacrificing the tree to extract it, the concentration of the compound in yew bark is low, its extraction is complex and expensive, and the Pacific yew is a limited resource that grows very slowly. Approximately 4000 trees were required to provide 360 g of taxol for the early clinical trials, and 38,000 trees were necessary to isolate 25 kg of taxol to treat 12,000 cancer patients after approval of the use of taxol for treating advanced ovarian cancer in 1992. Fortunately, it was subsequently discovered that the twigs and needles of the European yew, *Taxus baccata*, are a high-yielding (1 g/kg) and renewable source of a related compound—10-deacetylbaccatin III, lacking the C-13 side chain and the C-10 acetyl group—that could be transformed through a relatively simple semisynthetic route into paclitaxel and also into its more soluble and potent analog, docetaxel (Taxotere®), which was approved for advanced breast cancer in 1996 (Figure 9.5). Some other suitable baccatin derivatives have been subsequently discovered in different *Taxus* species that can serve as alternative starting materials in the semisynthesis of taxoids. Production of taxol in *T. baccata* suspension cultures[57] and by other biotechnological approaches[58] is also under consideration.

Paclitaxel arrests cells at the G_2/M stage of the cell cycle by stabilizing the spindle microtubules and thus arresting mitosis (Figure 9.6). It binds specifically at the 1–31 and 217–233 sequences of the

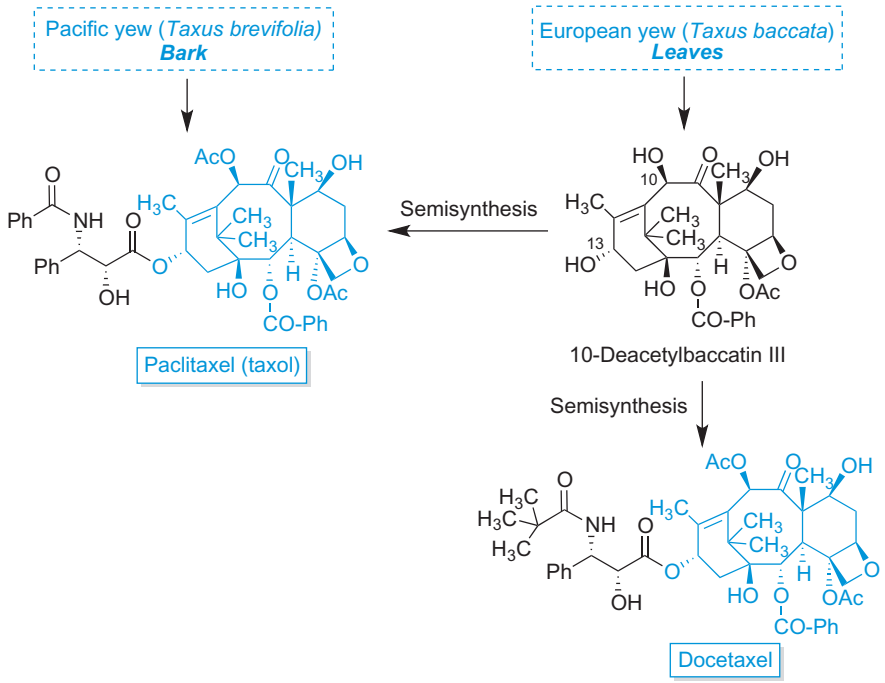


FIGURE 9.5

Semisynthesis of taxanes.

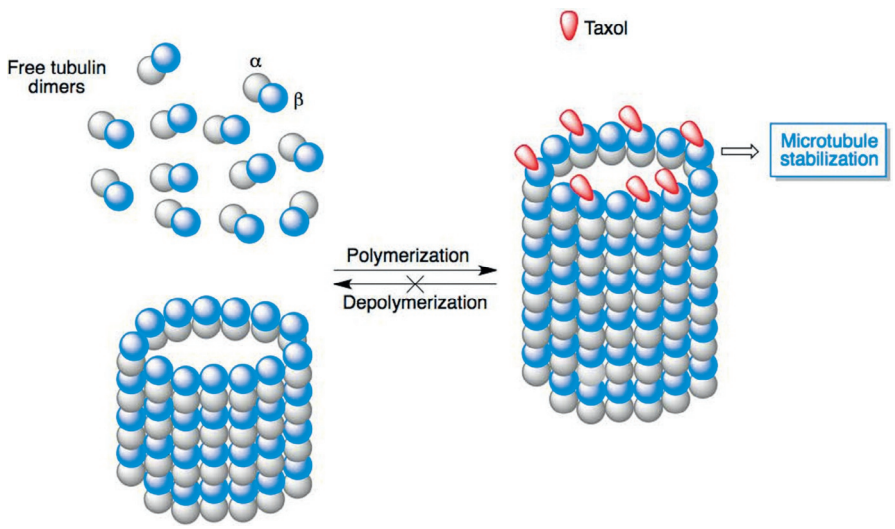


FIGURE 9.6

Mechanism of action of taxol.

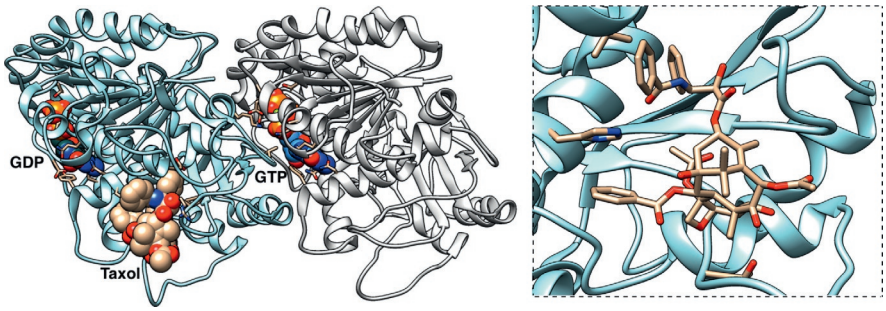
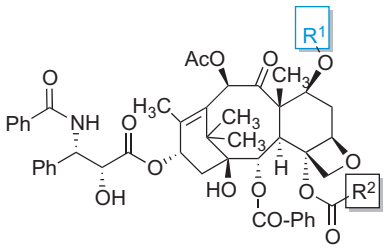


FIGURE 9.7

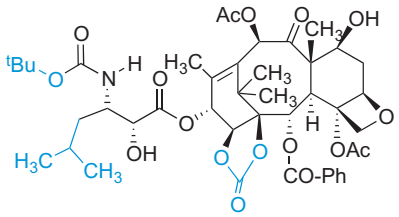
The taxol–tubulin complex. Generated from Protein Data Bank entry 1JFF and displayed with Chimera 1.8.1.

β-tubulin subunit, at the inner surface of the microtubule lumen (Figure 9.7), and it shows much higher affinity for tubulin in microtubules than for free tubulin in solution. Paclitaxel also increases the microtubule polymer mass, a phenomenon known as microtubule "bundling."

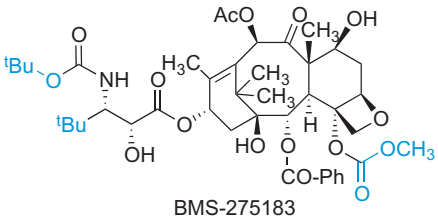
Although paclitaxel and docetaxel are widely used for the therapy of a variety of solid tumors and are being investigated clinically for numerous other cancers, they have some limitations. The main ones are the impossibility of oral administration and the frequent development of resistance mediated by tubulin mutation, leading to weaker interactions, or by overexpression of the Pgp-170 transport pump, leading to efflux from the cell. Another problem is the need to associate them with formulation vehicles to allow their administration. Thus, paclitaxel, very insoluble in water, is generally formulated using polyoxyethylated castor oil (Cremophor EL), whereas docetaxel, more soluble in water, is formulated using Tween 80 and ethanol. Cremophor EL is responsible for many hypersensitivity reactions, and Tween 80, albeit less toxic than Cremophor, may also be responsible of some toxic effects.[59] These problems have stimulated the search for new taxoids, several of which are under clinical evaluation.[60] Among the first-generation analogs are BMS-188797 and BMS-184476, which have improved pharmacokinetic properties. More substantial variations can be observed in ortataxel, in which the aromatic rings of paclitaxel have been replaced by other lipophilic substituents and the hydroxyl at the bridgehead position is part of a cyclic carbonate structure. Ortataxel shows increased potency with respect to paclitaxel and is the first orally active taxoid. Another structurally related, orally active, semisynthetic taxane is BMS-275183. Both ortataxel[61] and BMS-275183[62] have reached clinical trials for solid tumors. Cabazitaxel (XRP-6582, Jevtana®) is a potent tubulin ligand and a poor substrate for glycoprotein P-170, and therefore it is suitable for docetaxel-resistant tumors. This compound was first approved by the FDA in 1996 for the treatment of locally advanced or metastatic breast cancer. Among other applications, it was also approved in 2010 for the treatment of hormone-refractory prostate cancer.[63] Larotaxel (XRP-9881, RPR-109881) has been involved in several clinical studies, including a phase III trial in combination with cisplatin as first-line treatment for locally advanced or metastatic urothelial tract or bladder cancer.[64] Tesetaxel (DJ-927) is undergoing clinical trials for several types of cancer, including advanced or metastatic breast cancer, advanced gastric cancer, and other solid tumors.[65] Finally, milataxel is also being clinically studied for several indications, including advanced colorectal cancer in previously treated patients[66] and recurring or progressive malignant mesothelioma following previous chemotherapy.[67]



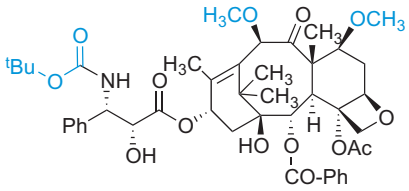
R^1	R^2	Compound
H	H_3CO	BMS-188797
H_3C-S-CH_2	CH_3	BMS-184476



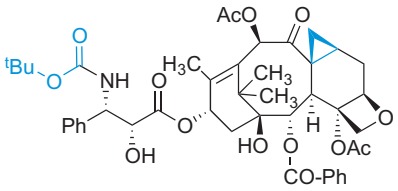
Ortataxel (BAY 59-8862, IDN-5109)



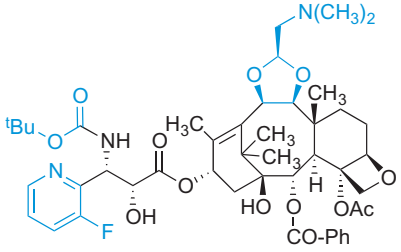
BMS-275183



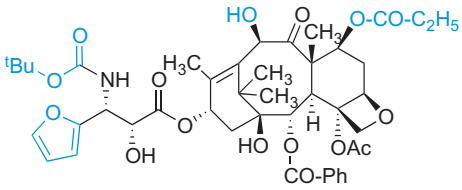
Cabazitaxel (XRP-6258, Jevtana)



Larotaxel (XRP9881, RPR109881)



Tesetaxel (DJ-927)



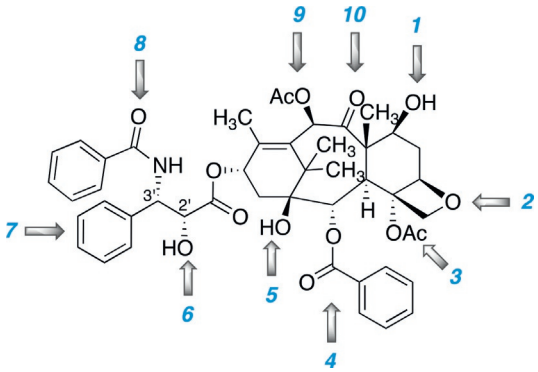
Milataxel (MAC-31, TL-139)

The large number of taxol analogs that have been synthesized has allowed the establishment of several SARs,[57] which are summarized as follows:[68]

1. The hydroxyl group ***1*** is not essential and can be removed, epimerized, or esterified.
2. The oxetane ring ***2*** (or a small-ring analog) is essential for activity.

3. The presence of acyl substituents ***3***, ***4***, and ***8*** is essential. Other acetoxy and benzoyloxy groups present in the natural product may be replaced by other acyls or removed.
4. Removal of the hydroxyl ***5*** leads only to a slight decrease in activity.
5. A free hydroxyl group in the side chain (***6***) is required. Esterification is possible if the ester group is easily hydrolyzable, leading to a variety of water-soluble and cell-specific paclitaxel prodrugs.
6. The phenyl group at the end of the side chain (***7***), or a close analog, is required for activity.
7. Reduction of the carbonyl ***10*** leads to slightly improved activity.

In summary, the northern half of the molecule allows more structural variations than the southern portion. The 2′*R*-3′*S*-isoserine side chain is also a key element in the antitubulin activity.



Structural and molecular modeling studies, as well as the evaluation of conformationally restricted analogs, have been undertaken to explain these SARs. The taxane core has a rigid conformation, and the side chain is the only portion of the molecule with rotational freedom. It can adopt a variety of conformations, two of which were identified as the potential active conformations and differ only in the value of the H2′–C2′–C3′–H3′ dihedral angle. Further research based on electron crystallographic analysis of tubulin sheets has led to evidence showing that taxol adopts a T-shaped conformation when it is bound to tubulin.[69] This binding model has been confirmed by the synthesis of a macrocyclic analog that adopts the T-Taxol conformation and is significantly more active than paclitaxel in both cytotoxicity and tubulin polymerization assays;[70] it is being used in the design of new taxanes.[71]

The clinical success of taxanes, many of which are under clinical development,[72] continues to promote new synthetic efforts[73] and has prompted an intensive search for drugs with a related mechanism of action. This search has led to the identification of several families of natural products that bind to the taxane site and share the ability of taxol to promote microtubule assembly and induce mitotic arrest. These are discussed in the next two sections.

3.2 EPOTHILONES

Epothilones A and B (a name derived from their molecular features—epoxide, thiazole, and ketone) are naturally occurring 16-membered macrolides that were isolated in 1993 from the myxobacterium

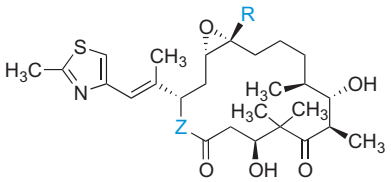
Sorangium cellulosum and first employed as agrochemical antifungal agents. In 1995, their taxol-like mechanism of action was discovered,[74] and subsequently they have been shown to have a number of advantages over taxoids, including a higher potency in some cases, activity against taxol-resistant cell lines because they seem to be poor P-170 substrates, higher aqueous solubility and simpler structures, leading to easier access to analogs.[75,76] Epothilone B (EPO-906, patupilone) has been granted orphan designation by the European Commission for the treatment of ovarian cancer.[77] Epothilone D, also known as desoxyepothilone B, KOS-862, or NSC-703147, displays a much more promising therapeutic index than epothilone B despite its slightly decreased *in vitro* cytotoxicity,[78] and it is under clinical assays as second-line therapy in non-small cell lung cancer.[79] Resistences to epothilone are known, but their mechanism seems to involve mutations in tubulin rather than upregulation of drug efflux pumps.[80]

One of the limitations of the natural epothilones is their metabolic lability, resulting from the easy hydrolysis of their lactone ring by esterases. This led to the design of metabolically more stable lactam analogs, among which ixabepilone (BMS-247550, Ixempra®) underwent clinical trials in colorectal, prostate, metastatic breast, and non-small cell lung cancers, among others,[81,82] and became the first epothilone to be approved by the FDA for the treatment of metastatic or locally advanced breast cancer as monotherapy or in combination with capecitabine after failure of other treatments.

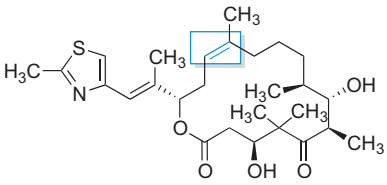
Another limitation of the epothilones is their poor water solubility, which requires its formulation with cosolubilizers. For instance, ixabepilone is formulated in Cremophor, leading to hypersensitivity reactions that require the prophylactic administration of oral histamine blockers.[83] This has stimulated the development of water-soluble analogs such as the semisynthetic amino derivative of epothilone B known as BMS-310705, which is under clinical assays in patients with advanced solid cancer.[84]

Sagopilone (ZK-EPO) is a promising, fully synthetic epothilone that was designed to overcome multidrug resistance.[85] This compound, currently under clinical assays,[86] exhibited significant activity across a broad spectrum of preclinical tumor models, including those resistant to widely used chemotherapeutic agents, because it is not recognized by cellular efflux pumps. It is also more water soluble than taxanes and does not require a formulating agent such as Cremophor. Another compound modified at the thiazole substituent is ABJ-879, which also proves the feasibility of replacing the epoxide oxygen by a methylene group. ABJ-879 is slightly more potent than epothylone B or paclitaxel at inducing tubulin polymerization, but it is much more potent as an antiproliferative agent and has entered clinical studies.[87]

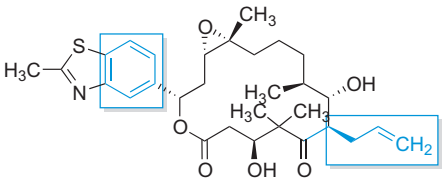
The discovery by the Danishefsky group of (*E*)-9,10-dehydroepothilones[88] as promising anticancer candidates prepared by totally synthetic means[89] paved the way for the preparation of several modified epothilones that have reached clinical status. Two of them are dehydelone (KOS-1584), the 9,10-dehydro derivative of epothilone B, and fludelone, containing a trifluoromethyl substituent instead of a methyl and showing better activity than taxol in cancer xenografts.[90] It was later discovered that replacement of the 2-methyl-4-thiazolyl group by a 5-methylisoxazolyl led to improved solubility along with better potency and thus to the discovery of iso-fludelone (KOS-1803), which entered clinical studies in patients with advanced solid tumors.[91]



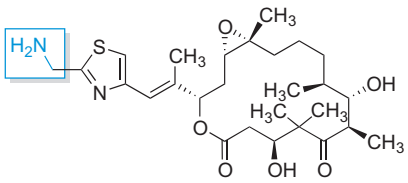
Compound	Z	R
Epothilone A	O	H
Epothilone B (EPO-906, patupilone)	O	CH_3
Ixabepilone (BMS-247550)	NH	CH_3



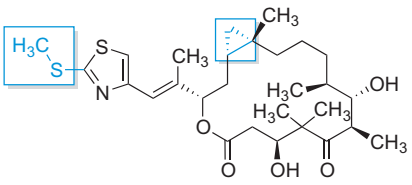
Epothilone D (KOS-862, NSC-703147)



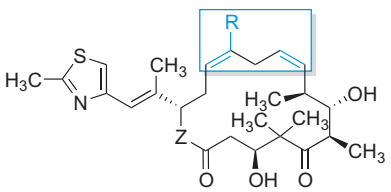
Sagopilone (ZK-EPO)



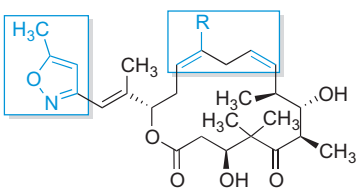
BMS-310705



ABJ-879



R = CH_3 Dehydelone (KOS-1584)
R = CF_3 Fludelone (KOS-1591)

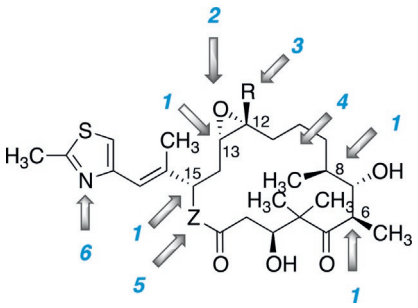


R = CH_3 KOS-1724
R = CF_3 Iso-fludelone (KOS-1803)

Hundreds of epothilone analogs have been prepared using conventional solution chemistry or combinatorial strategies. Their screening has allowed the establishment of a detailed SAR profile,[74,92,93] which is summarized as follows:

1. The configuration at the stereocenters C-6, C-8, C-13, and C-15 is important and must be that of the natural products (***1***).
2. The epoxide function is not essential, and it may be replaced by a C_{12}–C_{13} double bond or a cyclopropane ring (***2***), with diminished toxicity. Analogs incorporating a *trans* epoxide or *trans* olefin structure at C_{12}–C_{13} appear to be almost equipotent with the corresponding *cis* isomers.
3. A methyl group at C-12 enhances activity (***3***).

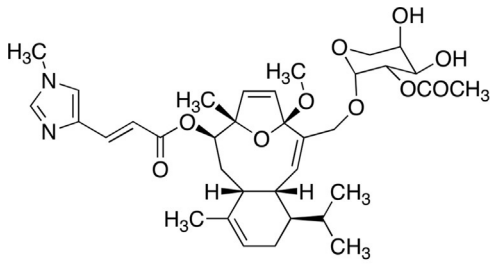
4. Expansion to a 17-membered ring, created by the presence of a *trans* C_{11}–C_{12} double bond and an additional methylene, leads to a compound in which antiproliferative activity is substantially maintained (***4***).
5. Z can be O or NH. In the latter case, the molecule is metabolically more stable.
6. A correct location for the nitrogen in the side chain at C-15 is significant for activity. However, the replacement of the thiazole ring either by an oxazole or by various pyridine moieties is well tolerated (***6***).



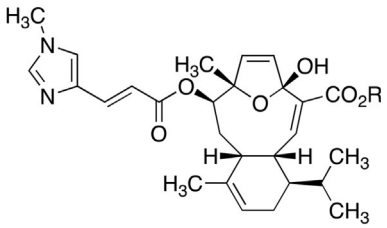
The epothilone binding site in tubulin has been studied by three-dimensional quantitative SAR models[94] and by electron crystallography, which has allowed identification of a common binding site on tubulin for paclitaxel, epothilone A, and eleutherobin.[95] Prior to these studies, a common pharmacophore had been proposed for the taxanes, the epothilones, and the sarcodictyines.[96] The interaction of epothilone A with the β subunit of the tubulin dimer is shown in Figure 9.8.

3.3 MISCELLANEOUS MARINE COMPOUNDS THAT BIND TO THE TAXANE SITE

Eleutherobin is a natural product isolated from an *Eleutherobia* marine soft coral that is extremely potent for inducing tubulin polymerization *in vitro* and is cytotoxic *in vitro* for cancer cells, with an IC_{50} value lower than that of paclitaxel.[97] Like paclitaxel, eleutherobin is a substrate for P-glycoprotein, and both compounds show cross-resistance in *MDR1*-expressing lines. The related sarcodictyins were also isolated from the Mediterranean coral *Sarcodictyon roseum* and seem more promising than eleutherobin, despite their lower activities, because of the MDR sensitivity of the latter.



Eleutherobin



R = CH_3 Sarcodictyin A
R = C_2H_5 Sarcodictyin B

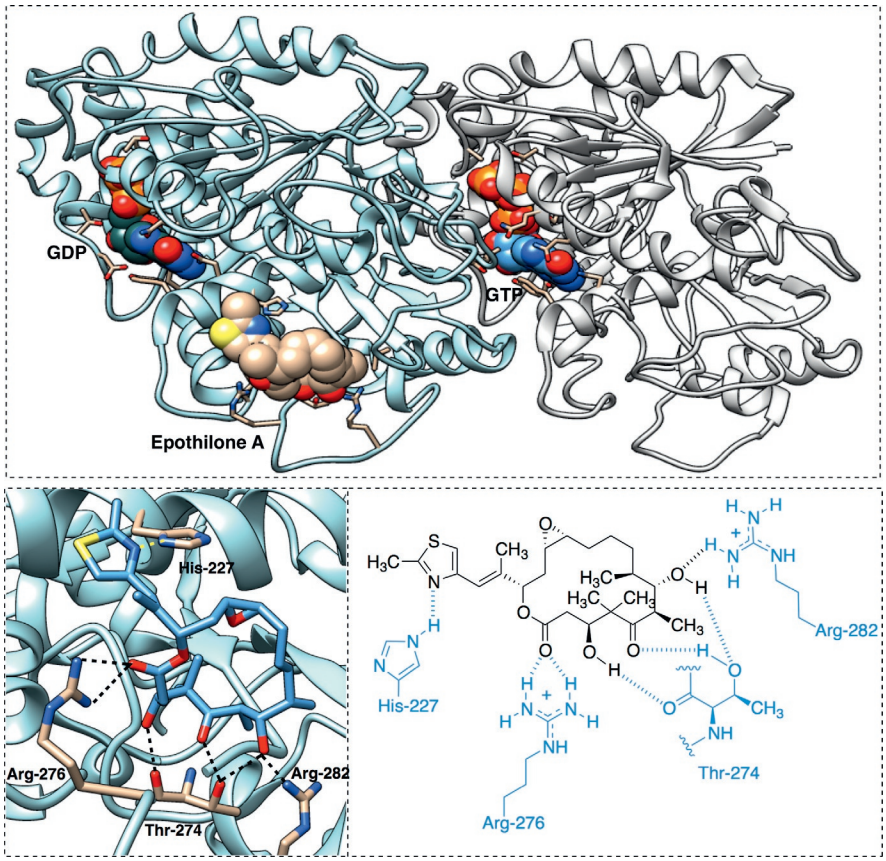
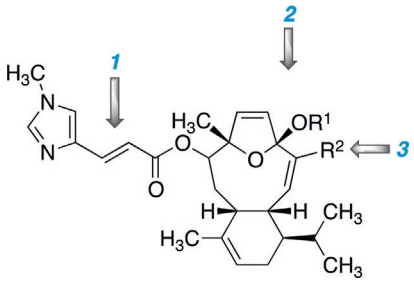


FIGURE 9.8

Interaction of epothilone A with the β subunit of the tubulin dimer, determined by electron diffraction. The epothilone–tubulin complex was generated from Protein Data Bank entry 1TVK and displayed with Chimera 1.8.1.

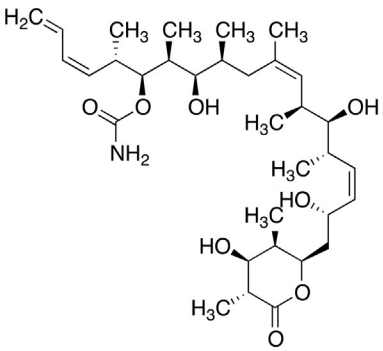
The eleutherobins and sarcodictyins have been extensively modified using conventional and combinatorial chemistry techniques, which have also allowed the formation of hybrid molecules of the two base structures. These studies have led to several conclusions regarding their SARs:[69,98]

1. The side chain is essential for activity. Both nitrogen atoms of the imidazole ring are important (***1***).
2. Both OH (hemiketal) and OCH_3 (ketal) R^1 groups are tolerated, with little difference in activity.
3. In eleutherobin, removal or modification of the sugar moiety (R^2) alters the cytotoxicity and resistance pattern. In the sarcodictyins, esters are more active than amides (***3***).

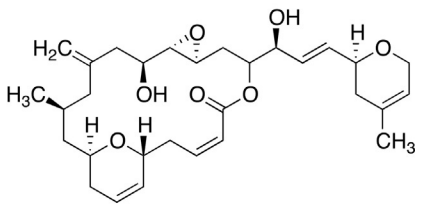


Discodermolide is a polyketide from the sponge *Discodermia dissoluta* with a mechanism of action similar to those of both paclitaxel and the epothilones. Discodermolide has several promising features, such as its broad-spectrum antitumor activity, its potent inhibition of taxane- and epothilone-resistant tumors in cell cultures and in animal models, and its synergic effect when combined with paclitaxel.[99] Early clinical evaluations with discodermolide have begun in patients with various advanced solid malignancies,[100] but because only small amounts are available from natural sources, all discodermolide used for preclinical activities as well as for the ongoing clinical trials has been supplied by total synthesis[101,102] using an impressive 39-step process that has been described by a leading synthetic chemist as "probably the best piece of synthetic work to come out from an industrial company."[103]

The Okinawan ocean sponge *Fasciospongia rimosa* and other Pacific sponges produce a potent microtubule-stabilizing agent called laulimalide or figianolide B. Although laulimalide is less potent than paclitaxel in drug-sensitive laboratory cell lines, it is up to 100 times more potent in MDR cell lines, again because it is a very poor substrate of Pgp-170. Another similarity with discodermolide is its synergistic action with paclitaxel.[104]



Discodermolide

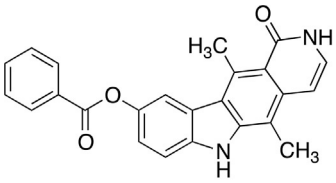


Laulimalide

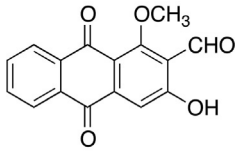
3.4 INHIBITORS OF LIM KINASE

Some proteins that interact with microtubule growing tips may be interesting targets for microtubule stabilization. The binding of these proteins to tubulin is regulated by phosphorylation/dephosphorylation processes or by tubulin modifications such as the tubulin tyrosination cycle.[105]

Actin cytoskeletal dynamics and remodeling are central to a variety of cell activities, including cell migration, division, morphogenesis, and gene expression. Among numerous actin-regulatory proteins, the actin-depolymerizing factor cofilin plays an essential role in regulating actin cytoskeletal dynamics and reorganization by severing and disassembling actin filaments. The binding of actin with cofilin is inhibited by phosphorylation of its serine residue at position 3 (Ser-3) near the N-terminus. An enzyme that phosphorylates and inactivates cofilin is LIM kinase 1 (LIMK1). Among the discovered inhibitors of this enzyme, Pyr-1 inhibits cell migration and invasion, being active in MDR cells. It was found in a cell-based assay that recognizes microtubule polymerization status to screen for chemicals that interact with regulators of microtubule dynamics. Pyr-1 reversibly stabilizes microtubules and blocks actin microfilament dynamics; therefore, it represents a potential approach to cancer treatment.[106] Damnacanthal is an anthraquinone extracted from *Morinda citrifolia* with antitumorigenic activity that inhibits several kinases, showing a preferential interaction with LIMK1.[107]



Pyr-1



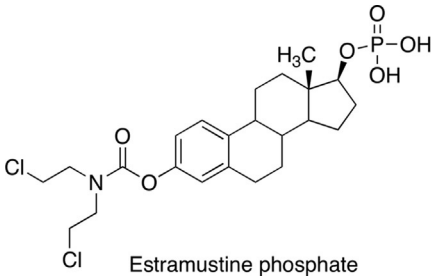
Damnacanthal

4 MISCELLANEOUS ANTICANCER DRUGS ACTING ON NOVEL SITES OF TUBULINE

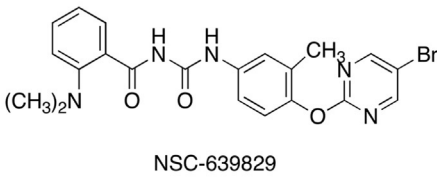
Estramustine phosphate, previously mentioned in Section 2.4 of Chapter 5 as an alkylating agent, is used for the palliative treatment of advanced methastatic prostate cancer, alone or in combination with other antitubulin agents such as vinblastine, paclitaxel, or ixabepilone.[108] Its transport into these tumors is due to the existence in the prostate of an estramustine binding protein (EMBP), which facilitates its uptake.[109] The fact that, on exposure to estramustine, cells were arrested in metaphase and the mitotic spindle was absent suggested that it interacts with microtubules to promote their disassembly. It was subsequently proven that this drug estramustine depolymerizes microtubule networks, inhibiting cell growth and inducing mitotic arrest, by a direct interaction with microtubule-associated proteins and with tubulin.[110,111]

Estramustine is administered as a phosphate prodrug, which is inactive because it does not enter the cells but is rapidly metabolized to the active species. Estramustine depolymerizes microtubule networks, inhibiting cell growth and inducing mitotic arrest, by binding to a site different from other drugs.[112,113] It has only moderate activity, which requires its combination with other antitubulin agents such as vinblastine, paclitaxel, or ixabepilone.[114]

NSC-639829 is a representative of the benzoylphenylurea (BPU) class of compounds,[115] which were developed initially as insecticides but showed antitumor activity in random screening. It inhibits tubulin polymerization by binding to a novel site and is being evaluated in clinical trials in patients with refractory metastatic cancer.[116] NSC-639829 is also a potent inhibitor of DNA polymerase.

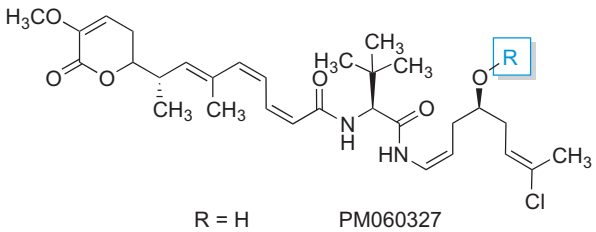


Estramustine phosphate



NSC-639829

A new family of tubulin-binding agents isolated from the marine sponge *Lithoplocamia lithistoides* includes PM-060327 and PM-050489. The first of these compounds, which is currently produced by total synthesis, demonstrated a very strong antitumor activity in preclinical models as well as an acceptable toxicology profile in preclinical evaluation,[117] and it is currently being assessed in phase I clinical studies.



R = H PM060327
R = $OCONH_2$ PM050489

Unlike vinblastine, these ligands induce tubulin self-association only weakly. Interestingly, the study of the interaction of these compounds with tubulin by a number of techniques has revealed a previously unknown binding mode at the association interface between tubulin heterodimers, probably at Asn residues (Figure 9.9).

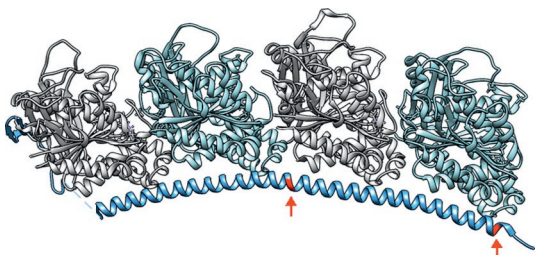


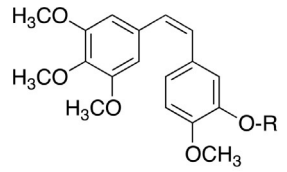
FIGURE 9.9

Scheme showing the approximate position of the β-tubulin Asn residues where PM-050489 binds. Generated from Protein Data Bank entry 3UT5 and displayed with Chimera 1.8.1.

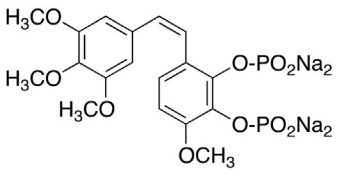
5 ANTIVASCULAR EFFECTS OF MICROTUBULE-TARGETED AGENTS

The tumor vasculature is an attractive target for tumor therapy.[118] The main approach to inhibiting vascular function in tumors is antiangiogenic therapy, which is discussed in Chapters 10 and 11. However, it has been shown that some compounds, especially microtubule-targeted agents, have the ability to shut down existing vasculature at tumors due to depolymerization of the microtubule cytoskeleton at the endothelial cells (vascular-targeting agents).[119,120] Furthermore, the compounds of this group that are under development seem to damage tumor vasculature with preference to normal tissues. This selectivity for the microvessels of tumors may reflect, in part, variability in the cytoskeletal makeup of rapidly proliferating endothelial cells inherent to microvessels feeding tumor cells versus the normally proliferating endothelial cells of microvessels serving healthy cells.[121]

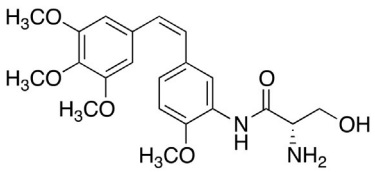
Among the tubulin-targeted agents previously discussed, the most efficient at harming tumor vasculature are the ones targeting the colchicine site.[122] Several compounds of this type have entered clinical trials, including some derivatives of the combretastatins, such as combretastatin A-4-3-*O*-phosphate, combretastatin A-2 phosphate, AVE-8062A,[123] the *N*-acetylcolchinol phosphate ZD-6126,[124,125] and the flavonoid 5,6-dimethylxanthenone acetic acid (DMXAA, AS-1404). The previously mentioned TZT-1027, which binds in the *Vinca* domain, is also in clinical trials as a small-molecule vascular disrupting agent.



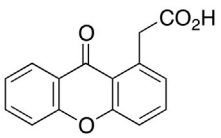
R = H Combretastatin A-4
R = PO_2Na_2 CA-4P



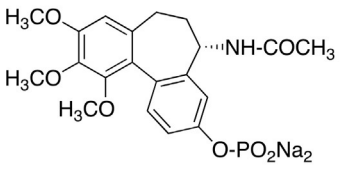
OXI-4503



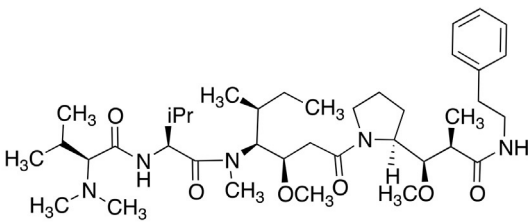
AVE-8062 (AC-7700)



DMXAA (AS-1404)



ZD-6126



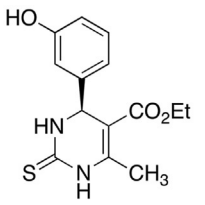
TZT-1027

Another field of interest for these drugs that is also under clinical evaluation is the therapy of various retinopathies such as the wet form of age-related macular degeneration, in which inhibition of the formation of eye vasculature is beneficial, and other vascular diseases.

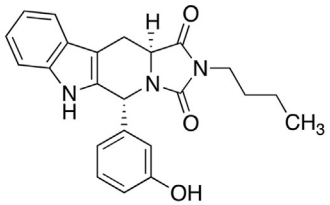
6 MITOTIC KINESIN INHIBITORS

Despite the diverse array of essential spindle proteins that could be exploited as targets for the discovery of novel cancer therapies, all spindle-targeted therapeutics in clinical use today that were mentioned in this chapter act on only one protein, tubulin. Kinesins are motor proteins that function to transport organelles within cells, and one group of them (mitotic kinesins) move chromosomes along microtubules during cell division, playing essential roles in the assembly and function of the mitotic spindle. Mitotic kinesins have an ATPase site that allows them to convert chemical energy into mechanical energy for the transport of DNA. They represent the first novel class of drug targets within mitosis to emerge in nearly 20 years.[126]

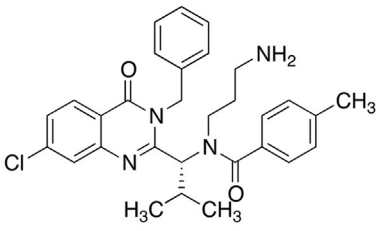
The most studied mitotic kinesin is the so-called kinesin spindle protein (KSP, Eg5), which functions at the earliest stages of mitosis to mediate centrosome separation and formation of a bipolar mitotic spindle. Eg5 localizes to microtubules in mitosis but not to interphase microtubules, suggesting that its inhibitors may specifically target proliferating tumor tissue, thereby avoiding dose-limiting neuropathy observed with other antimicrotubule agents such as taxanes or *Vinca* alkaloids. The first small-molecule inhibitor of the motor protein Eg5 to be characterized was monastrol, which is an allosteric inhibitor of the ATPase function of Eg5 that prevents ADP release by forming a ternary complex. The β-carboline derivative monastroline (HR-22C16) was identified through a high-throughput microscopy-based forward-chemical-genetic screen.[127] Another class of inhibitors of Eg5 function are quinazoline derivatives, which function via an allosteric mechanism similar to that of monastrol. Among them, ispinesib (SB-715992) has reached clinical trials in patients with a variety of refractory solid tumors.[128] Filanesib (ARRY-520) is a kinesin spindle protein inhibitor that, after several clinical trials, has been proposed as a cancer treatment, specifically for multiple myeloma.[129]



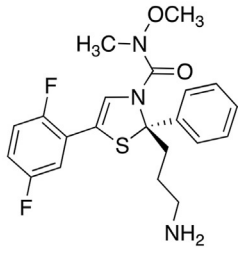
Monastrol



Monastroline (HR-22C16)



Ispinesib (SB-715992, CK-0238273)



Filanesib (ARRY-520)

REFERENCES

1 Hameroff S, Penrose R. *Phys Life Rev* 2014;**1**:39.
2 Hamel E. *Med Res Rev* 1996;**16**:207.
3 Wood KW, Cornwell WD, Jackson JR. *Curr Opin Pharmacol* 2001;**1**:370.
4 Kuppens IELM. *Curr Clin Pharmacol* 2006;**1**:57.
5 For reviews, see. (a) Jordan MA. *Curr Med Chem Anticancer Agents* 2002;**2**:1; (b) Jordan MA, Wilson L. *Nature Rev Cancer* 2004;**4**:253.
6 Montero A, Fossella F, Hortobagyi G, Valero V. *Lancet Oncol* 2005;**6**:229.
7 Gascoigne KE, Taylor SS. *Cancer Cell* 2008;**14**:111.
8 Huang HC, Shi J, Orth JD, Mitchison TJ. *Cancer Cell* 2009;**16**:347.
9 Fahy J. *Curr Pharm Des* 2001;**7**:1181.
10 (a) Hill BT. *Curr Pharm Des* 2001;**7**:1199; (b) Bellmunt J, Théodore C, Demkov T, Komyakov B, Sengelov L, Daugaard G, et al. *J Clin Oncol* 2009;**27**:4454.
11 For representative reviews, see. (a) Newman CJ, Cragg GM. *Curr Med Chem* 2004;**11**:1693; (b) Mayer AMS, Glaser KB, Cuevas C, Jacobs RS, Kem W, Little RD, et al. *Trends Pharmacol Sci* 2010;**31**:255; (c) Radjasa OK, Vaske YM, Navarro G, Vervoort HC, Tenney K, Linington RG, et al. *Bioorg Med Chem* 2011;**19**:6658; (d) Petit K, Biard J-F. *Anticancer Agents Med Chem* 2013;**13**:603; (e) Newman CJ, Cragg GM. *Mar Drugs* 2014;**12**:255.
12 Aicher TD, Buszek KR, Fang FG, Forsyth CJ, Jung SH, Kishi Y, et al. *J Am Chem Soc* 1992;**114**:3162.
13 Towle MJ, Salvato KA, Budrow J, Wels BF, Kuznetsov G, Aalfs KK, et al. *Cancer Res* 2001;**61**:1013.
14 Cortes J, O'Shaughnessy J, Loesch D, Blum JL, Vahdat LT, Petrakova K, et al. *Lancet* 2011;**377**:914.
15 Molife R, Cartwright TH, Loesch DM, Garbo LE, Sonpavde G, Calvo E, et al. *J Clin Oncol* 2007;**25** (18S):15513.
16 Schöffski P, Ray-Coquard IL, Cioffi A, Bin Bui N, Bauer S, Hartmann JT, et al. *Lancet Oncol* 2011;**12**:1045.
17 Jordan MA, Kamath K, Manna T, Okouneva T, Miller HP, Davis C, et al. *Mol Cancer Ther* 2005;**4**:1086.
18 For a monograph on bioactive peptides from marine sources, see Kim S-K. *Marine proteins and peptides: biological activities and applications*. New York: Wiley; 2013.
19 Vaishampayan H, Glode M, Du W, Kraft A, Hudes G, Wright J, et al. *Clin Cancer Res* 2000;**6**:4205.
20 Margolin K, Longmate J, Synold TW, Gandara DR, Weber J, González R, et al. *Invest New Drugs* 2001;**19**:335.
21 Schöffski P, Thate B, Beutel G, Bolte O, Otto D, Hofmann M, et al. *Ann Oncol* 2004;**15**:671.
22 Kerbrat P, Dieras V, Pavlidis N, Ravaud A, Wanders J, Fumoleau P. *Eur J Cancer* 2003;**39**:317.
23 (a) Ebbinghaus S, Hersh E, Cunningham CC, O'Day S, McDermott D, Stephenson J, et al. *J Clin Oncol* 2004;**22**:7530; (b) Mita AC, Hammond LA, Bonate PL, Weiss G, McCreery H, Syed S, et al. *Clin Cancer Res* 2006;**12**:5207.
24 Cormier A, Marchand M, Ravelli RBG, Knossow M, Gigant B. *EMBO Rep* 2008;**9**:1101.
25 Cruz-Monserrate Z, Mullaney JT, Harran PG, Pettit GR. *Eur J Biochem* 2003;**270**:3822.
26 Bai R, Durso NA, Sackett DL, Hamel E. *Biochemistry* 1999;**38**:14302.
27 Ayral-Kaloustian S, Zask A. *Drugs Fut* 2005;**30**:254.
28 Hadaschik BA, Ettinger S, Sowery RD, Zoubeidi A, Andersen RJ, Roberge M, et al. *Int J Cancer* 2008;**122**:2368.
29 Subbaraju GV, Golakoti T, Patterson GML, Moore RE. *J Nat Prod* 1997;**60**:302.
30 Shih C, Teicher BA. *Curr Pharm Des* 2001;**7**:1259.
31 Panda D, Ananthnarayan V, Larson GS, Shih C, Jordan MA, Wilson L. *Biochemistry* 2000;**39**:14121.
32 For a review, see Gerber-Lemaire S, Vogel P. *Comp Rend Chimie* 2008;**11**:1382.
33 Uckun FM, Mao C, Jan S-T, Wanh H, Vassilev AO, Navara CS, et al. *Curr Pharm Des* 2001;**7**:1291.

34 Bai R, Taylor GF, Cichacz ZA, Herald CL, Kepler JA, Pettit GR, et al. *Biochemistry* 1995;**34**:9714.
35 Uckun FM. *Curr Pharm Des* 2001;**7**:1627.
36 Dorléans A, Gigant B, Ravelli RBG, Mailliet P, Mikol V, Knossow M. *Proc Natl Acad Sci U S A* 2009;**106**:13775.
37 For a review of tubulin inhibitors that interact with the colchicine binding site, see Lu Y, Chen J, Xiao M, Li E, Miller DD. *Pharm Res* 2012;**29**:2943.
38 Goto H, Yano S, Zhang H, Matsumori Y, Ogawa H, Blakey DC. *Cancer Res* 2002;**62**:3711.
39 Hsieh HP, Liou JP, Mahindroo N. *Curr Pharm Des* 2005;**11**:1655.
40 Cirla A, Mann J. *Nat Prod Rep* 2003;**20**:558.
41 Hadimani MB, Hua J, Jonklaas MD, Kessler RJ, Sheng Y, Olivares A, et al. *Bioorg Med Chem Lett* 2003;**13**:1505.
42 Rustin GJ, Shreeves G, Nathan PD, Gaya A, Ganesan TS, Wang D, et al. *Br J Cancer* 2010;**102**:1355.
43 http://clinicaltrials.gov/ct2/results?term=AVE8062.
44 Nowak AK, Brown C, Millward MJ, Creaney J, Byrne MJ, Hughes B, et al. *Lung Cancer* 2013;**81**:422.
45 Galmarini CM. *Curr Opin Invest Drugs* 2005;**6**:623.
46 Yee KWL, Hagey A, Verstovsek S, Cortes J, García-Manero G, O'Brien SM, et al. *J Clin Cancer Res* 2005;**11**:6615.
47 Shan B, Medina JC, Santha E, Frankmoelle WP, Chou T-C, Learned RM, et al. *Proc Natl Acad Sci U S A* 1999;**96**:5686.
48 For a review of diketopiperazine-derived antimicrotubule/vascular disrupting agents, see Hayashi Y, Yamazaki-Nakamura Y, Yakushiji F. *Chem Pharm Bull* 2013;**61**:889.
49 http://clinicaltrialsfeeds.org/clinical-trials/results/term=Indibulin.
50 Kim LJ, Chamberlain MC, Zhu J, Raizer JJ, Grimm SA, Phuphanich S, et al. *J Clin Oncol* 2011;**29** (Suppl.):2088.
51 http://www.australiancancertrials.gov.au/search-clinical-trials/search-results/clinical-trials-details.aspx?TrialID=82702&ds=1.
52 Ricart A, Ashton E, Cooney M, Sarantopoulos J, Brell J, Feldman M, et al. *Cancer Chemother Pharmacol* 2011;**68**:959.
53 Thomas JP, Moore T, Kraut EH, Balcerzak SP, Galloway S, Vandre DD. *Cancer Invest* 2002;**20**:192.
54 Desbène S, Giorgi-Renault S. *Curr Med Chem Anticancer Agents* 2002;**2**:71.
55 (a) Lakhani NJ, Sarkar MA, Venitz J, Figg WD. *Pharmacotherapy* 2003;**23**:165; (b) Matei D, Schilder J, Sutton G, Perkins S, Breen T, Quon C, et al. *Gynecol Oncol* 2009;**115**:90.
56 Pasquier E, Sinnappan S, Muñoz MA, Kavallaris M. *Mol Cancer Ther* 2010;**9**:1408.
57 Malika S, Cusidó R, Mirjalilic MH, Moyano E, Palazón J, Bonfill M. *Process Biochem* 2011;**46**:23.
58 Guéritte F. *Curr Pharm Des* 2001;**7**:1229.
59 Immordino ML, Brusa P, Arpicco S, Stella B, Dosio F, Cattel L. *J Control Release* 2003;**91**:417.
60 Bissery MC. *Curr Pharm Des* 2001;**7**:1251.
61 Gurtler JS, Von Pawel J, Spiridonidis CH, Grossi F, Larriba JL, Moscovici M, et al. *J Clin Oncol* 2004;**22**:7136.
62 Broker LE, De Vos FY, Gall H, Gietema JA, Voi M, Cohen MB, et al. *J Clin Oncol* 2004;**22**:2029.
63 For a review of cabazitaxel, see Paller CJ, Antonarakis ES. *Drug Des Dev Ther* 2011;**5**:117.
64 Sternberg CN, Skoneczna IA, Castellano D, Theodore C, Blais N, Voog E, et al. *Oncology* 2013;**85**:208.
65 Saif MW, Sarantopoulos J, Patnaik A, Tolcher AW, Takimoto C, Beeram M. *Cancer Chemother Pharmacol* 2011;**68**:1565.
66 Ramanathan RK, Picus J, Raftopoulos H, Bernard S, Lockhart AC, Frenette G, et al. *Cancer Chemother Pharmacol* 2008;**61**:453.
67 http://www.cancer.gov/clinicaltrials/search/view?cdrid=597371&version=HealthProfessional.
68 Nicolaou KC, Valiulin RA. *Org Biomol Chem* 2013;**11**:4154.

69 Ganesh T, Guza RC, Bane S, Ravindra R, Shanker N, Lakdawala AS, et al. *Proc Natl Acad Sci U S A* 2004;**101**:10006.
70 Ganesh T, Guza RC, Bane S, Ravindra R, Shanker N, Lakdawala AS, et al. *Proc Natl Acad Sci U S A* 2004;**101**:10006.
71 Ganesh T, Norris A, Sharma S, Bane S, Alcaraz A, Snyder JP, et al. *Bioorg Med Chem* 2006;**14**:3447.
72 Cragg GM, Newman DJ. *J Nat Prod* 2004;**67**:232.
73 Nicolaou KC, Valiulin RA. *Org Biomol Chem* 2013;**11**:4154.
74 Bollag DM, McQueney PA, Zhu J, Hensens O, Koupal L, Liesch J, et al. *Cancer Res* 1995;**55**:2325.
75 For reviews, see. (a) Stachel SJ, Biswas K, Danishefsky SJ. *Curr Pharm Des* 2001;**7**:1277; (b) Nicolaou KC, Snyder SA. *Classics in total synthesis II*. Weinheim, Germany: Wiley-VCH; 2003 [chapter 7]; (c) Watkins EB, Chittiboyina AG, Jung J-C, Avery MA. *Curr Pharm Des* 2005;**11**:1615; (d) Trivedi M, Budihardjo I, Loureiro K, Reid TR, Ma JD. *Future Oncol* 2008;**4**:483.
76 For a monograph, see Kinghorn AD, Falk H, Kobayashi J, editors. *The epothilones—an outstanding family of antitumor agents: from soil to the clinic*. *Progress in the chemistry of organic natural products*, vol. 90. New York: Springer; 2009.
77 For a review, see Altmann KH. *Mini Rev Med Chem* 2003;**3**:149.
78 Chou TC, O'Connor OA, Tong WP, Guan Y, Zhang Z-G, Stachel SJ, et al. *Proc Natl Acad Sci U S A* 2001;**98**:8113.
79 Yee L, Lynch T, Villalona-Calero M, Rizvi N, Gabrail N, Sandler A, et al. *J Clin Oncol* 2005;**23**:7127.
80 Wartmann M, Altmann K-H. *Curr Med Chem Anticancer Agents* 2002;**2**:123.
81 Kolman A. *Curr Opin Invest Drugs* 2004;**5**:657.
82 Low JA, Wedam SB, Lee JJ, Berman AW, Brufsky A, Yang SX, et al. *J Clin Oncol* 2005;**23**:2726.
83 De Jonge M, Verweeij J. *J Clin Oncol* 2005;**23**:9048.
84 Höfle G, Glaser N, Leibold T, Karama U, Sasse F, Steinmetz H. *Pure Appl Chem* 2003;**75**:167.
85 Klar U, Buchmann B, Schwede W, Skuballa W, Hoffmann J, Lichtner RB. *Angew Chem Int Ed* 2006;**45**:7942.
86 (a) Schmid P, Kiewe P, Kuehnhardt D, Korfel A, Lindemann S, Giurescu M, et al. *J Clin Oncol* 2005;**23**:2051; (b) Beer TM, Smith DC, Hussain A, Alonso M, Wang J, Giurescu M, et al. *Br J Cancer* 2012;**107**:808.
87 Wartmann M, Loretan J, Reuter R, Hattenberger M, Muller M, Vaxelaire J. *Proc Am Assoc Cancer Res* 2004;**45**, abstract #5440.
88 For a review, see Chou TC, Zhang X, Zhong ZY, Li Y, Feng L, Eng S, et al. *Proc Natl Acad Sci U S A* 2008;**105**:13157.
89 Rivkin A, Yoshimura F, Gabarda AE, Cho YS, Chou T-C, Dong H, et al. *J Am Chem Soc* 2004;**126**:10913.
90 For an account of the discovery of fludelone, see Rivkin A, Chou T-C, Danishefsky SJ. *Angew Chem Int Ed* 2005;**44**:2838.
91 https://clinicaltrials.gov/ct2/show/NCT01379287.
92 Altmann KH. *Org Biomol Chem* 2004;**2**:2137.
93 Altmann KH. *Curr Pharm Des* 2005;**11**:1595.
94 Manetti F, Maccari L, Corelli F, Botta M. *Curr Topics Med Chem* 2004;**4**:203.
95 Nettles JH, Li H, Cornett B, Krahn J, Snyder JP, Downing KH. *Science* 2004;**305**:866.
96 Giannakakou P, Gussio R, Nogales E, Downing KH, Zaharevitz D, Bollbuck B, et al. *Proc Natl Acad Sci U S A* 2000;**97**:2904.
97 Long BJ, Carboni JM, Wasserman AJ, Cornell LA, Casazza AM, Jensen PR, et al. *Cancer Res* 1998;**58**:1111.
98 Kingston DGI, Newman DJ. *Curr Opin Drug Discov Devel* 2002;**5**:304.
99 Giannakakou P, Fojo T. *Clin Cancer Res* 2000;**6**:1613.
100 Mani S, Macapinlac M, Goel S, Verdier-Pinard D, Fojo T, Rothenberg M, et al. *Anticancer Drugs* 2004;**15**:553.

101 Mickel SJ, Niederer D, Daeffler R, Osmani A, Kuesters E, Schmid E, et al. *Org Process Res Dev* 2004;**8**:122, and four proceeding papers.
102 Mickel SJ. In: Harmata M, editor. *Strategies and tactics in organic synthesis*, vol. 6. New York: Elsevier; 2005 [chapter 9].
103 Freemantle M. *Chem Eng News* 2004;**82**:33.
104 Gapud EJ, Bai R, Ghosh AK, Hamel E. *Mol Pharmacol* 2004;**66**:113.
105 Peris L, Wagenbach M, Lafanechere L, Brocard J, Moore AT, Kozielski F, et al. *J Cell Biol* 2009;**185**:1159.
106 Prudent R, Vassal-Stermann E, Nguyen Ch-H, Pillet C, Martínez A, Prunier Ch, et al. *Cancer Res* 2012;**72**:4429.
107 Ohashia K, Sampeia K, Nakagawaa M, Uchiumia N, Amanumaa T, Aibab S, et al. *Mol Biol Cell* 2014;**25**:828.
108 Smaletz O, Galsky M, Scher HI, De la Cruz A, Slovin SF, Morris MJ, et al. *Ann Oncol* 2003;**14**:1518.
109 Walz PH, Bjork P, Gunnarsson PO, Edman K, Hartley-Asp B. *Clin Cancer Res* 1998;**4**:2079.
110 Laing N, Dahlöff B, Hartley-Asp B, Ranganathan S, Tew KD. *Biochemistry* 1997;**36**:871.
111 Panda D, Miller HP, Islam K, Wilson L. *Proc Natl Acad Sci U S A* 1997;**94**:10560.
112 Laing N, Dahlöff B, Hartley-Asp B, Ranganathan S, Tew KD. *Biochemistry* 1997;**36**:871.
113 Panda D, Miller HP, Islam K, Wilson L. *Proc Natl Acad Sci U S A* 1997;**94**:10560.
114 Smaletz O, Galsky M, Scher HI, De la Cruz A, Slovin SF, Morris MJ, et al. *Ann Oncol* 2003;**14**:1518.
115 Hallur G, Jimeno A, Dalrymple S, Zhu T, Jung MK, Hidalgo M, et al. *J Med Chem* 2006;**49**:2357.
116 Messerschmith WA, Baker SD, Donehower RC, Dolan S, Zabelina Y, Zhao M, et al. *Proc Am Soc Clin Oncol* 2003;**22**:203.
117 Pera B, Barasoain I, Pantazopoulou A, Canales A, Matesanz R, Rodríguez-Salarichs J, et al. *ACS Chem Biol* 2013;**8**:2084.
118 Thorpe PE. *Clin Cancer Res* 2004;**10**:415.
119 Marx MA. *Exp Opin Ther Pat* 2002;**12**:769.
120 Dhanabal M, Jeffers M, LaRochelle WJ. *Curr Med Chem Anticancer Agents* 2005;**5**:115.
121 Kanthou C, Tozer GM. *Blood* 2002;**99**:2060.
122 Lawrence NJ, McGown AT. *Curr Pharm Des* 2005;**11**:1679.
123 Hori K, Saito S. *Br J Cancer* 2003;**89**:1334.
124 Davis PD, Dougherty GJ, Blakey DC, Galbraith SM, Tozer GM, Holder AL, et al. *Cancer Res* 2002;**62**:7247.
125 Soltau J, Drevs J. *IDrugs* 2004;**7**:380.
126 Wood KW, Bergnes G. *Annu Rep Med Chem* 2004;**39**:173.
127 Hotha S, Yarrow JC, Yang JC, Garrett S, Renduchintala KV, Mayer TU, et al. *Angew Chem Int Ed* 2003;**42**:2379.
128 http://www.clinicaltrials.gov/ct/show/NCT00097409.
129 Ocio EM, Mitsiades CS, Orlowski RZ, Anderson KC. *Exp Rev Hematol* 2014;**7**:127.

CHAPTER

DRUGS THAT INHIBIT SIGNALING PATHWAYS FOR TUMOR CELL GROWTH AND PROLIFERATION

KINASE INHIBITORS

10

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00010-7

1 INTRODUCTION

Conventional anticancer drugs have been traditionally focused on targeting DNA processing and cell division. They can be very efficacious, but their lack of selectivity for tumor cells usually leads to serious side effects. By the late 1980s, advances in molecular biology begun to provide a greatly increased understanding of regulatory and signaling networks in normal cells that control fundamental cellular processes such as vascularization, growth, and proliferation. All these processes are greatly enhanced in tumor cells in response to different factors through complex mechanisms in which several signaling pathways are responsible for transforming normal cells into malignant cancers.[1]

These findings have provided the basis for seeking inhibitors of macromolecular targets essential to the malignant tumor phenotype but not utilized in vital organs and tissues, which in principle should lead to a better selectivity in comparison to traditional cytotoxic drugs. These anticancer drugs are usually known as "molecularly targeted agents"—a name that is perhaps not completely appropriate because many drugs developed in the first era of cancer chemotherapy, such as the cytotoxic antifolate thymidylate synthase inhibitors, were also molecularly targeted. Alternative names for this new class of anticancer drugs, one of the fastest growing areas of research in cancer chemotherapy, are "signal transduction inhibitors" or "secondary messenger inhibitors."

2 ONCOGENES AND SIGNAL TRANSDUCTION

Mutations in at least several hundred human genes (out of a total of approximately 25,000 genes) become drivers of the abnormal cell growth and division process that generates human cancer. These oncogenes encode the protein components of signal transduction pathways that enable external signals (growth and survival factors) to move from the cell surface receptors to key promoter–enhancer regions along human chromosomes. There, they promote the expression of genes needed for cell growth and division, as well as the evasion of programmed cell death, which is very important in the ever-growing resistance of late-stage aggressive cancer cells to radio- and chemotherapies.

The first human oncogene was discovered in1982,[2] but today more than 500 are known, which can be categorized as follows:

1. Activated oncogenes (e.g., *RAS*, *RAF*, and *PI3KCA*) and deactivated tumor suppressor genes (e.g., *P53* and *PTEN*)
2. Genes that when inactivated or mutated lead to DNA repair defects (e.g., *BRCA1* and *BRCA2*, whose acronym stands for Berkeley, California)
3. Genes that support oncogenic pathways such as those encoding the chaperone HSP90 and histone deacetylases, which are involved in post-translational modification of proteins, chromatin modification, and control of gene expression
4. Genes controlling the tumor microenvironment, including cancer–host interactions

Normal cells undergo genetic alterations at the nucleotide and chromosomal levels as they divide, but as a defense mechanism, they are programmed to undergo cell death in response to such alterations. However, cancer cells evolve by acquiring mutations in genes such as the tumor-suppressor protein p53 (known as "guardian of genome").[3] More than 100 driver genes affected by subtle mutations confer a selective growth advantage to cancer cells through different pathways that participate in cellular processes such as cell fate, cell survival, and genome maintenance.[4]

The precise balance between cell differentiation and division is mainly controlled by the adenomatous polyposis coli (APC), Notch, and HH signal pathways, as well as by genes encoding chromatin-modifying enzymes such as chaperone HSP90 and histone deacetylases. In normal development, the heritable switch from division to differentiation is not determined by mutation but, rather, by epigenetic alterations affecting DNA and chromatin proteins, whereas in cancer cells many genetic alterations favor the division.

Cancer cell survival is dependent on the abnormal vasculature of tumors due to VHL gene mutations, whose product stimulates angiogenesis through the secretion of vascular endothelial growth

factor, and on mutations of *EGFR*, *HER2*, *FGFR2*, *PDGFR*, *TGF-αR2*, *MET*, *KIT*, *RAS*, *RAF*, *PI3KCA*, and *PTEN* genes, which encode receptors for the growth factors themselves or, when activated, relay the signal from the growth factor to the interior of the cell, leading to stimulated growth (e.g., mutations in *K-RAS* or *B-RAF* genes confer on cancer cells the ability to grow in low glucose concentrations). Other driver genes that are often mutated in cancers, such as *MYC* and *BCL2*, regulate progression through the cell cycle or apoptosis.

Deficiencies in genome maintenance contribute significantly to the onset of cancer because cells make mistakes while replicating their DNA and are also exposed to a variety of toxic substances such as reactive oxygen species. Under these circumstances, checkpoints slow down the cycle of such cells or lead them to programmed death (apoptosis), but if tumor cells can survive this damage, they will have a selective growth advantage. Therefore, genes whose mutations abrogate these checkpoints, such as *p53* and *ATM*, or genes that control point mutation rates are frequently mutated in cancers or in the germline of patients predisposed to them.

3 THE ROLE OF PROTEIN KINASES IN CANCER: SIGNALING PATHWAYS RELATED TO KINASES

Modern anticancer drug research has become increasingly focused on signal transduction therapy, and many of the validated targets are transduction-related macromolecules, especially kinases. Protein kinases (PTKs) are enzymes that regulate the biological activity of proteins by phosphorylation of specific amino acids with ATP as the source of phosphate, thereby inducing a conformational change from an inactive to an active form of the protein (Figure 10.1).

There are three main types of PTKs, which are classified according to the amino acid side chain that they phosphorylate:

1. Tyrosine kinases (TKs), which phosphorylate the Tyr phenolic hydroxyl
2. Serine–threonine (Ser–Thr) kinases, which phosphorylate the hydroxy group of these two amino acids
3. Histidine kinases, which phosphorylate the nitrogen of His residues

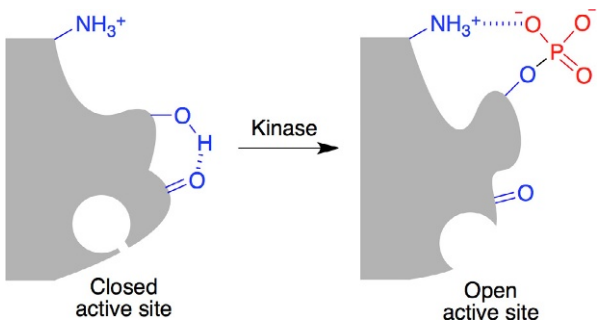


FIGURE 10.1

Schematic representation of the control of protein activity by phosphorylation reactions.

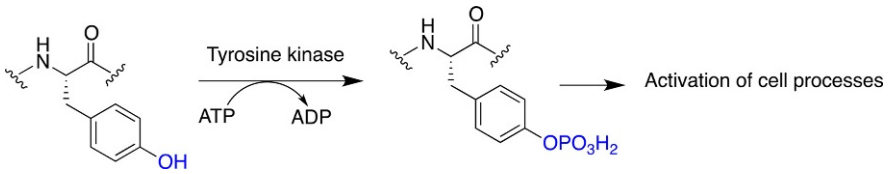


FIGURE 10.2

Reaction catalyzed by tyrosine kinases.

As an example of the types of reactions catalyzed by kinases, the phosphorylation of tyrosine residues by tyrosine kinases is shown in Figure 10.2.

Protein phosphorylation is one of the most significant signal transduction mechanisms by which intercellular signals regulate crucial intracellular processes such as ion transport, cellular proliferation and differentiation, and hormone responses. The Human Genome Project revealed that 20% of human genes encode proteins involved in signal transduction. Among them, there are more than 500 protein kinases and approximately 150 protein phosphatases, which are enzymes that catalyze the inverse process of protein dephosphorylation. Targeting protein kinases is a compelling approach to cancer chemotherapy because in many cancers there is an overexpression of these enzymes or their associated messengers.[5–7] It is important to bear in mind that due to their different effects, cellular growth inhibition or cell death may be achieved through either inhibition or activation of different kinases and that these effects can be reversed through activation of protein phosphatases that remove the phosphate groups from the activated kinases.

Most PTKs are related to oncogenes, and approximately 16 of them are considered as possible therapeutic targets. Based on their localization and structure, these enzymes are classified as receptor- or non-receptor PTKs. Receptor protein kinases (RPTKs) have dual roles: as receptors and as enzymes. They have a hydrophobic domain that transverses the cell membrane, an extracellular ligand-binding domain that recognizes an external messenger (growth hormones or growth factors),[8] and a cytoplasmatic kinase domain that becomes activated upon binding of the external messenger, triggering a signaling cascade that ultimately controls the transcription of specific genes related to cellular proliferation and differentiation. Non-receptor PTKs are activated by upstream signaling molecules such as G protein-coupled receptors, immune system receptors, or RPTKs. They have no transmembrane or extracellular domains and are not covalently bound to a membrane receptor, nor anchored to the phospholipid membrane via a lipid modification.

Ligand binding to a RPTK induces its dimerization or oligomerization, leading to interactions between adjacent cytoplasmic domains with accompanying activation of the kinase moiety. Activation of a non-receptor kinase is similarly induced in response to the appropriate extracellular signal, but dimerization may or may not be necessary for activation. The activated kinase then initiates a cascade of phosphorylation reactions resulting in the activation of other proteins, as well as the production of secondary messengers that transmit the signal to the nucleus.

All protein kinases have a region in their active site that recognizes ATP, which is the phosphorylating agent in all cases, as well as another region for their substrates. Most clinically used inhibitors interact with these ATP recognition sites that, despite having a common substrate, are relatively different for different kinases, making possible some selectivity in the inhibition. Very often, structurally close

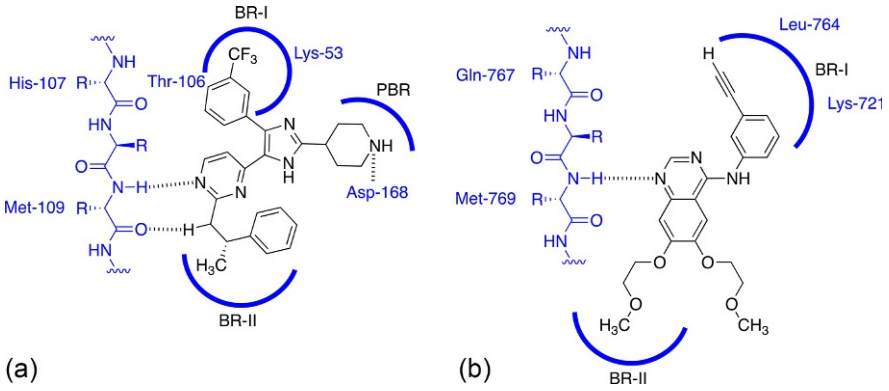


FIGURE 10.3

Binding modes of inhibitors at the ATP site of PTKs.

compounds bind to the ATP site in different topologies and are able to recognize different kinases. For this reason, chemical similarity between kinase inhibitors often fails to correlate with target specificity. Binding to an adjacent allosteric site or to an inactive form of the kinase has also been exploited.[9]

These compounds target a highly conserved structural determinant of the ATP binding site in the kinase family, namely an alternating hydrogen bonding pattern present in the so-called hinge peptide portion that connects the N- and C-terminal domain of kinases. Inhibitors form hydrogen bonds with the protein backbone while their peripheral groups are oriented toward two hydrophobic pockets called BR-1 and BR-2 (binding region-1 and -2) or toward the phosphate-binding region (PBR). Two typical binding modes of inhibitors of PTKs at the ATP site are shown in Figure 10.3.

The main kinase inhibitors that are discussed in this chapter are summarized in Table 10.1, classified according to their main target.

Although a more detailed explanation is given in the individual sections, a simplified pictorial summary of some of the most important signaling pathways targeted by drugs described in this chapter is given in Figure 10.4.

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development

Type	Target	Agents
Tyr kinases	EGFR (HER-1)	Small-molecule inhibitors Gefitinib (ZD-1839, Iressa®) Erlotinib (OSI-774, Tarceva®) Lapatinib (GW-2016, Tyverb®) Canertinib (CI-1033) Afatinib (BIBW-2992, Gilotrif®) EKI-785 Pelitinib (EKB-569) Neratinib (HKI-272) AZD-9291 CO-1686

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
		Monoclonal antibodies Cetuximab (IMC-C225, Erbitux®) Panitumumab (ABX-EGF, Vectibix®) Matuzumab (EMD-72000) Nimotuzumab MDX-447
	HER-2 (ErbB2)	Small-molecule inhibitor ARRY-380 (ONT-380) Monoclonal antibodies Trastuzumab (Herceptin®) Pertuzumab (2C4, Perjeta®)
	HER-3	Monoclonal antibodies MM-121 U3-1287 LJM716
	Pan-HER	Small-molecule inhibitor Varlitinib (ARRY-543, ASLAN001)
	IGF-1R	Small-molecule inhibitors AEW-541 INSM-18 (nordihydroguaiaretic acid, NDGA) BVP-51004 (cyclolignan PPP) Antibodies Figitumumab (CP-751871) Ganitumab (AMG-479)
	HGFR (c-Met)	Small-molecule inhibitors Tivantinib (ARQ 197) JNJ-38877605 PF-04217903 NC280 (INCB-28060)
	VEGF, VEGFR, and related receptors	Small-molecule inhibitors Semaxanib (SU-5416) SU-6668 Sunitinib (SU-11248, Stutent®) Vatalanib (PTK-787, ZK-222584) Cediranib (AZD-2171, Recentin®) Foretinib (EXEL-2880, GSK-1363089, XL-880) Cabozantinib (Cometriq®, XL184) Tivozanib (AV-951) Lenvatinib (E-7080, Lenvima®) Linifanib (ABT-869) Pazopanib (GW-786034, Votrient®) Axitinib (AG-013736, Inlyta®) Nintedanib (BIBF1120, Vargatef®) CEP-5214

Continued

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
		CEP-7055 Monoclonal antibody Bevacizumab (Avastin®) Soluble decoy receptor Ziv-aflibercept (Zaltrap®) Ribozyme Angiozyme (RPI.4610)
	PDGFRs	Small-molecule inhibitor Suramin (Metaret®)
	FGFRs	Small-molecule inhibitors Dovitinib (TKI258) BGJ398 (NVP-BGJ398) Monoclonal antibody PRO-001
	FLT3 (CD135)	Small-molecule inhibitors Tandutinib (MLN-518, CT-53518) Lestaurtinib (CEP-701) Midostaurin (PKC-412)
	Bcr-Abl	Small-molecule inhibitors ATP mimics Imatinib (STI-571, Glivec®) Nilotinib (AMN-107, Tasigna®) Radotinib (Supect®) Ponatinib (AP24534, Iclusig®) Tyrosine mimics Adaphostin (NSC-680410) ON-012380
	Bcr-Abl/Src	Small-molecule inhibitors Dasatinib (BMS-354825, Sprycel®) Bosutinib (SKI-606, Bosulif®) Saracatinib (AZD-0530)
	Bcr-Abl biosynthesis	Small-molecule inhibitor Omacetaxine mepesuccinate (Synribo®)
	ALK	Small-molecule inhibitors Crizotinib (PF-02341066, Xalkori®) X-376 X-396 Ceritinib (LDK-378, Zykadia®) AP26113 CEP-37440 ASP-3026 Alectinib PF-06463922 RXDX-101 (NMS-E628) TSR-011

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
	JAK–STAT and PRL	Small-molecule inhibitors Ruxolitinib (INCB018424, Jakafi®) FLLL-32 Monoclonal antibody LFA102
	BTK	Small-molecule inhibitor Ibrutinib (PCI-32765, Imbruvica®)
	Trk	Small-molecule inhibitor LOXO-101
Ser–Thr kinases	CDK	Small-molecule inhibitors Alvocidib (flavopiridol, HMR-1275) Riviciclib (P776-00) Seleciclib (roscovitine, CYC-202) SNS-032 (BMS-387032) AT7519 Indisulam (E-7070®) Palbociclib (PD-0332991, Ibrance®) LEE011 Dinaciclib (SCH-727965) SB-1317 (TG-02) BAY-1000394 RGB-286638 Terameprocol (EM-1421) Milciclib (PHA-848125) AG-24332 ZK-304709 R-547 AZD-5438
	PLK1	Small-molecule inhibitors Volasertib
	PLK4	Small-molecule inhibitor CFI-400945
	CHKs	Small-molecule inhibitors UCN-01 LY 2603618 LY 2606368 PF-00477736 SCH900776 AZD-7762 XL-844 (EXEL-9844) GDC-0575 (ARRY-575) GDC-0425

Continued

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
	PI3K	Small-molecule inhibitors BEZ235 (NVP-BEZ235) Buparlisib (BKM-120) Alpelisib (BYL-719) XL-765 (SAR-245409) GDC-0032 Idelalisib (Zydelig®)
	PDPK1	Small-molecule inhibitors UCN-01
	AKT	Small-molecule inhibitors A-443654 Perifosine (KRK-0401)
	mTOR	Small-molecule inhibitors Temsirolimus (CCI-779, Torisel®) Everolimus (RAD-001, Afinitor®) Ridaforolimus (AP-23573) Deferasirox (DBO-1609, Exjade®)
	SGK1	Small-molecule inhibitor GSK-650394
	Aurora kinases	Small-molecule inhibitors Tozasertib (VX-680, MK-0457) Barasertib (AZD-1152) Danusertib (PHA-739358) AT-9283
	PKC	Small-molecule inhibitors UCN-01 Midostaurin (PKC-412, CGP-41251) Ruboxistaurin (LY-333531, Arxxant®) Enzastaurin (LY-317615) Sotrastaurin (AEB071) Bryostatin 1 Antisense oligonucleotide ISIS-3521 (LY-900003, Affinitac®)
	Pim kinases	Small-molecule inhibitor LGH447
Ras/Raf/MEK pathway	Ras	Antisense oligonucleotides inhibitor of Ras expression ISIS-2503 Farnesyltransferase inhibitors L-744832 AZD-3409 FTI-276 FTI-277 BMS-214662 Tipifarnib (R-115777, Zarnestra®) L-778123 Lonafarnib (SCH-66366) SCH-226374

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
	Farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase	Small-molecule inhibitors Risedronate Zoledronate Minodronate
	Raf	Small-molecule inhibitors Sorafenib (BAY43-9006, Nexavar®) Regorafenib (BAY73-4506, Stivarga®) Vemurafenib (PLX-4032, Zelboraf®) Dabrafenib (GSK-2118436, Tafinlar®) RAF265 (CHIR-265) Encorafenib (LGX818) Antisense oligonucleotide ISIS-5132
	MEK	Small-molecule inhibitors CI-1040 (PD-184352) PD-0325901 Cobimetinib (GDC-0973, XL-518) ARRY-424704 (AZD-8330) Trametinib (GSK-1120212, Mekinist®) Selumetinib (ARRY-142886, AZD-6244) Binimetinib (MEK162, ARRY-162) RO4927350
MAPK pathways	ERK1/2	Small-molecule inhibitors Hyphothemycin FR148083 FR180204 GDC-0994
	JNK	Small-molecule inhibitors CC-401 GNK-IN-8 Aplidine (dihydrodidemnin B, Aplidin®)
	P38	Small-molecule inhibitors LY228820 Doramapimod (BIRB-796)
	TGF-β_2	Antisense oligonucleotides Trabedersen (AP-12009) AP-11014 Allogenic tumor cell vaccines Glionix® Lucanix® Monoclonal antibodies Lerdelimumab (CAT-152, Trabio®) Metelimumab (CAT-192) Fresolimumab (GC-1008)

Continued

Table 10.1 Selected Kinase Inhibitors in the Market or That Have Entered Clinical Development—cont'd

Type	Target	Agents
		2G7 Small-molecule inhibitors Tasisulam (LY573636) LY2157299
Kinases and other enzymes involved in anaerobic glycolysis	Hexokinase	Small-molecule inhibitors Lonidamine 3-Bromopyruvate 2-Deoxy-D-glucose
	PDK	Dichloroacetate (DCA)
	ACL	SB-204990

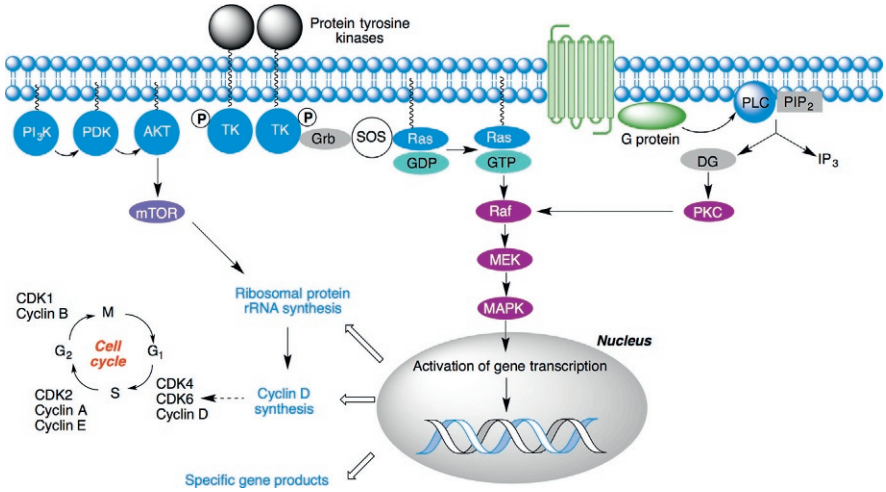


FIGURE 10.4

Some signaling pathways related to kinases.

4 INHIBITORS OF TYROSINE KINASES

The development of specific tyrosine kinase inhibitors started by the synthesis of hydroxyphenyl compounds as tyrosine mimics. Some of them were derivatives of itaconic acid, a compound that inhibited the insulin receptor with no effect on Ser–Thr kinases. Another source of inspiration was the natural product erbstatin, an inhibitor of epidermal growth factor receptor (EGFR) and other kinases. The first potent inhibitor to arise from this work was tyrphostin (AG-213). Conformational restriction strategies by cycle formation in this compound eventually led to the identification of the quinoxaline system as a very useful pharmacophore in the design of tyrosine kinase inhibitors (Figure 10.5). Interestingly, they act as ATP mimics rather than as substrate analogs, which was the original rationale behind this work.[10,11]

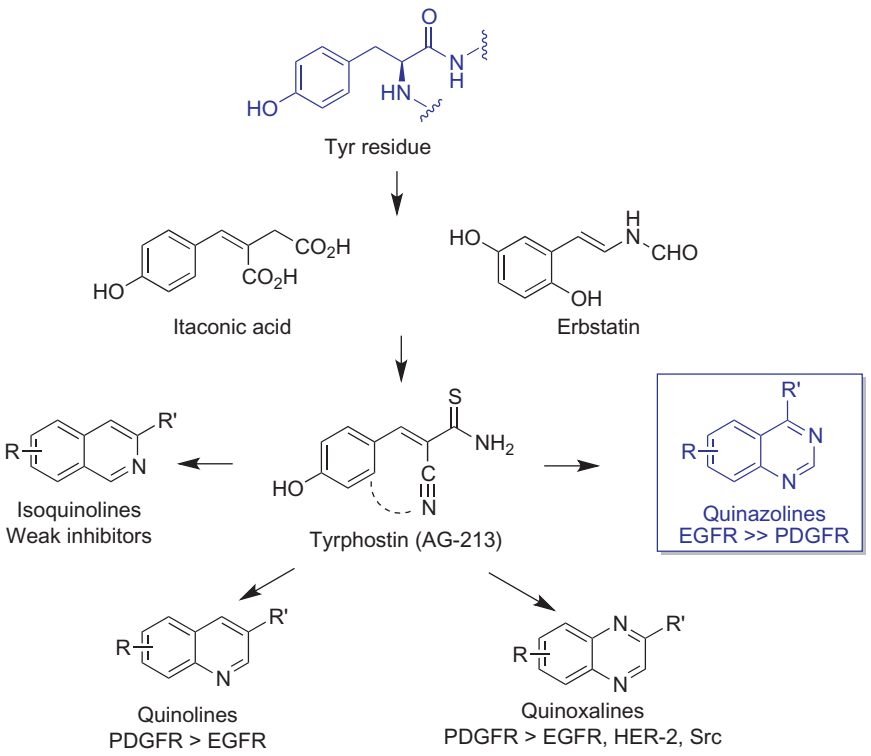


FIGURE 10.5

Representative tyrosine kinase inhibitors.

Initially, tyrosine kinase inhibitors (TKIs) were developed as targeted therapies that would solely interfere with aberrant tyrosine kinase activation in malignant cells. However, several TKIs, such as gefitinib, erlotinib, lapatinib, sorafenib, and dasatinib, also exhibit "off-target" effects that are not mediated by the assumed mechanisms of action.[12]

4.1 INHIBITORS OF EGFR (HER-1)

Epidermal growth factor receptors (EGFRs) are overexpressed or mutated (due to the transformation of a normal gene to an oncogene) in several cancers, and many tumors overexpress these receptors as well as their ligands: epidermal growth factors (EGFs) and transforming growth factor-α (TGF-α), which are involved in several human cancers. The best understood among several types of EGFRs are HER-1 (normally used as a synonym to EGFR) and HER-2 (synonym to ErbB2). The HER-3 receptor has gained interest as a potential new target for cancer therapy.[13]

EGFR is considered as a suitable target for lung cancer, colorectal cancer, myeloid leukemia, and hormone-dependent or -independent breast cancer.[14] It is also altered in 50% of glioblastomas, which are very aggressive tumors resistant to conventional chemo- and radiotherapy. It has been recently

found that inhibition of DYRK1A (a dual-specificity kinase regulated by tyrosine phosphorylation) has a fundamental role in regulating EGFR in these tumors.[15] The most selective and potent known inhibitor of DYRK1A is the β-carboline alkaloid harmine,[16] but it is highly toxic.

Ligand binding to EGFRs leads to activation of its kinase activity through homodimerization or heterodimerization with a receptor belonging to the same family, followed by autophosphorylation at the Tyr-1068 residue, which in turn leads to the activation of a range of cell signaling pathways, especially Ras–Raf–MEK–MAPK(ERK) and PI3 kinase–AKT signaling (see Figure 10.3). Transduction of signals to the nucleus and activation of gene transcription by several factors lead to the induction of a number of processes that are essential for tumor cell growth, including cell proliferation, survival, angiogenesis, invasion, and metastasis (Figure 10.6).

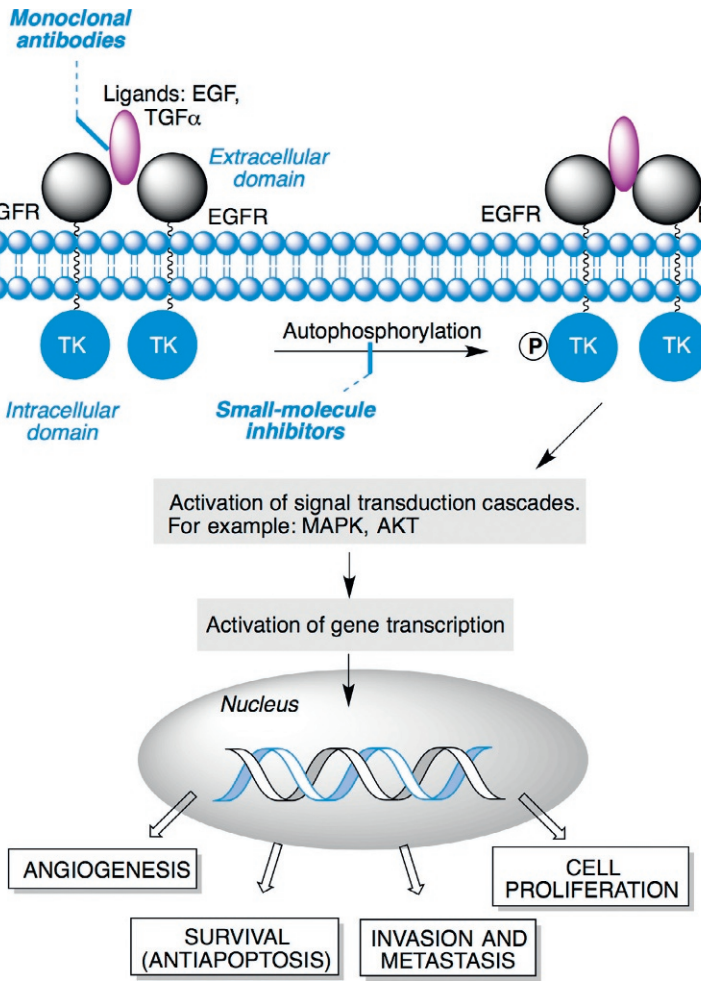


FIGURE 10.6

Events triggered following activation of the EGFRs.

Many anti-EGFR agents are known, and some of them are used in clinical practice or are under clinical development. They can be classified in the following two groups:

1. Small molecules that compete with ATP binding to the TK domain of the receptor, inhibiting autophosphorylation and blocking signal transduction
2. Monoclonal antibodies (mAbs), which are directed at the extracellular portion of the EGFR and compete with the receptor ligands EGF and TGF-α inhibiting receptor dimerization

4.1.1 Small-Molecule EGFR Inhibitors

During studies aimed at characterizing the catalytic domain of EGFR using high-throughput screening techniques, it was discovered that 4-anilinoquinazolines were promising inhibitors,[17] and investigation of substituent effects on their biological activity led to the conclusions summarized in Figure 10.7.[18]

Among 4-anilinoquinazolines, the first small-molecule anticancer drug acting as an anti-EGFR agent was gefitinib (ZO-1839, Iressa®), developed by AstraZeneca Pharmaceuticals in the mid- to late 1990s. In 2003, it was the first "noncytotoxic" compound approved for clinical use as a monotherapy for the treatment of patients with locally advanced non-small lung cell cancer (NSCLC) following failure of platinum and docetaxel treatments.[19,20] However, a subsequent large randomized study failed to demonstrate a survival advantage for gefitinib in the treatment of this cancer, and its combination with platinum agents did not show any clinical benefit.[21,22] These limitations, together with the report of lethal pulmonary toxicity from studies in Japan, led to its replacement by the closely related compound erlotinib (OSI-774, Tarceva®). Interestingly, the lack of clinical response to gefitinib in some patients is associated with mutations in various positions of EGFR, which exemplifies the possibilities of treatments based on pharmacogenomics or personalized medicine.[23] Erlotinib was approved by the U.S. Food and Drug Administration (FDA) in 2004 for EGFR-positive patients with advanced or metastatic NSCLC after failure of prior chemotherapy. This means that, ideally, all potential patients to be treated with this drug should be prescreened for their EGFR status, but this has time and cost implications and a tumor biopsy may not be feasible for some types of tumors. Its analog icotinib (BPI-2009H, Conmana®) is another targeted drug for patients with NSCLC whose tumors are positive for *EGFR* mutations. In 2011; this compound received CFDA approval for use in China as a treatment

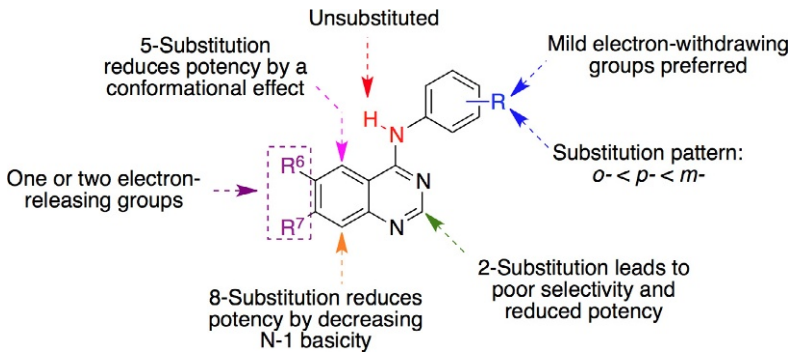
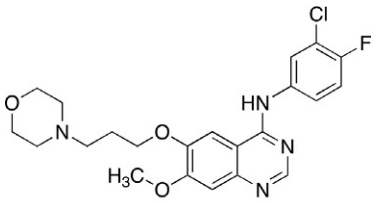


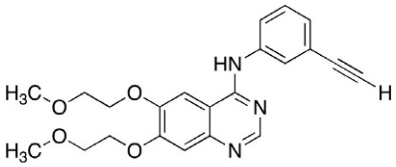
FIGURE 10.7

Structure–activity relationships in 4-anilinoquinazolines as EGFR inhibitors.

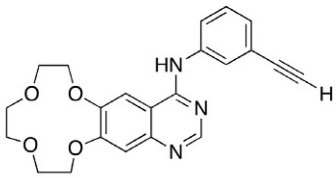
for NSCLC.[24] Another quinazoline derivative that inhibits EGFR with similar efficacy is lapatinib (GW-2016, Tyverb®), a dual inhibitor of EGFR and the closely related receptor HER-2, which is another important therapeutic target in a number of cancers in which it is overexpressed. Lapatinib was approved by the FDA in 2007 for breast cancer and is under clinical assays for several solid tumors.[25]



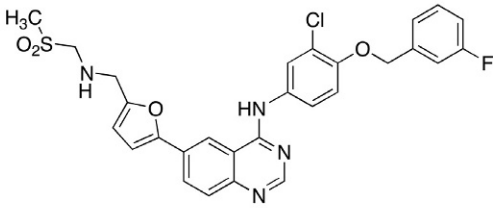
Gefitinib (ZD-1839)



Erlotinib (OSI-774)



Icotinib (BPI-2009H)



Lapatinib (GW-2016)

The binding of ATP to its site at the TK domain of EGFR was initially studied by molecular modeling techniques, based on the X-ray crystal structure of the complex between the related cAMP-dependent protein kinase, an inhibitor, Mg, and ATP. This binding involves, among other interactions, two hydrogen bonds at the Gln-767 and Met-769. The ribose unit binds to its own pocket, and the triphosphate chain is placed in a cleft that leads to the surface of the enzyme (Figures 10.8a and 10.9a). This active site also contains unoccupied spaces, especially a hydrophobic pocket opposite to the place where the ribose binds that shows slight differences between the different kinases, allowing the design of relatively selective inhibitors. Because the ATP binding site is quite large, several orientations are possible for inhibitors, even those belonging to the same structural class.

The interaction between gefitinib and the EGFR catalytic domain, which was studied by X-ray crystallography,[26] is summarized in Figures 10.8b and 10.9b. The N-1 atom of the quinazoline ring acts as a hydrogen bond acceptor in an interaction with the Met-769 NH, the N-3 atom interacts with Thr-830 through a bridging water molecule, and the aniline ring occupies the normally empty hydrophobic pocket. Replacement of the Met-790 residue by Thr leads to resistance to gefitinib and erlotinib due to steric hindrance to binding of the inhibitor.[27]

Because ATP competitive EGFR inhibitors compete with the high endogenous concentrations of ATP, they are rapidly cleared from tumors. To overcome this problem, intensive efforts have been directed toward the development of a second generation of EGFR inhibitors that bind irreversibly. The therapeutic success of this class of compounds is dependent on whether or not the covalent bond can be confined solely to the protein kinase of interest.[28] Canertinib (CI-1033),[29] afatinib (BIBW-2992, Gilotrif®), EKI-785, pelitinib (EKB-569), and neratinib (HKI-272) are representative examples under clinical evaluation. Some of them are dual EGFR–HER-2 inhibitors.[30,31] Afatinib is being developed as

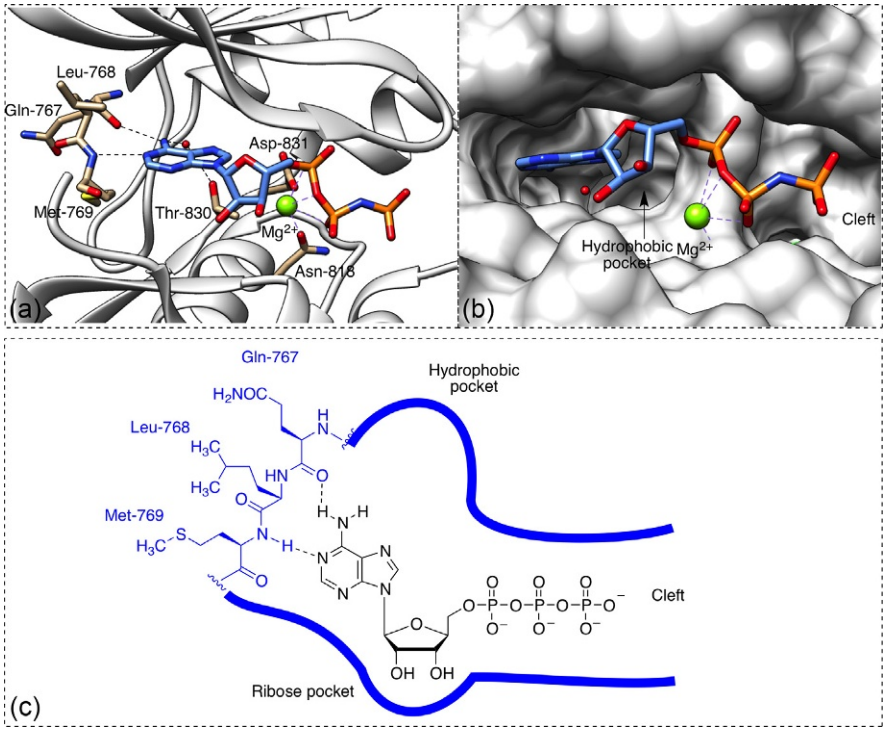


FIGURE 10.8

Binding of ATP to the EFGR active site. The structures were generated from Protein Data Bank reference 2GS7 and displayed with Chimera 1.8.1.

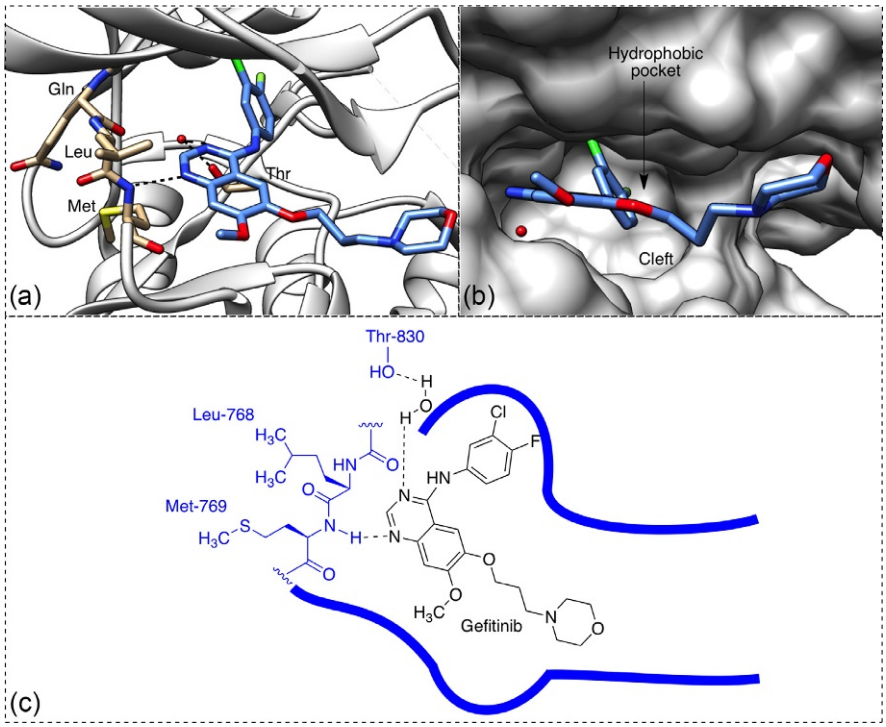
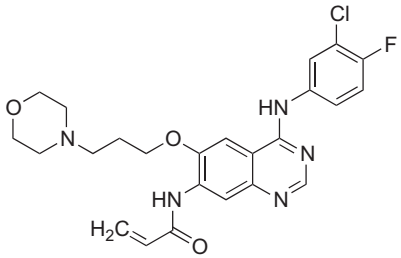


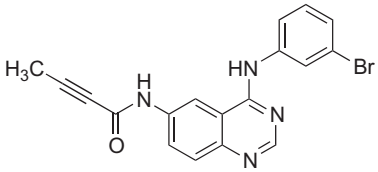
FIGURE 10.9

Binding of gefitinib to the EFGR active site. Generated from Protein Data Bank reference 2ITY and displayed with Chimera 1.8.1.

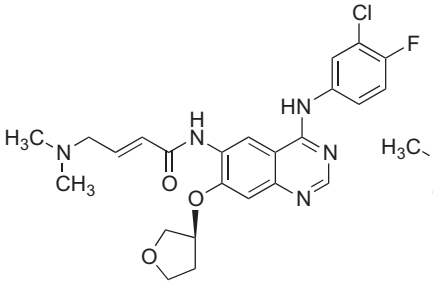
second-line therapy for NSCLC,[32] and it is also in clinical trials for breast, prostate, head, and neck cancer and glioma.



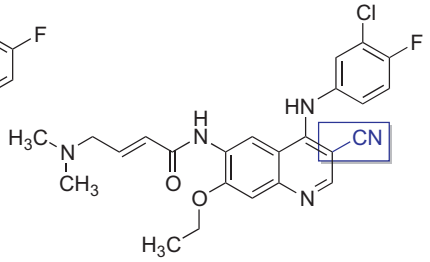
Canertinib (CI-1033)



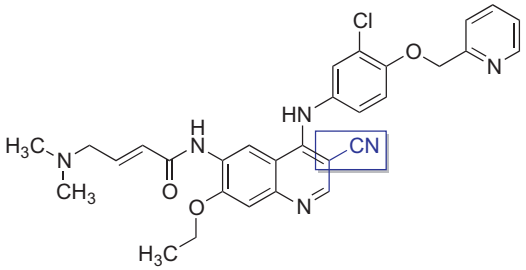
EKI-785



Afatinib (BIBW-2992))



Pelitinib (EKB-569)



Neratinib (HKI-272)

These compounds can be considered as active site-directed irreversible inhibitors because they contain a 4-anilinoquinazoline structural fragment (replaced in some of them by a 3-cyanoquinoline) that can be recognized by the ATP site and also an electrophilic α,β-unsaturated carbonyl moiety, responsible for covalent binding to the enzyme. The conserved cysteine residue Cys-773 within the ATP binding pocket seems to be responsible for the nucleophilic attack to these Michael substrates, as shown in Figure 10.10 for the case of EKI-785.[33]

Mutations of EGFR confer a drug-resistant state that does not diminish the kinase activity of the receptor but enhances its affinity for ATP while decreasing the affinity for the EGFR inhibitors. The most relevant mutation is T790M, which is present in 50–60% of patients who develop resistance to EGFR inhibitors (see Chapter 14, Section 9.2). Although there are currently no approved treatments for these patients, some investigational third-generation EGFR inhibitors have shown activity in them.

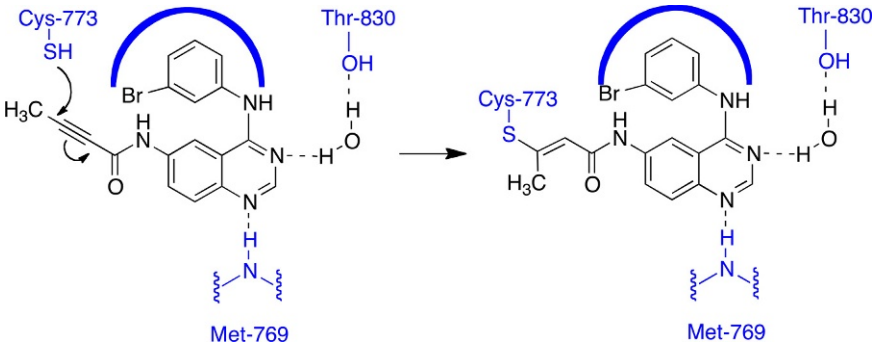
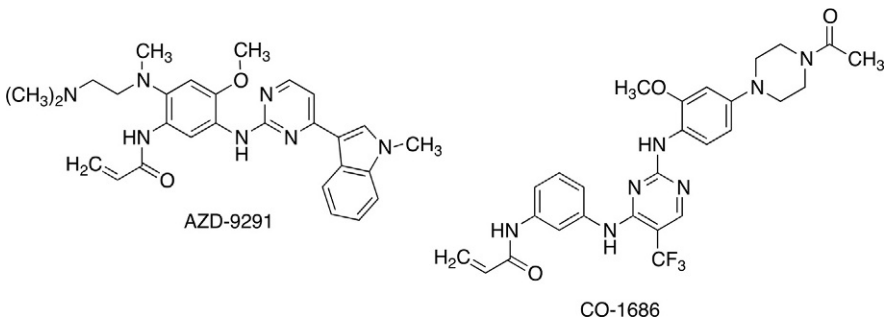


FIGURE 10.10

Binding of EKI-785, an irreversible EGFR inhibitor.

They include AZD-9291 and CO-1686, both of which are irreversible inhibitors of the T790M-mutated enzyme.[34] AZ-5104, a metabolite of AZD-9291, is also a potent inhibitor of mutated EGFR and may contribute to the efficacy of the latter.[35]



4.1.2 Monoclonal Antibodies Acting as Inhibitors of EGFR

Because antibodies recognize specific proteins with high specificity, they can be used as antagonists of the binding of an overexpressed protein to its ligands, although they are not devoid of toxic side effects (antibody-mediated cellular cytotoxicity). The role of monoclonal antibodies (mAbs) in cancer treatment is analyzed in Section 2 of Chapter 12, but some examples appear previously because they are discussed together with their specific targets.

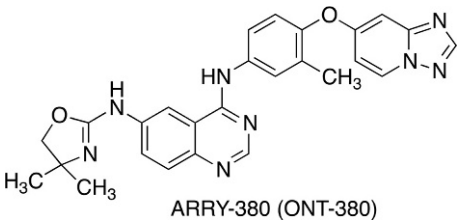
Antibodies for EGFR prevent the binding of EGF or TGF-α, and hence receptor dimerization and signal transduction, in addition to causing receptor internalization and proteosomal degradation. Cetuximab (IMC-C225, Erbitux®) is a chimeric monoclonal antibody* approved in 2004 for EGFR-expressing metastatic colorectal carcinoma. This approval was later extended for other

*A chimeric protein is one that is encoded by a nucleotide sequence made by splicing together two or more complete or partial genes, which can even be from different species.

indications. It has been recently found that cetuximab downregulates lactate dehydrogenase A (LDH-A) and inhibits glycolysis in an HIF-1α downregulation-dependent manner.[36] In 2006, the FDA approved panitumumab (ABX-EGFR, Vectibix®) for the treatment of patients with EGFR-expressing, metastatic colorectal cancer with disease progression following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens.[37] Other antibodies directed to EGFR that are under clinical evaluation are MDX-447[38] and nimotuzumab.[39] The development of matuzumab (EMD-72000) was halted in 2008 because of disappointing clinical results.

4.2 INHIBITORS OF HER-2

As previously mentioned, HER-2 is a member of the EGFR family identified as an important therapeutic target because it is overexpressed in approximately 20–30% of patients with aggressive breast cancer. In addition to the previously mentioned EGFR/HER-2 dual inhibitors, ARRY-380 (ONT-380) is an anilinoquinazoline derivative that acts as a reversible and selective HER-2 inhibitor, which has the additional advantage of being orally active due to its small-molecule nature. This compound is in phase I clinical studies.



ARRY-380 (ONT-380)

Some monoclonal antibodies are also directed at this receptor. The most important one is trastuzumab (Herceptin®), a humanized monoclonal antibody that targets the extracellular region of the HER-2 receptor, leading to its internalization and degradation. Interaction of trastuzumab with the human immune system via its human immunoglobulin G1 Fc domain may potentiate its antitumor activities. The mechanism of action of trastuzumab includes antagonizing the constitutive growth-signaling properties of the HER-2 system and enlisting of immune cells to attack and kill the tumor target, augmenting chemotherapy-induced cytotoxicity.[40]

Trastuzumab was approved in 1998 by the FDA, being used as part of a treatment regimen containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant treatment of women with node-positive, HER-2-overexpressing breast cancer. In 2010, it was also approved in combination with cisplatin and fluoropyrimidine for the treatment of patients with HER-2-overexpressing metastatic gastric or gastroesophageal (GE) junction adenocarcinoma. The antibody–drug conjugates trastuzumab emtansine or ado-trastuzumab emtansine (Kadcyla®), which combine trastuzumab with the potent antimicrotubule agent emtansine (DM-1), are discussed in Section 4.6 of Chapter 13.

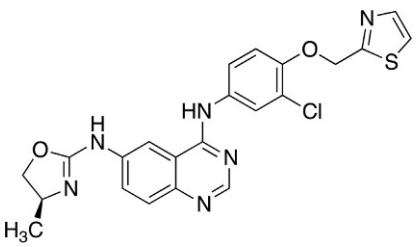
Pertuzumab (2C4, Perjeta®) is another antibody that was approved by the FDA in 2012 for use in combination with trastuzumab and docetaxel in the treatment of patients with HER-2-positive metastatic breast cancer.

4.3 INHIBITORS OF HER-3

Recently, the use of more sensitive methods to analyze protein interactions has uncovered the relevance of the cell surface receptor HER-3, which can be up to 10 times more effective than HER-2 in recruiting accessory proteins that drive the rapid proliferation, enhanced survival, and distant spread of cancers.[41] HER-3 lacks a fully functional tyrosine kinase domain, but upon ligand binding, it heterodimerizes with other receptors of the EGFR family, forming a functional oncogenic signaling unit in many HER-2-driven breast cancers. Compared to the other EGFRs, HER-3 has a number of direct binding sites for the p85 subunit of phosphoinositide-3-kinase (PI3K), which enables more efficient signaling via the PI3K–AKT pathway. Overactivation of HER-3 accounts for some of the resistance to EGFR and HER-2 inhibitory agents via either increased receptor phosphorylation and cell surface localization or overexpression of the receptor or upregulation of the ligands. Therefore, the HER-3 receptor is an interesting target for new antitumor therapeutics, and currently several antibodies, including MM-121, U3-1287, and LJM716,[42] are in clinical trials. Affibody molecules, which are small three-helix proteins originally derived from one of the subunits of staphylococcal protein A, are promising candidates for future HER-3-targeted cancer therapy.[43]

4.4 PAN-HER INHIBITORS

Varlitinib (ARRY-543, ASLAN 001) is another anilinoquinazoline that acts as a HER inhibitor, in this case without selectivity. It has shown clinical activity in both HER2-positive and EGFR-positive tumors and is currently in clinical studies for gastric cancer, both alone and in combination.



Varlitinib (ARRY-543, ASLAN 001)

4.5 INHIBITORS OF INSULIN-LIKE GROWTH FACTOR RECEPTORS (IGF-1R)

The insulin-like growth factors (IGFs) are peptides with a high sequence homology with insulin which are part of a complex system (often referred to as the IGF "axis") that has a role in the promotion of cell proliferation and in the inhibition of apoptosis. Insulin-like growth factor-1 receptor (IGF-1R) is a membrane tyrosine kinase receptor with a 70% homology to the insulin receptor that, when activated by its ligands IGF-1, IGF-2, or insulin at supraphysiological concentrations, transmits a signal to its two major substrates, insulin receptor substrate-1 (IRS-1) and Shc, and the signal is subsequently transduced to the nucleus. Reduction of tumor invasion upon blockade of IGF-1R by several inhibitors indicated the critical function of this signaling for the acquisition of a malignant phenotype[44] and in chemotherapy resistance, but it has proven to be a tough target. It has been shown

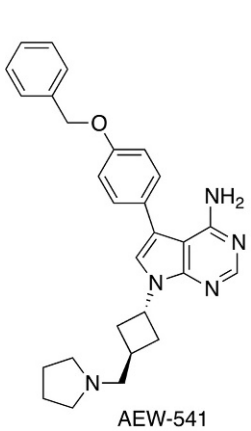
that dual targeting of HER-2 and IGF-1R improves response in cell line models of acquired trastuzumab resistance.[45]

Various strategies, such as anti-IGF-1R antibodies, IGF-1 mimetic peptides, antisense strategies, IGF-1R-specific peptide aptamers, targeted degradation of IGF-1R, and expression of dominant-negative IGF-1R mutants, have been explored to inhibit IGF-1R signaling. Targeting the intracellular kinase domain of IGF-1R with small molecules (most commonly ATP antagonists) has gained considerable attention,[46] although the high sequence homology of the kinase domains of IGF-1R and insulin receptor (IR) may be associated with metabolic adverse effects because they can affect both IR and IGF-1R signaling. This similarity has also complicated the design process for IGF-1R-specific low-molecular-weight TKIs.

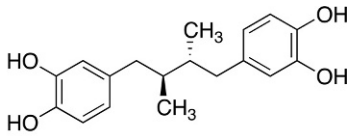
AEW-541 is a small-molecule inhibitor of the autophosphorylation of IGF-1 receptor whose antineoplastic efficacy has been shown in experimental models of several cancers, including musculoskeletal tumors, multiple myeloma and biliary cancers.[47]

INSM-18 (nordihydroguaiaretic acid, NDGA) is an antioxidant component of the creosote bush (*Larrea tridentata*) that inhibits IGF-1R and human epidermal growth factor receptor (Her2/Neu). It has demonstrated antitumor activity in preclinical studies of breast, lung, pancreatic, and prostate tumors, and preliminary clinical studies are encouraging.

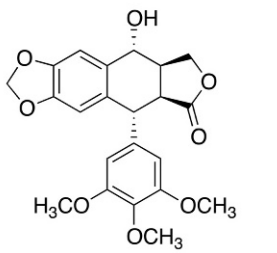
BVP-51004, also known as cyclolignan PPP, is an ATP noncompetitive IGF-IR inhibitor that inhibits the phosphorylation of Tyr-1136 in the activation loop of IGF-IR kinase, which contributes to stabilize the conformation of the activation loop. A study in human colorectal cancer cells indicated that BVP-51004 is a selective IGF-1R kinase inhibitor that is highly effective in IGF-2-driven tumors.[48]



AEW-541



INSM-18

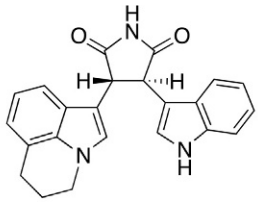


BVP-51004 (cyclolignan PPP)

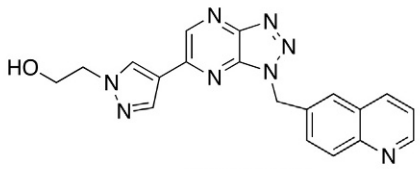
Among anti-IGF-1R monoclonal antibodies, figitumumab (CP-751871) was developed for the treatment of various forms of cancer, including lung, prostate, breast, and colorectal cancers and Ewing's sarcoma, but its development was halted in 2011 after the failure of two phase III studies in NSCLC. Another antibody, ganitumab (AMG-479), was positioned for a number of malignancies, but the results obtained for pancreatic cancer led to halting further development.[49]

4.6 INHIBITORS OF HEPATOCYTE GROWTH FACTOR RECEPTOR (HGFR, c-MET)

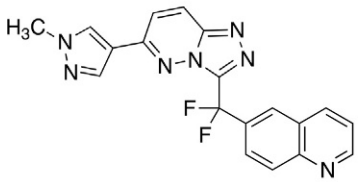
The hepatocyte growth factor (HGF) is a heparin-binding glycoprotein that binds to the tyrosine kinase receptor (HGFR), also known as c-Met (mesenchymal–epithelial transition factor). It is a receptor tyrosine kinase that is normally expressed in stem and progenitor cells, which allows them to grow invasively in order to generate new tissues in an embryo or regenerate damaged tissues in an adult. However, this protein is overexpressed or mutated in many tumor cell types playing key roles in tumor cell proliferation, survival, invasion, metastasis, and tumor angiogenesis. Many patent applications associated with inhibition of the HGF/c-Met axis have been published, and some small-molecule c-Met inhibitors and monoclonal antibodies directed against HGF and c-Met, as well as multitargeted therapies, have entered clinical trials with encouraging results.[50] Tivantinib (ARQ 197), a selective kinase inhibitor that is non-ATP competitive, as well as the ATP competitive inhibitors JNJ-38877605, PF-04217903, and the Novartis-developed compound INC280 (formerly INCB-28060),[51] are examples of small-molecule c-Met inhibitors in clinical trials.



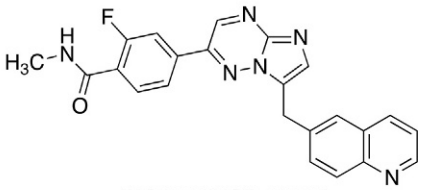
Tivantinib (ARQ 197)



PF-04217903



JNJ-38877605



INC280 (INCB-28060)

4.7 INHIBITORS OF PRO-ANGIOGENIC TYROSINE KINASES: VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR AND RELATED KINASE RECEPTORS

Angiogenesis is defined as the growth of new blood vessels from preexisting microvasculature. Solid tumors can surpass their supply of nutrients and oxygen as they grow, resulting in metabolic stress. Tumor cells must then undergo a period of adaptation, inducing angiogenesis and neovascularization or apoptosis. The "angiogenic switch" is a discrete step that can occur at different stages in the tumor-progression pathway, depending on its nature and microenvironment. Most tumors start growing as avascular nodules until they reach a steady-state level of proliferating and apoptosing cells in which the angiogenic switch ensures exponential tumor growth. The process begins with perivascular detachment and vessel dilation, followed by angiogenic sprouting, new vessel formation and maturation, and the recruitment of perivascular cells. In healthy adults, angiogenesis is triggered only locally and

transiently in processes such as wound healing, and changes in the equilibrium between pro- and anti-angiogenic factors are associated with a number of disease states. Tumors express many angiogenic factors, and as the tumor cells proliferate, hypoxic conditions lead to increased expression and activity of hypoxia-inducible factor (HIF), epidermal growth factor (EGF), and IGF-1, which induce the activation of PI3K and MAPK signaling.

Some pro-angiogenic growth factors, such as the vascular endothelial growth factor (VEGF) family, the platelet-derived growth factor (PDGF), and the fibroblast growth factor (FGF), bind to and activate the cell-surface tyrosine kinase receptors VEGFR-1 (FLT-1), VEGFR-2 (KDR), and VEGFR-3 (FLT-4). The previously mentioned EGF also has activity as a pro-angiogenic growth factor. VEGFR-1 was the first receptor tyrosine kinase to be identified, and its signaling can be important in tumor growth and metastasis, including the induction of matrix metalloproteinases such as MMP9 (M-phase phosphoprotein 9). VEGFR-2 is expressed in endothelial cells and is the principal receptor through which VEGFs exert their mitogenic, chemotactic, and vascular permeabilizing effects on the host vasculature (Figure 10.11). Activation of VEGFR-3 promotes lymphangiogenesis.

Because VEGF signaling is critical for blood vessel formation and is involved in all stages of angiogenesis, its inhibition is an attractive therapy target in a wide range of tumor types, and disruption of the VEGF signal has become one of the dominant strategies for the angiogenesis-related treatment of cancer.[52] There are two main classes of approved drugs in this area: monoclonal antibodies such as bevacizumab (Avastin®) and orally active small-molecule TKIs such as sunitinib (Sutent®), vandetanib (Caprelsa®), sorafenib (Nexavar®), cabozantinib (Cometriq®), pazopanib (Votrient®), axitinib (Inlyta®), and nintedanib (Vargatef®). Whereas bevacizumab binds circulating and local VEGF and hence neutralizes its biologic activity, the TKIs inhibit the intracellular catalytic function of VEGF receptors expressed by vascular endothelial cells, particularly VEGFR-2, the major signaling receptor for VEGF-mediated (tumor) angiogenesis. They are not totally specific for VEGF receptors and also antagonize the function of other RTKs similar in structure, such as platelet-derived growth factor receptors (PDGFRs), c-kit, Flt-3, and Raf.

4.7.1 VEGFR Inhibitors

Semaxanib (SU-5416) was the first indolinone derivative that inhibited VEGFR-1 and PDGFRs. It was identified in a high-throughput library screening and reached phase III clinical trials for colorectal cancer, but its development was discontinued at this stage.[53] SU-6668,[54] obtained by introduction of a

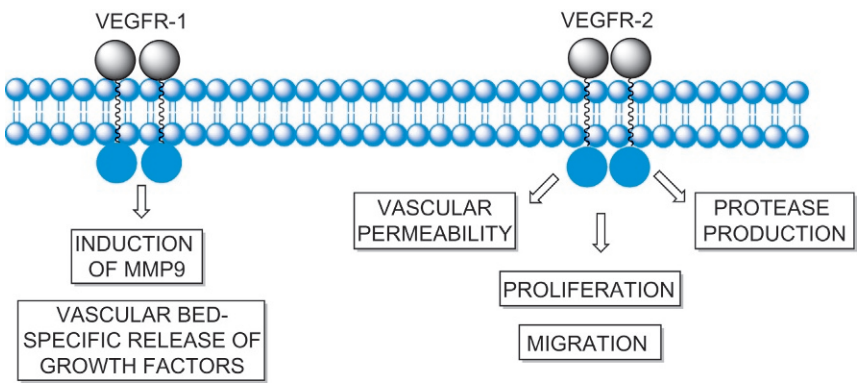


FIGURE 10.11

Events triggered after activation of VEGFR-1 and VEGFR-2.

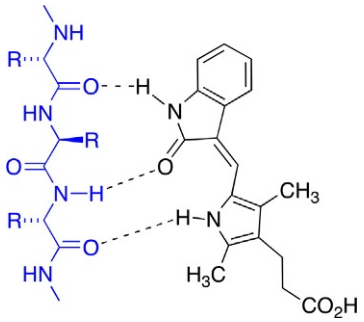
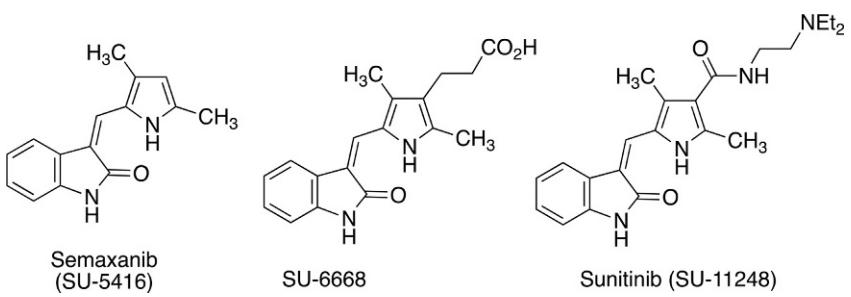


FIGURE 10.12

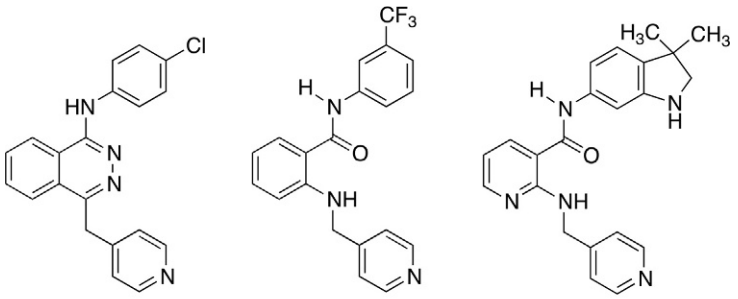
Binding of the indolinone sunitinib (SU-6668) to VEGFR-1.

propionic acid chain at the C-4′ position of semaxanib, was also a disappointment in clinical trials, but the (diethylaminoethyl)carbamoyl derivative sunitinib (SU-11248, Sutent®)[55] was approved by the FDA in 2006 and 2011 for gastrointestinal and renal cancer and for neuroendocrine pancreatic tumors, respectively. X-ray crystallographic studies of these pyrrole-derived indolinones co-crystallized with VEGFR-1 showed that these inhibitors occupy the same region as ATP and establish several hydrogen bond interactions in a side chain of the receptor as shown for SU-6668 (Figure 10.12).

Sunitinib inhibits multiple receptor kinases, including VEGFRs, PDGFRs, and c-Kit (CD117).[56] Because the latter kinase, when improperly activated by mutation, drives the majority of gastrointestinal stromal cell tumors,[57] sunitinib has been recommended as a second-line therapy for patients who become intolerant to imatinib.[58]



Vatalanib (PTK-787, ZK-222584) is an orally available aminophthalazine derivative that was identified through a screen of a chemical library against VEGFR-1.[59] It potently inhibits several VEGFR kinases, and also the tyrosine kinase activity of c-Kit and PDGFR, and has shown promising results in patients with metastatic colorectal cancer.[60] It is active in patients diagnosed with imatinib- or sunitinib-resistant gastrointestinal stromal tumors,[61] and it has been used as a starting point for the development of second-generation VEGFR inhibitors. Based on its binding mode to the receptors, an anthranilamide scaffold was selected for optimization, leading to the identification of AAL-993 as a potent and selective VEGFR-2 inhibitor. Motesanib (AMG-706) is a related inhibitor of VEGFR and PDGFR that has undergone clinical testing for non-squamous non-small cell lung carcinoma and breast cancer.[62]



Vatalanib (PTK-787, ZK-222584) AAL-993 (ZK-260255) Motesanib (AMG 706)

The crystal structure of the drug–protein complex showed that, similarly to imatinib (see later), AAL-993 targets the inactive conformation of the enzyme. The binding involves three hydrogen bond interactions (Figure 10.13) and several hydrophobic interactions. The phenyl ring of the anthranilamide unit is sandwiched between the hydrophobic side chains of Val-916 and Lys-868, and the trifluoromethylphenyl substituent fits a lipophilic pocket.[63]

Quinazolines were initially developed as EGFR tyrosine kinase inhibitors and later refined to give VEGFR-2-selective compounds. Among other members of this family, vandetanib (ZD-6474, Zactima®, Caprelsa®) demonstrated therapeutic efficacy in a phase III trial of patients with advanced medullary thyroid cancer,[64] having been approved for this indication in 2011 by the FDA and in 2012 by the European Medicines Agency (EMA). Vandetanib occupies the ATP adenine binding site, where it forms a single hydrogen bond involving its N-1 nitrogen and the Cys-912 residue of the protein. Several structure–activity relationships (SARs) have been deduced for this family, including an increased activity for the 2-fluoro and 5-hydroxy derivatives, the latter effect being attributed to the formation of an additional hydrogen bond.[65] Another promising quinazoline derivative that acts on VEGFR signaling is cediranib (AZD-2171, Recentin®), which is undergoing a number of clinical trials (phase I and phases II/III) to evaluate its potential role in the treatment of a range of solid tumors.[66] Recent clinical trials have shown that its combination with the PARP inhibitor olaparib is significantly active in recurrent platinum-sensitive ovarian cancer.

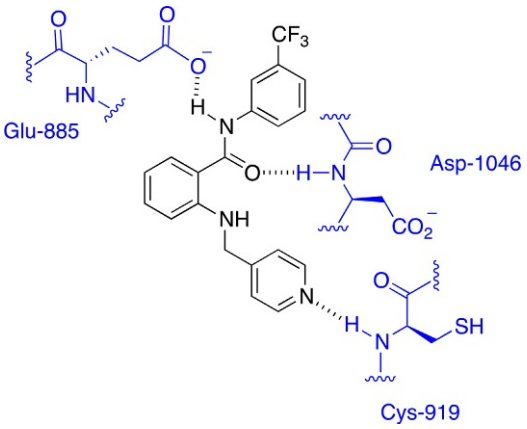
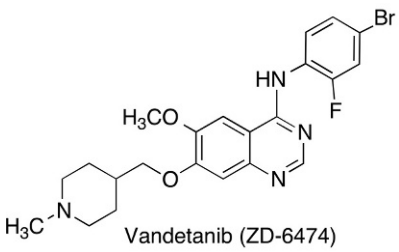
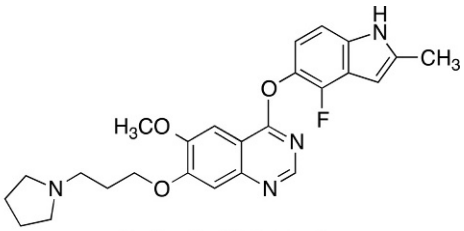


FIGURE 10.13

Binding of AAL-993 to VEGFR-2.

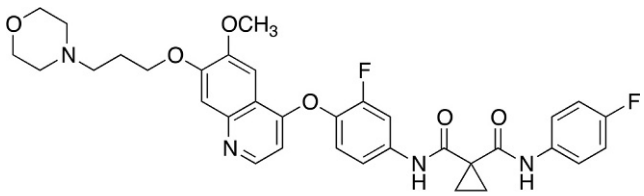


Vandetanib (ZD-6474)

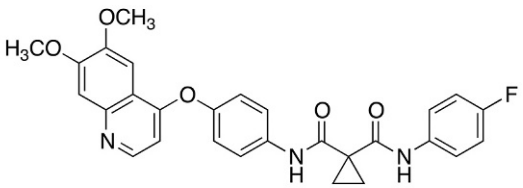


Cediranib (AZD-2171)

Because VEGF and HGF–c-Met signaling are activated in angiogenesis, the combined inhibition of both signaling has a major effect on the induction of endothelial cell apoptosis and reduction in the formation of capillaries as well as on the decreased microvessel density within tumors. Among a family of quinoline derivatives, foretinib (EXEL-2880, GSK-1363089, XL-880) inhibits several receptors, mainly VEGFR-2 and the hepatocyte growth factor receptor (HGFR, c-Met). Foretinib has entered phase II clinical trials in patients with estrogen, progesterone, and HER-2 receptor negative recurrent/metastatic breast cancer,[67] metastatic gastric cancer, and squamous cell cancer of the head and neck.[68] Its analog, cabozantinib (Cometriq®, XL184), is also a potent inhibitor of both c-Met and VEGFR-2 that showed promising signs of antitumor activity at doses not associated with toxicity in its early clinical experience. It was approved by the FDA in 2012 for the treatment of medullary thyroid cancers and is also in clinical trials for other malignancies, in which it has shown encouraging activity in castration-resistant prostatic cancers.



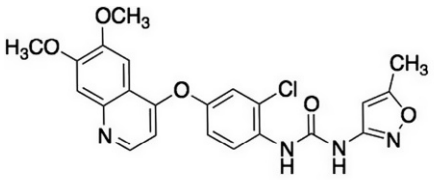
Foretinib (GSK-1363089, XL-880)



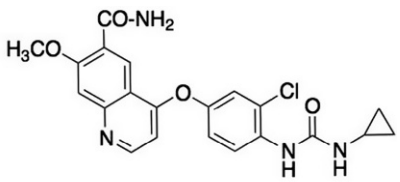
Cabozantinib (XL184)

Tivozanib (AV-951), which bears a high degree of structural similarity with foretinib and cabozantinib but has a urea group instead of a malonamide, is an orally active inhibitor that was designed to target all three VEGF receptors, and it also has shown high potency against c-Kit and PDGFR. This compound displayed promising activity in renal carcinomas, reaching phase III clinical studies, but it showed inferior overall survival rates in comparison with sorafenib. The closely related lenvatinib (E-7080, Lenvima®) is another quinoline/*N*-phenylurea hybrid that inhibits both VEGFR-2 and VEGFR-3. This compound was approved in 2012-2013 by several agencies as an orphan drug for several types of thyroid cancer not sensitive to radioiodine. In early 2015, it finally received FDA approval for radioactive iodine-refractary differentiated thyroid cancer. Linifanib (ABT-869), another urea derivative, is a multitargeted receptor tyrosine kinase inhibitor

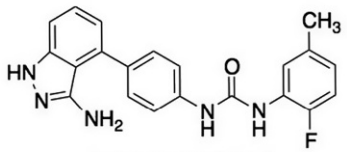
that has high potency against VEGF-2, PDGF, colony-stimulating factor 1 receptor (CSF-1R), and several tyrosine kinases, and is under phase III clinical studies for advanced hepatocellular carcinoma.[69]



Tivozanib (AV-951)

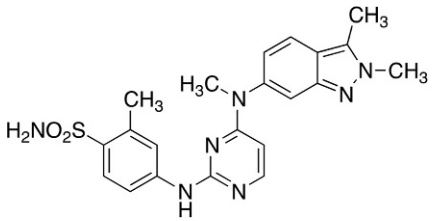


Lenvatinib (E-7080)

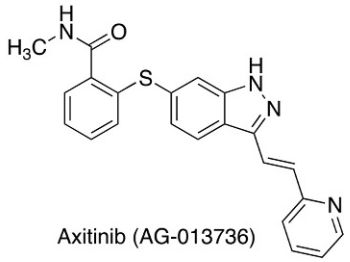


Linifanib (ABT-869)

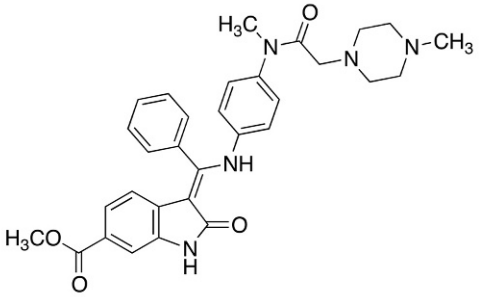
Pazopanib (GW-786034, Votrient®) is a pyrimidine derivative orally active, potent, and selective multitargeted receptor tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α/β, and c-Kit that blocks tumor growth and inhibits angiogenesis. It was approved in 2009 by the FDA for renal cell carcinoma and in 2012 for soft tissue sarcoma, being also active in ovarian cancer, NSCLC, and other cancers.[70] The indazole derivative axitinib (AG-013736, Inlyta®) is another orally available inhibitor of VEGFR, PDGFR, and c-Kit (CD117) tyrosine kinases that was approved by the FDA in 2012 for metastatic renal cell carcinoma. It is also in clinical development for other tumors.[71,72] Nintedanib (BIBF1120, Vargatef®) is a potent inhibitor of the receptor tyrosine kinases VEGFR, FGFR, and PDGFR, which are crucially involved in angiogenesis. It is in phase III clinical trials for NSCLC, ovarian cancer, and idiopathic pulmonary fibrosis (IPF), and it will be further evaluated in a number of other tumor types, including hepatocellular, renal, and colorectal cancers.[73] In 2013, it was granted orphan drug designation for the treatment of IPF by the EMA.



Pazopanib (GW-786034)

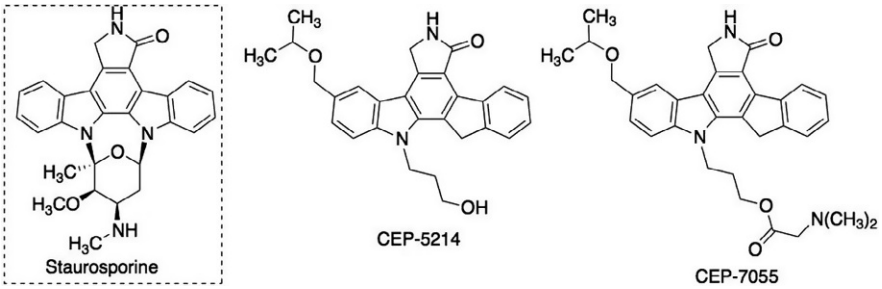


Axitinib (AG-013736)



Nintedanib (BIBF-1129)

Another family of VEGFR inhibitors has been designed as analogs of the natural product staurosporine, a nonselective kinase inhibitor. CEP-5214, which was identified from SAR studies of approximately 2000 analogs as the best candidate, has a potent pan-VEGFR kinase inhibitory activity. Its *N*,*N*-dimethylglycine ester CEP-7055 is a water-soluble prodrug that can be orally administered and has entered clinical trials.[74]



4.7.2 Other Types of Anti-VEGF Therapy

Bevacizumab (Avastin®) is a recombinant humanized monoclonal IgG1 antibody that inhibits all isoforms of VEGF-A, thereby blocking their binding to VEGFR-1 and VEGFR-2.[75] It was the first approved agent to target tumor angiogenesis, in 2004 by the FDA and in 2005 in Europe, to be used in combination with other drugs such as 5-fluorouracil or irinotecan for the first-line treatment of patients with metastatic colorectal cancer. It has since been approved for other tumors, such as NSCLC, renal cell cancer, and glioblastoma multiforme. The provisional approval for metastatic breast cancer given by the FDA in 2008 was revoked in 2011, although the drug remains approved for breast cancer in some countries. It is also being studied for other indications.[76] On the other hand, in December 2011, bevacizumab was approved as front-line treatment for women with newly diagnosed, advanced ovarian cancer in Europe, enabling the use of Avastin® in combination with carboplatin and gemcitabine for treatment of adult patients with first recurrence of platinum-sensitive epithelial ovarian, fallopian tube, or primary peritoneal cancer who have not received prior therapy with bevacizumab or other VEGF inhibitors or VEGF receptor-targeted agents.

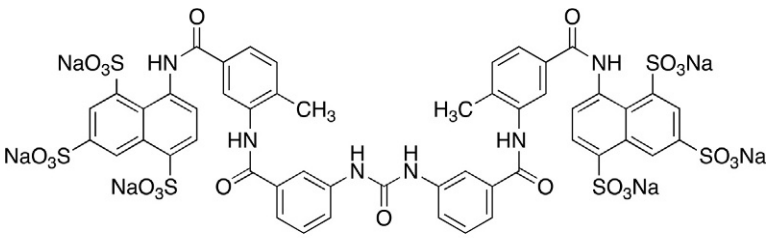
Ziv-aflibercept (Zaltrap®) is a chimeric protein comprising segments of the extracellular domains of VEGFR-1 and VEGFR-2 fused to the constant region (Fc) of human IgG1 with potential antiangiogenic activity. Its binding to these pro-angiogenic factors and to the placental growth factor prevents the binding of these factors to their cell receptors. This disruption may result in the inhibition of tumor angiogenesis, metastasis, and ultimately tumor regression. Ziv-aflibercept was approved in 2011 by the FDA for the treatment of wet macular degeneration and in 2012 (in combination with 5-fluorouracil, leucovorin, and irinotecan) to treat adults with metastatic colorectal cancer that is resistant to or has progressed following an oxaliplatin'containing regimen.[77]

Although the majority of known enzymes are proteins, other molecules such as ribozymes also display catalytic activity. Ribozymes (from *ribonucleic acid enzymes*) are RNA molecules involved in a variety of cellular processes, but their most interesting property from the standpoint of cancer therapy is their ability to catalyze the cleaving of messenger RNA (mRNA) molecules that can no

longer be translated to produce protein. By targeting the mRNAs encoding proteins with pathological roles in cancer, ribozymes can slow or inhibit cancerous growth. Inhibition of the VEGF receptor activity can be accomplished using ribozymes that cleave the mRNAs for the primary VEGF receptors. Angiozyme (RPI.4610)[78] is one of these ribozymes. It was undergoing clinical studies for the treatment of solid tumors, but the clinical responses were not very good. For instance, a recent phase II trial aimed at the treatment of metastatic breast cancer showed that although angiozyme had a well-tolerated safety profile, it lacked sufficient clinical efficacy to justify its further development.[79]

4.7.3 Inhibitors of PDGFRs

Some inhibitors of this angiogenic signal transduction, such as sunitimib, vatalanib, pazopanib, axitinib, and nontedanib, have been previously mentioned. Another important compound is suramin (Metaret®), a polysulfonated naphthylurea originally developed for the treatment of trypanosomiasis and onchocerciasis that blocks the activity of several angiogenic factors, especially PDGF and FGF. After its internalization into the cell, it may affect the activity of various key enzymes involved in the intracellular transduction of mitogenic signals, including protein kinase C (PKC). Furthermore, it inhibits the enzyme heparanase, an endo-β-D-glucuronidase that is strongly implicated in tumor metastasis and angiogenesis (see Chapter 11, Section 3). Suramin has demonstrated a variety of biological effects including antitumor activity against several cancers, being evaluated in clinical trials in combination with several other chemotherapeutic agents in patients with distinct solid tumors.[80] It was submitted to the FDA for the treatment of hormone-refractory prostate cancer in 1997, but its approval was rejected in 1998, receiving instead approval for the "List of Orphan Designations and Approvals." A study showed that suramin had a palliative effect in terms of improvement in quality of life and decreased levels of depression in patients with metastatic hormone-refractory prostate carcinoma.[81]



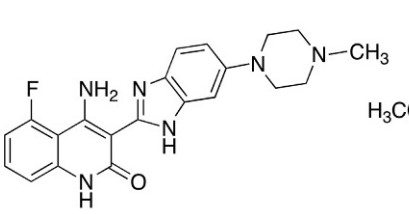
Suramin

4.7.4 Fibroblast Growth Factor Receptor (FGFR) Inhibitors

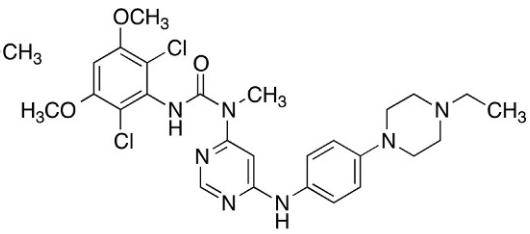
Activating mutations or overexpression of FGFRs or their ligands have been associated with neoplastic progression and tumor vascularization in multiple cancer types, including breast cancer, bladder cancer, multiple myeloma (MM), hepatocellular, and renal cell carcinoma.

Dovitinib (TKI258) is an inhibitor of tyrosine kinases VEGFR, PDGFR, and FGFRs that is in phase III trials for renal cell carcinoma and in phase II trials for advanced breast cancer,

hepatocellular carcinoma, and endometrial cancer.[82] BGJ398 (NVP-BGJ398) is an orally bioavailable selective inhibitor of the FGFRs[83] that is in ongoing phase I studies in advanced solid tumors.



Dovitinib (TKI-258)



BGJ398 (NVP-BGJ398)

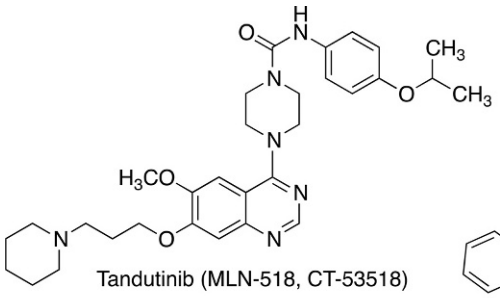
PRO-001 is a cytotoxic monoclonal antibody of the fibroblast growth factor receptor 3 (FGFR3), a receptor tyrosine kinase whose expression is associated with MM.[84,85]

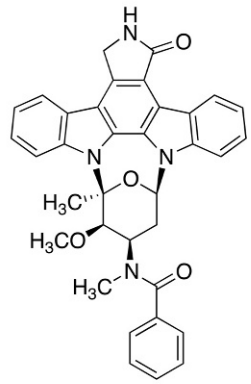
4.8 INHIBITORS OF FLT3 (CD135)

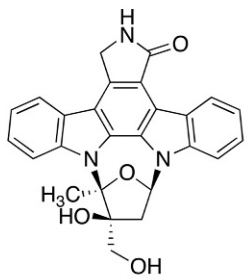
FLT3 (CD135) is a membrane receptor tyrosine kinase of type III related to PDGFR and c-Kit. When this receptor binds its ligand (FLT3L), it forms a homodimer that activates its tyrosine kinase activity, phosphorylating and activating several signal transduction molecules. This signaling plays a role in cell survival, proliferation, and differentiation of lymphocytes. Because mutations of FLT3 are present in approximately 30% of acute myeloid leukemia (AML) patients and are associated with lower cure rates from standard chemotherapy, this kinase has become a very popular target for the design of drugs against AML.

The 4-anilinoquinazoline tandutinib[86] (MLN-518, CT-53518) is a potent, ATP-competitive inhibitor of FLT3 and PDGFR tyrosine kinases that exhibited limited activity as a single agent in phase I and II clinical trials in patients with AML and myelodysplastic syndrome, but it displayed promising antileukemic activity in a phase I/II trials in patients with newly diagnosed AML when administered in combination with cytarabine and daunorubicin. Phase II clinical trials for tandutinib were ongoing in patients with AML or metastatic renal cell carcinoma (mRCC),[87] but it was concluded that due to excessive toxicity, it should not be further developed for the last indication.[88]

Other FLT3 inhibitors have been designed as analogs of staurosporine, the most studied being lestaurtinib (CEP-701)[89] and midostaurin (PKC-412).[90] Both compounds inhibit several kinases besides FLT3 and are under clinical evaluation for AML and other tumors. In 2006, the FDA granted for lestaurtinib (CEP-701) orphan drug designation for the treatment of AML. Additional targets and indications were later found for this compound.[91]







4.9 INHIBITORS OF Bcr-Abl TYROSINE KINASE (ABELSON KINASE)

In normal cells, the *Bcr* (breakpoint cluster region) and *Abl* genes are in different chromosomes and encode different proteins, but in chronic myeloid leukemia (CML) there is an exchange of genetic material between chromosomes 9 and 22 whereby the latter is altered and becomes the so-called Philadelphia chromosome. This chromosome codifies the oncogenic protein Bcr-Abl, also known as the Abelson tyrosine kinase, which is a hybrid PTK with deregulated and high ABL kinase activity, resulting in a high leukocyte count. The TK domain contained in the ABL portion of this hybrid protein is therefore the target for the design of drugs for the treatment of CML.[92]

4.9.1 Compounds Acting as ATP Mimics

Imatinib (STI-571, from *s*ignal *t*ransduction *i*nhibitor; Glivec®) was the first protein kinase inhibitor to be approved as a cancer treatment. After a particularly rapid clinical development, it was approved in 2001 for patients with Philadelphia chromosome-positive CML.[93] The lead compounds in the development of imatinib (Figure 10.14) were 2-anilinopyrimidine derivatives **10.1**, identified by random screening as inhibitors of the serine–threonine kinase PKC (see Section 5.6). All attempts to modify the guanidine portion shown in bold were unsuccessful, which was later explained by its involvement in two hydrogen bonds with the active site of kinases. Optimization work led to compound **10.2**, bearing a 3-pyridyl substituent, as a potent inhibitor of PKC and to the discovery that the addition of an amide group to the anilino substituent led to compounds, such as **10.3**, that are dual inhibitors of PKC and

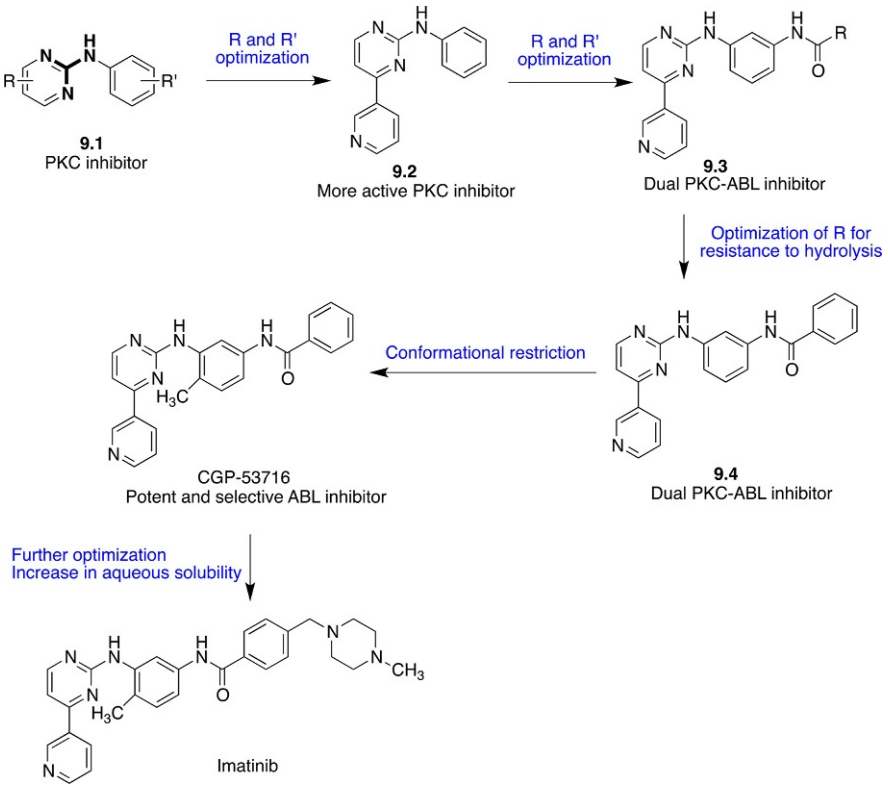


FIGURE 10.14

Development of imatinib.

ABL. One potential problem with these compounds was their hydrolysis *in vivo* to aniline derivatives, which are known to be mutagens through their metabolic activation. For this reason, the amide moiety had to be optimized for resistance to hydrolysis, and the benzamido group shown in compound **10.4** was chosen for this purpose. In efforts to eliminate the PKC inhibitory activity, a number of analogs were prepared, and it was found that an *ortho*-methyl substituent led to a selective ABL inhibitor (CGP-53716), which can be explained by assuming that the conformational restriction imposed by this substitution forces the molecule into a conformation that is suitable only for the ABL active site. Finally, further modifications were carried out in order to improve aqueous solubility by the introduction of basic side chains that would allow the preparation of salts, leading to the preparation of STI-571 (imatinib).[94] Unexpectedly, it was later shown that the piperazine ring added for this purpose also contributed to binding at the active site (see later).

X-ray crystallography of a simplified model compound[95] and of imatinib[96] in the active site of ABL and related kinases[97] has shown that imatinib binds at the ATP binding site of ABL, showing specificity for an inactive conformation of the kinase. This inactive form contains the amino terminus of the activation loop folded into the ATP binding site and mimics a bound peptide substrate. The fact that imatinib binds to an unusual conformation of the kinase may explain its high selectivity. The drug is

sandwiched between the N and C lobes of the kinase domain and penetrates through the central region of the protein. In this arrangement, the pyridine and pyrimidine rings of imatinib occlude the region where the adenine ring of ATP binds. The rest of the compound wedges itself between the activation loop and helix αC, whereby the kinase is maintained in an inactive conformation. The piperazine ring lies along a hydrophobic pocket on the surface, making Van der Waals interactions reinforced by hydrogen bonds with the carbonyl oxygen atoms of Ile-360 and His-361., Imatinib makes a total of six hydrogen bond contacts (Figure 10.15), with a large number of complementary Van der Waals interactions.

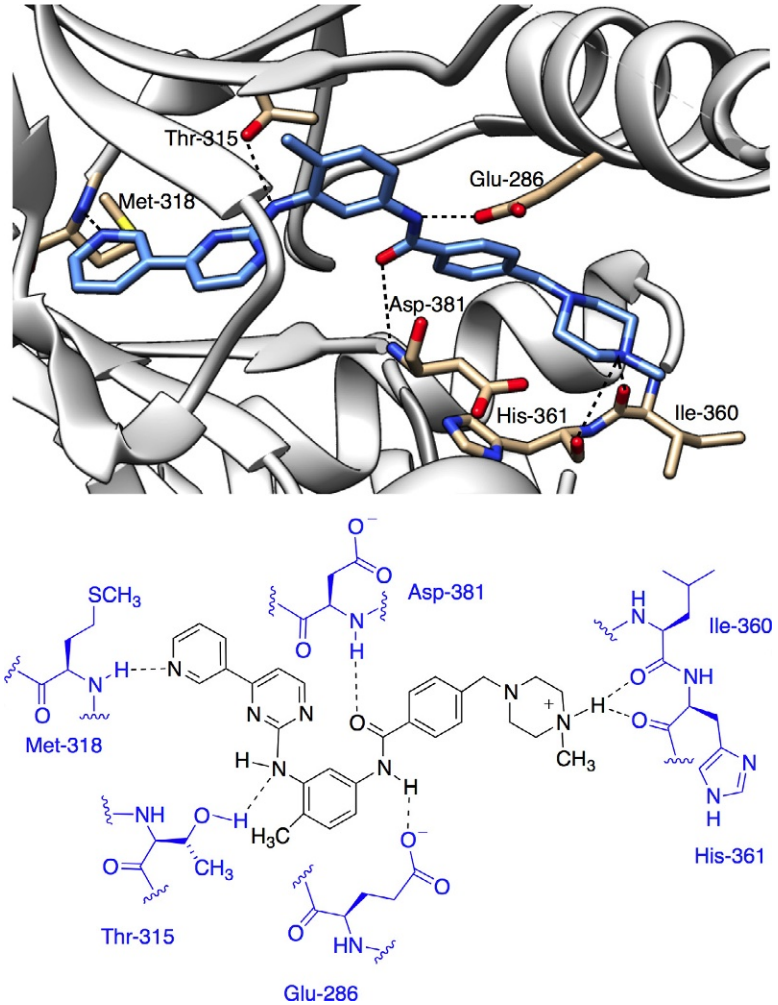


FIGURE 10.15

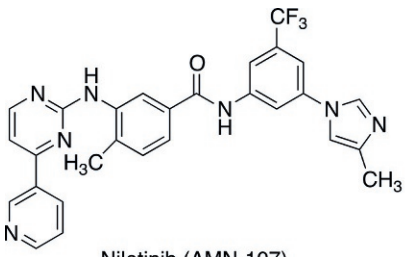
Interaction of imatinib with human Abelson kinase. The three-dimensional structure was generated from Protein Data Bank reference 2HYY and displayed with Chimera 1.8.1.

In addition to Bcr-Abl, imatinib inhibits other kinases including c-Kit, which is mutated in a rare subset of gastrointestinal soft tissue sarcomas known as gastrointestinal stromal tumors (GISTs). It also inhibits (PDGF)-receptor tyrosine kinase, which has an important role in tumorigenesis, especially in chronic myeloproliferative diseases. On the basis of a series of phase II studies, imatinib was granted approval in 2002 for the treatment of advanced or metastatic GISTs, and it was granted approval for adjuvant use in patients with resectable GISTs in 2008. Its activity in glioma, prostate cancer, and small cell lung cancer is under active research.

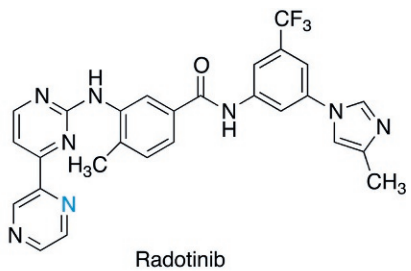
Resistance against imatinib is increasingly being encountered, but the molecular basis of this resistance remains somewhat controversial. Together with other mechanisms involving transport by Pgp-170 and others, this resistance has been associated with mutations in the Bcr-Abl[98] and c-Kit[99] kinase domains, which impair the ability of the kinase to adopt the specific conformation to which imatinib binds.

Nilotinib (AMN-107, Tasigna®)[100] is an imatinib analog in which an imidazole ring replaces the piperidine moiety and the amide function is reversed. It has a high affinity and specificity for Bcr-Abl. In addition to being more potent than imatinib against wild-type Bcr-Abl, nilotinib is also significantly active against most imatinib-resistant Bcr-Abl mutants and seems to be superior to imatinib in terms of the development of resistance.[101] It received FDA and EMA approval for patients with resistance or intolerance to existing therapies in 2007, and in 2010 it received approval for newly diagnosed CML patients. The closely related radotinib (Supect®) is a dual inhibitor of Bcr-Abl kinase and of PDGFR that has been approved in South Korea for use as a second-line treatment of CML.

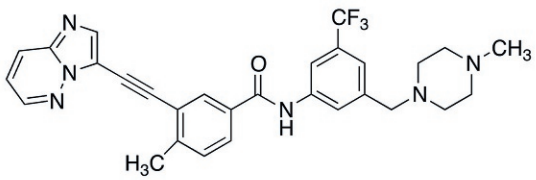
Since the T315I mutant of Bcr-Abl kinase emerged as resistant to these agents, the structure-guided design of novel series of potent pan-inhibitors of Bcr-Abl (including the T315I mutation) showed that a key structural feature to achieve this aim was the presence of a carbon–carbon triple-bond linker. One of these compounds is ponatinib (AP24534, Iclusig®), a potent, orally active pan-kinase inhibitor that targets Bcr-Abl, but also the VEGFR and FGFR families of kinases,[102] which was approved by the FDA in 2012.



Nilotinib (AMN-107)



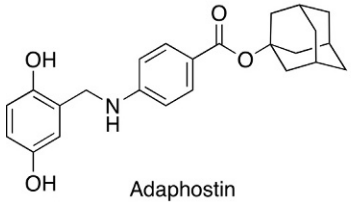
Radotinib



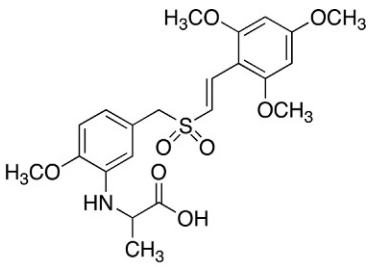
Ponatinib (AP24534)

4.9.2 Compounds Acting as Tyrosine Mimics

In contrast to the ATP-competitive compounds mentioned so far, another approach to the design of Bcr-Abl inhibitors has been analogy to its substrate—that is, tyrosine. Some of them, such as adaphostin and ON-012380, are being developed for *Bcr-Abl* mutants resistant to imatinib. The dihydroquinone derivative adaphostin (NSC-680410) is an adamantyl ester analog of thyrfostin AG957 that inhibits Bcr-Abl-mediated signaling much more slowly than imatinib mesylate, although it induces apoptosis more rapidly. Several studies have demonstrated that, in addition to its activity as a Bcr-Abl kinase inhibitor, it has a second cytotoxic mechanism causing oxidative stress through intracellular peroxide production followed by DNA strand breaks, indicating that it might have a broader spectrum of activity than originally predicted.[103] ON-012380 is another selective inhibitor of Bcr-Abl effective against cells in which resistance to imatinib is due to overexpression or activation of Lyn kinases, but its effectiveness and safety *in vivo* have not yet been confirmed.[104]



Adaphostin



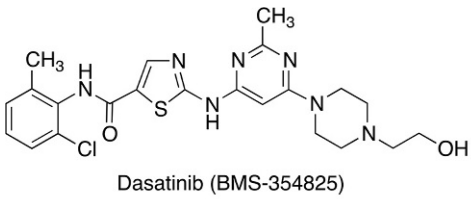
ON-012380

4.9.3 Dual Inhibitors of Bcr-Abl and Src Tyrosine Kinases

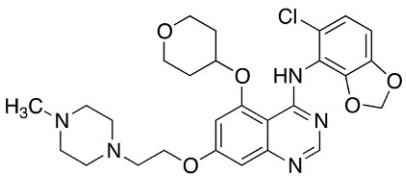
Src kinases are a family of non-receptor tyrosine kinases that modulate intracellular signal transduction whose kinase domain is approximately 47% identical in sequence with that of Bcr-Abl. They are highly regulated in most normal cells but are deregulated in several human tumors, including metastatic colon and breast cancers, in which elevated Src kinase activity has been linked to poor prognosis. Although they have most of the amino acids involved in the binding of imatinib to Bcr-Abl, Src kinases cannot recognize this drug, perhaps due to differences in the inactive conformations of both proteins.

Some dual inhibitors of Bcr-Abl and Src kinases have entered clinical trials. Among them are dasatinib (BMS-354825, Sprycel®), bosutinib (SKI-606, Bosulif®), and saracatinib (AZD-0530). In contrast to imatinib, dasatinib binds to both open and closed conformations of Bcr-Abl kinase, although in an opposite orientation and with the inhibitor in different conformations in both cases.[105] As a result, this compound inhibits not only the wild type of Bcr-Abl but also 14 of the 15 reported imatinib-resistant Bcr-Abl mutations.[106] Dasatinib was approved in 20 ALK tyrosine kinase receptor 06 by the FDA for second-line treatment in patients with CML who were not successfully treated using imatinib.[107]

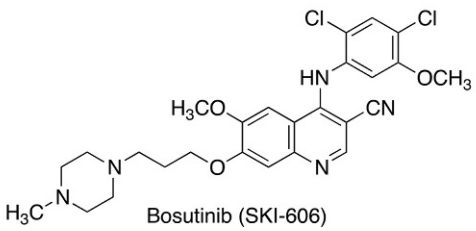
Bosutinib is a 4-anilinoquinoline-3-carbonitrile structurally related to pelitinib (EKB-569) and neratinib (HKI-272), which were discussed in Section 4.1.1. Bosutinib is similar to dasatinib, but it does not inhibit PDGFR and EGFR kinases, being less toxic. It was approved in 2012 by the FDA for CML.[108] The structurally related quinazoline derivative saracatinib (AZD-0530) is a highly selective, orally available, dual-specific Src/Abl kinase inhibitor that is in early clinical trials.[109] Other studies evaluating its effects on bone resorption in patients with prostate or breast cancers with metastatic bone disease and for the treatment of hormone receptor-negative metastatic breast cancers have been reported.[110]



Dasatinib (BMS-354825)



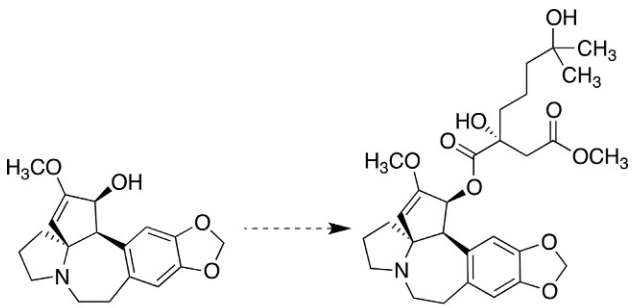
Saracatinib (AZD-0530)



Bosutinib (SKI-606)

4.9.4 Inhibitors of Bcr-Abl Biosynthesis

Omacetaxine mepesuccinate (Omapro, Synribo®) is an inhibitor of protein synthesis that was approved by the FDA in 2012 for the treatment of adult patients with chronic or accelerated phase CML with resistance and/or intolerance to two or more tyrosine kinase inhibitors. Although a detailed understanding of the mechanism of action of this compound remains to be established, it has been shown to prevent the production of specific proteins, including Bcr-Abl and Mcl-1, an apoptosis inhibitor. Omacetaxine mepesuccinate is a semisynthetic derivative of the alkaloid cephalotaxine.[111]



Cephalotaxine Omacetaxine mepesuccinate

4.10 ANAPLASTIC LYMPHOMA KINASE (ALK) INHIBITORS

The *ALK* gene, which encodes the enzyme known as anaplastic lymphoma kinase (ALK) and the ALK receptor (also known as CD246 or cluster of differentiation 246), can be oncogenic in several ways—for instance, by forming a fusion gene with other genes. Approximately 4% of patients with NSCLC have a chromosomal rearrangement that generates a fusion gene between those encoding ALK and

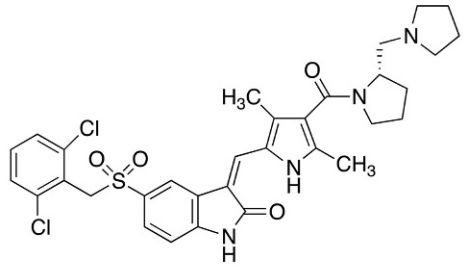
EML4 (echinoderm microtubule-associated protein-like 4) whose constitutive kinase activity contributes to carcinogenesis and seems to drive the malignant phenotype. Because chromosomal rearrangements involving the ROS1 tyrosine kinase receptor have also been recently found in NSCLC, there is much interest in the development of multitargeted tyrosine kinase inhibitors with activity against both ROS1 and ALK for their study against NSCLC.[112] This task is facilitated by the high homology between the human ALK and ROS1 receptors.

Crizotinib (PF-02341066, Xalkori®) is a potent and selective dual inhibitor of ALK and c-Met kinases by competitive binding within their ATP binding pockets. It was approved by the FDA for treatment of late-stage NSCLC in 2011.

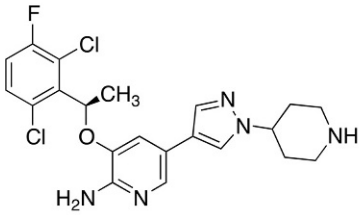
Crizotinib was the first ALK inhibitor acting competitively within the ATP binding pocket of the kinase to be employed in cancer therapy, and it revolutionized the treatment of ALK-positive NSCLC. However, it has limitations due to the following:

1. The high likelihood of acquired tumor resistance after a prolonged treatment.
2. Its poor penetration through the blood–brain barrier, preventing its effectiveness in brain metastases, which are relatively common in patients with primary NSCLC tumors.

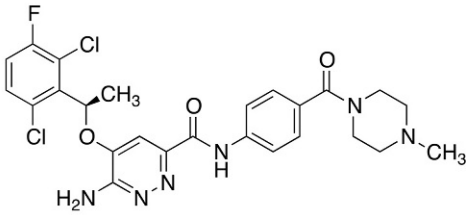
These limitations have spurred intense work on the development of new ALK inhibitors.[113] X-376 is structurally similar to crizotinib, having the same hydrophobic fragment, but it has a 6-aminopyridazine–6-carboxamide motif. It is 10 times more potent than crizotinib as an ALK inhibitor and has not entered clinical studies. The related compound X-396, whose structure has not been disclosed, entered phase I clinical trials in 2012.



PHA-665752



Crizotinib



X-376

Crizotinib was designed by considering a novel ATP site environment revealed in the co-crystal structure of the indolinone derivative PHA-665752, an analog of sunitinib, bound to the c-Met kinase domain (see Section 4.6). This knowledge was translated into the design[114,115] of a novel 2-amino-5-aryl-3-benzyloxypyridine series, following the process outlined in Figure 10.16. The 2-aminopyridine

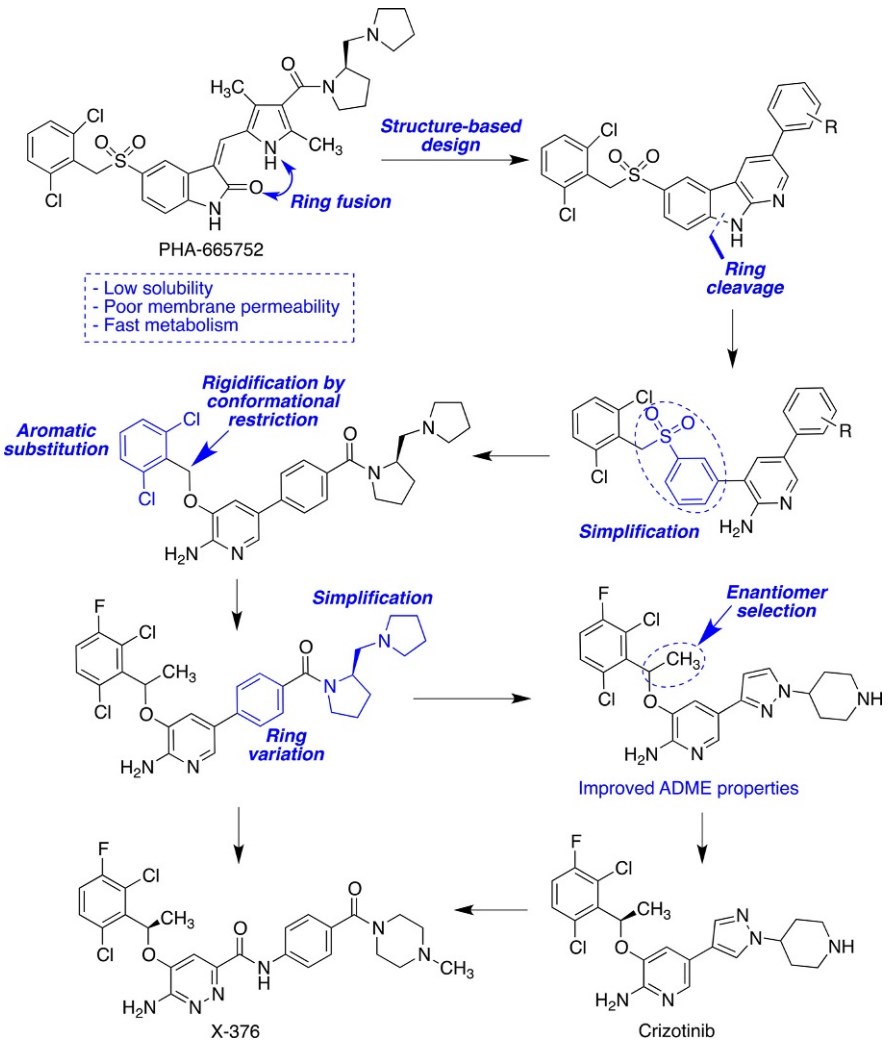


FIGURE 10.16

Design of crizotinib and X-376.

core thus designed allowed a 3-benzyloxy group to reach into the same pocket as the 2,6-dichlorophenyl group of the parent compound, but with a better ligand efficiency (Figure 10.17).

Ceritinib (LDK-378, Zykadia®) is a selective inhibitor of ALK that has shown a marked clinical response in ALK^+ metastatic NSCLC patients.[116] This compound was approved in 2014 for the treatment of ALK^+ metastatic NSCLC in case of failure of crizonitib due to intolerance or resistance. The structurally related AP-26113 is a dual inhibitor of ALK and EGFR that has shown activity in models of crizotinib resistance[117] and is undergoing phase I/II clinical trials. CEP-37440 is another member of

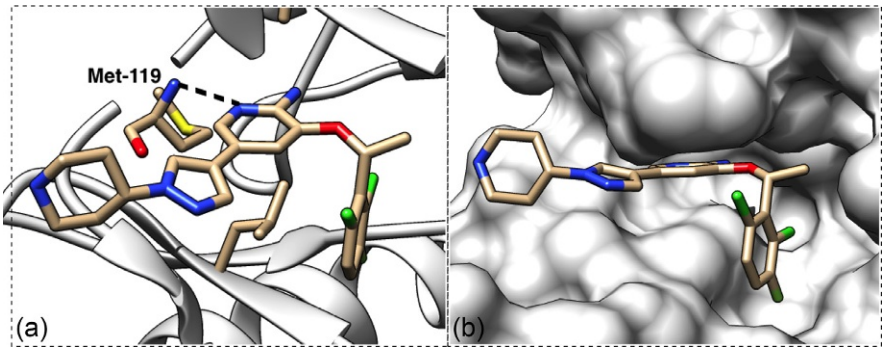
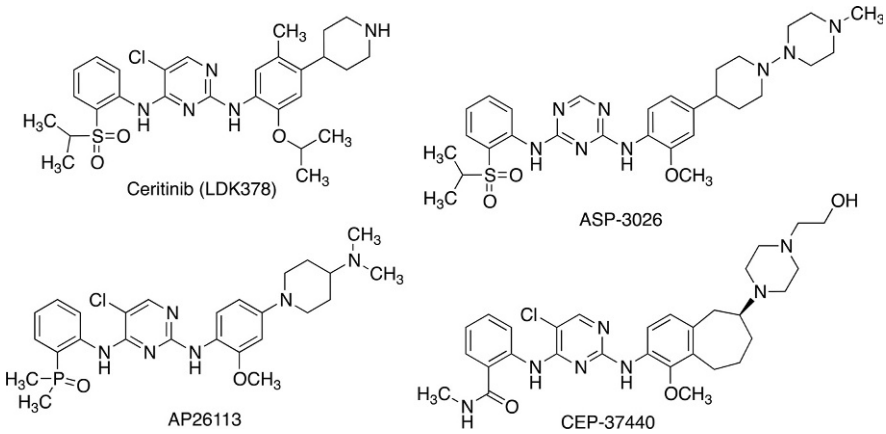


FIGURE 10.17

Crizotinib in its ALK binding site, highlighting its L-shaped binding conformation. The structures were generated from Protein Data Bank reference 2XP2 and displayed with Chimera 1.81.

the diaminopyrimidine ALK inhibitors; it entered phase I clinical trials in 2013. Also closely related, ASP-3026 is a sim-triazine derivative that showed high potency as both ALK and ROS1 inhibitor. A phase I clinical trial involving patients with advanced solid tumors showed that it has a good safety profile.



This family of compounds was designed by optimization of NVO-TAE684, a high-throughput screening hit, using classical criteria for structure manipulation, as shown in Figure 10.18 for the cases of AP-26113, ASP-3026, and ceritinib.

Alectinib is a potent and orally available second-generation ALK inhibitor that was granted breakthrough therapy designation by the FDA in 2013 and was then approved in 2014 in Japan for ALK^+ NSCLC. In addition to ALK, it also has activity against the LTK and GAK kinases. Like ceritinib, alectinib seems to be active in most patients with crizotinib-resistant ALK^+ NSCLC.

PF-06463922, which has a novel macrocyclic structure, is a potent inhibitor of both ALK and ROS1 and has shown high activity against all known ALK and ROS1 mutants identified in patients with crizotinib-resistant NSCLC. To increase its central nervous system penetration, which is relevant in cases of brain metastases, it was designed for good passive permeability and low propensity for P-glycoprotein 1-mediated efflux.[118] A phase I/II clinical study of PF-06463922 in ALK^+ and $ROS1^+$ NSCLC is in

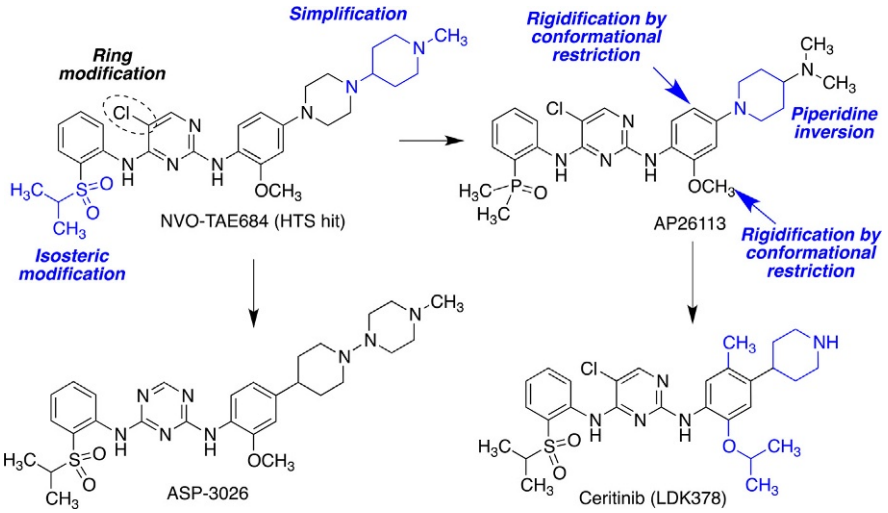
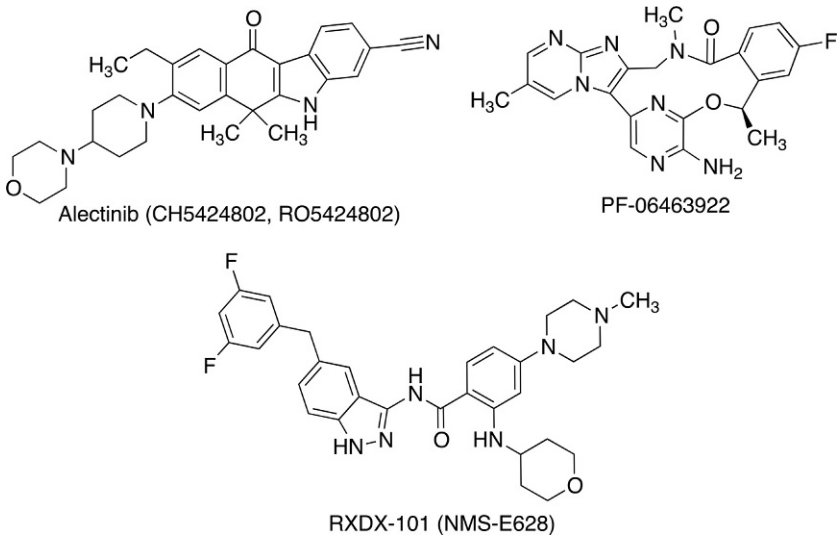


FIGURE 10.18

Design of ceritinib and some of its analogs.

progress. RXDX-101 (NMS-E628) is active against some crizotinib-resistant ALK mutants and has shown good tolerance, along with some early evidence of antitumor activity, in phase I trials. The structure of another ALK inhibitor under clinical assay (TSR-011) has not been disclosed.

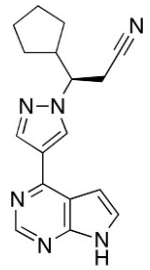


4.11 INHIBITORS OF JAK–STAT AND PRL PATHWAYS

Cytokine receptors (see Section 5.1) do not possess catalytic kinase activity and rely on intracellular, nonreceptor tyrosine kinases called Janus kinases (JAKs) to phosphorylate and activate downstream proteins involved in their signal transduction. The receptor undergoes a conformational change after

binding to its cytokine ligand, which brings together two JAKs that are close enough to phosphorylate each other. This autophosphorylation induces a conformational change leading to transduction of an intracellular signal by further phosphorylation of STATs (*s*ignal *t*ransducer and *a*ctivator of *t*ranscription or *s*ignal *t*ransduction *a*nd *t*ranscription), which are transcription factors that regulate many aspects of growth, survival, and differentiation in cells and are involved in the development and function of the immune system. The activated STATs translocate to the cell nucleus, where they regulate gene transcription that ultimately induces cell proliferation (Figure 10.19).

The discovery in 2004 of the JAK2V617F mutation in a significant proportion of patients with Bcr-Abl1-negative chronic myeloproliferative neoplasms spurred the development of several small-molecule JAK inhibitors.[119] Ruxolitinib (INCB018424, Jakafi®) was the first of these compounds, which went from phase I testing to drug approval in only 4 years. This compound is a JAK inhibitor with selectivity for subtypes 1 and 2 of this enzyme, which mediate the signaling of a number of cytokines and growth factors that are important for hematopoiesis and immune function. It was the first drug to receive FDA approval for the treatment of intermediate- and high-risk myelofibrosis in 2011.[120]



Ruxolitinib

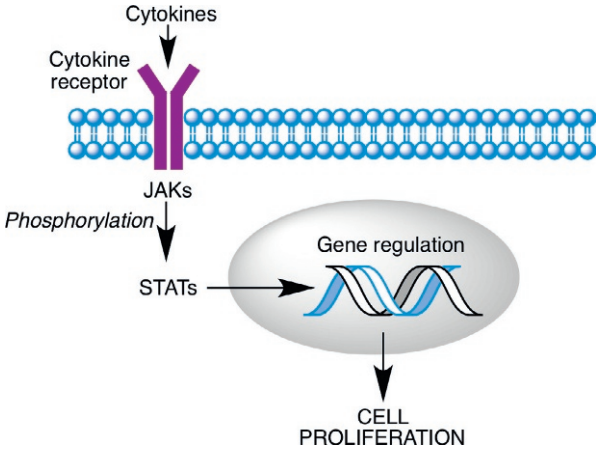
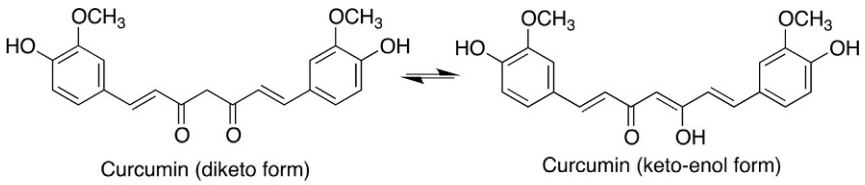
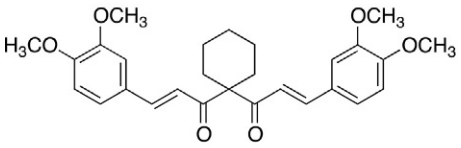


FIGURE 10.19

The JAK–STAT pathway.

Curcumin is the main bioactive component of turmeric (*Curcuma longa*), the main component of curry, and is known to inhibit several targets associated with the JAK2/STAT3 pathway, but it shows poor bioavailability and potency. Furthermore, it lacks specificity, and this problem was hypothesized to arise from the existence of two tautomeric forms. FLLL-32 was designed to lack the possibility of tautomerism because of disubstitution of the active methylene, and it was also designed to be more lipophilic by methylation of the two phenolic hydroxyls of curcumin. This compound has shown promising preclinical results in human rhabdomyosarcoma cells (see also Section 7.1 of Chapter 11 and Section 3.2 of Chapter 12).[121]

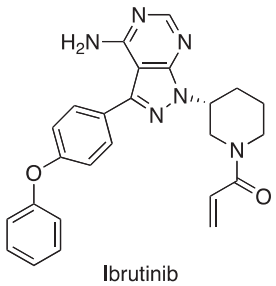




PRLR, another receptor that lacks catalytic kinase activity, has a single transmembrane domain that mediates the physiological effects of the polypeptide hormone prolactin (PRL). PRLR activates signaling through the JAK–STAT, PI3K–AKT, and MEK–ERK1/2 pathways, leading to cell proliferation and survival. Its overexpression has been correlated with increased breast and prostate cancer risk. LFA102, a humanized monoclonal antibody that binds to the dimerization region of PRLR and thereby inhibiting this signaling,[122] is currently undergoing a phase I study in patients with $PRLR^+$ castration-resistant prostate cancer or $PRLR^+$ metastatic breast cancer.

4.12 INHIBITORS OF BRUTON'S TYROSINE KINASE (BTK)

Ibrutinib (PCI-32765, Imbruvica®) is an anticancer drug targeting B-cell malignancies that was approved by the FDA in November 2013 for the treatment of mantle cell lymphoma and in February 2014 for the treatment of chronic lymphocytic leukemia. It is currently under study for additional B-cell malignancies, including diffuse large B-cell lymphoma and multiple myeloma. It is an orally administered, selective and covalent inhibitor of the enzyme known as Bruton's tyrosine kinase (BTK). Inhibition of BTK phosphorylation abrogates downstream survival pathways activated by this kinase, including ERK1/2, PI3K, and NF-κB. Ibrutinib was discovered in 2007 through a structure-based approach for creating a series of small molecules that inactivate BTK through covalent binding of a Michael acceptor (an α,β-unsaturated ketone in ibrutinib) to the cysteine-481 residue located near the ATP binding domain of the enzyme.[123]



Ibrutinib

4.13 INHIBITORS OF TROPOMYOSIN RECEPTOR KINASE (Trk)

Trk consists of a family of three membrane receptors—known as TrkA, TrkB, and TrkC—that are primarily found in neurons. Their ligands are the neurotrophins, and they have a role in the regulation of neural behavior in several situations, including pain, cognition, proprioception, and mood. Furthermore, a range of cancers, including lung, colon, and endometrial cancers, harbor Trk mutations or translocations. Trk seems to behave similarly to known validated oncogenes such as *ALK* (see Section 4.10), acting as a translocation partner in gene fusion events in a range a malignancies.

The search for Trk inhibitors is in its early stages, but it has already yielded a promising candidate. LOXO-101, a pan-Trk inhibitor whose structure has not currently been disclosed, is undergoing a phase I clinical study.

5 INHIBITORS OF SERINE–THREONINE KINASES

5.1 CYCLIN-DEPENDENT KINASES (CDKs)

Cyclin-dependent kinases (CDKs) are involved in the control of the cell cycle, together with the ubiquitin–proteasome system (see Chapter 11, Section 2.1). They are responsible for moving it from one phase to the next after activation by complexation with a group of associated proteins called cyclins. Successful progression through the cell cycle through G_1, S, G_2, and M phases is controlled by a number of different regulatory mechanisms termed checkpoints.[124] Specific checkpoints that are activated by changes in DNA structure and integrity induced by drug treatment or, in general, by genotoxic stress are frequently defective in cancer cells. Therefore, nonfunctional cell cycle checkpoints may greatly influence the efficacy of antitumor agents and may be associated with both drug resistance and oversensitivity to these drugs.[125]

Several types of cyclins and CDKs play roles at different stages of the cell cycle. For instance, in the G_1 phase, an increase in cyclins D followed by their binding to CDK4 and CDK6 leads to the phosphorylation of the tumor suppressor protein known as retinoblastoma (pRb), a molecule that is normally bound to the transcription factor E2F, which is thereby inactivated. Phosphorylation of pRb prevents this binding and leaves the transcription factor free to bind to DNA, leading to the synthesis of several proteins, including cyclin E. This cyclin binds to CDK2 to form a complex

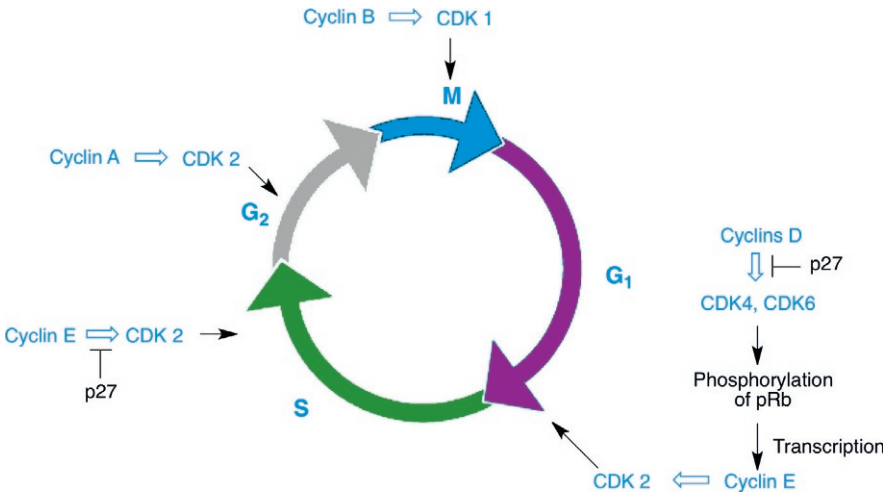


FIGURE 10.20

Control of cell cycle by cyclins and CDKs.

that is necessary for progression from the G_1 to the S phase. Other complexes that are required in subsequent stages for the progression of the cell cycle are cyclin A–CDK2 and cyclin B–CDK1 (Figure 10.20). The cell cycle is downregulated by natural CDK inhibitors (also known as CKIs) such as peptides p15, p21, or p27, which restrain the activity of CDKs. Overactivity of cyclins or CDKs, or insufficient activity of CKIs, is associated with several tumors, making these processes attractive cancer[126] and antiviral[127] targets.

Several strategies are currently being followed in the search for compounds targeting CDKs[128] and are summarized in Figure 10.21. To date, only the first one to be pursued—that is, competitive binding at the ATP site—has yielded compounds that have reached clinical status.

One of these competitive inhibitors is alvocidib (flavopiridol), a semisynthetic flavone related to a natural product extracted from two Indian plants (*Amoora rohituka* and *Dysoxylum binectariferum*). This compound is a nonselective CDK inhibitor, thus explaining the subsequent G_1 and G_2 arrest, and also an inhibitor of transcription.[129] It was the first CDK inhibitor to reach human clinical trials in patients with NSCLC in combination with paclitaxel; however, despite highly promising phase I trials, the results of phase II studies were rather disappointing in most cases. Nevertheless, encouraging data in one of these studies[130] prompted a phase III assay for the treatment of metastatic lung carcinoma, in combination with other chemotherapeutic agents. It is also under clinical development for the treatment of chronic lymphocytic leukemia, and in 2014, the FDA granted orphan drug designation for this compound for the treatment of patients with AML.

Riviciclib (P776-00) is a close analog of flavopiridol designed by contraction of its piperidine ring, which is more potent than the reference drug and has been shown to induce G_1–G_2 cell cycle arrest and also to induce apoptosis. This compound has undergone a number of phase I and phase II clinical studies for a variety of cancers.[131]

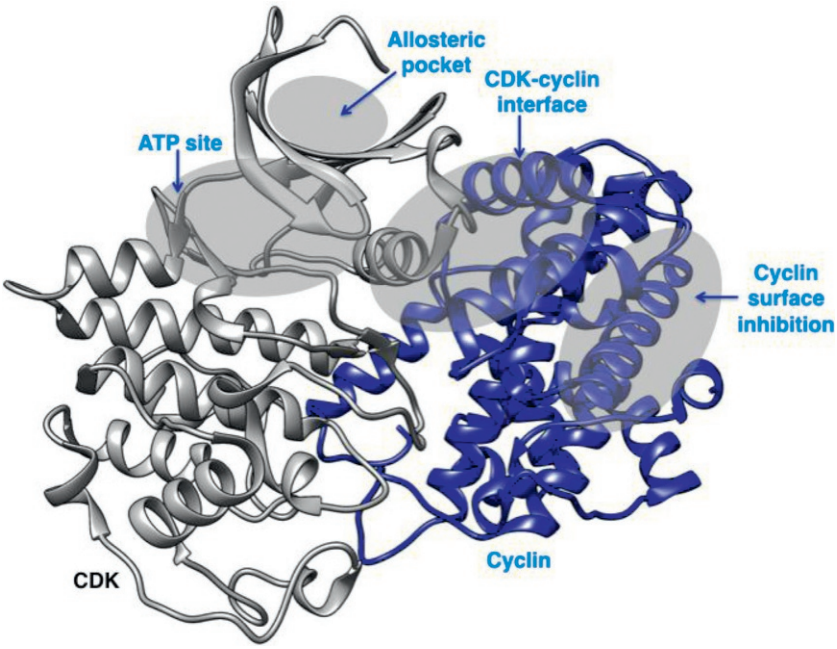
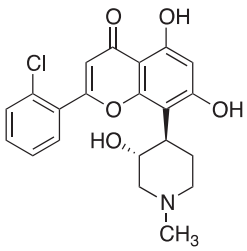
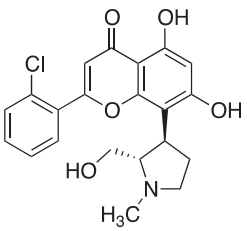


FIGURE 10.21

Schematic representation of the strategies used for the inhibition of cyclin-dependent kinases. The CDK2–cyclin E complex was generated from Protein Data Bank 1 W98 and displayed with Chimera 1.81.



Alvocidib (flavopiridol, HMR-1275)



Riviciclib (P776-00)

Alvocidib has been shown to bind to the ATP site, with the benzopyran ring lying in the adenine-binding region, and establishes the hydrogen-bonding network shown in Figure 10.22.[132]

Seleciclib [(*R*)-roscovitine, CYC-202], which was identified from a study of heterocycles with close analogy to the purine portion of ATP, is under clinical studies for lung and B-cell malignancies, including multiple myeloma.[133]

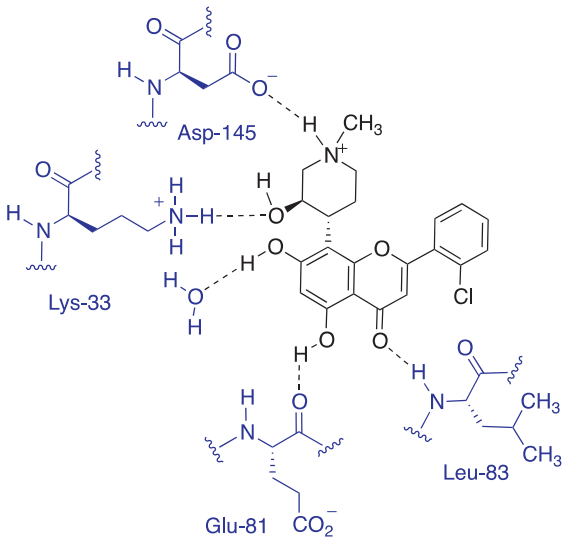
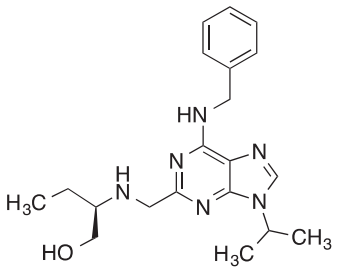


FIGURE 10.22

Binding of flavopiridol to CDKs.



Seleciclib
(*R*)-Roscovitine, CYC-202

Selecilib is rather selective for CDKs, especially CDK2, where it binds tightly in an essentially hydrophobic cavity, as shown in Figure 10.23. It does not affect most other kinases, although it also binds to pyridoxal kinase, a nonprotein enzyme responsible for phosphorylation and activation of vitamin B_6, where, rather unexpectedly, it recognizes the pyridoxal rather than the ATP site.[134]

SNS-032 (formerly BMS-387032) is a potent and selective inhibitor of CDK2, -7, and -9. It was developed from the lead compound BC-2626, which was identified as a selective CDK2 inhibitor by high-throughput screening but was inactive *in vitro*. When it was speculated that this inactivity was due to the facile hydrolysis of the ester group, BMS-239091 was designed as a metabolically stable bioisoster, which showed the expected cytotoxic activity against cancer cells. Replacement of the ethyl

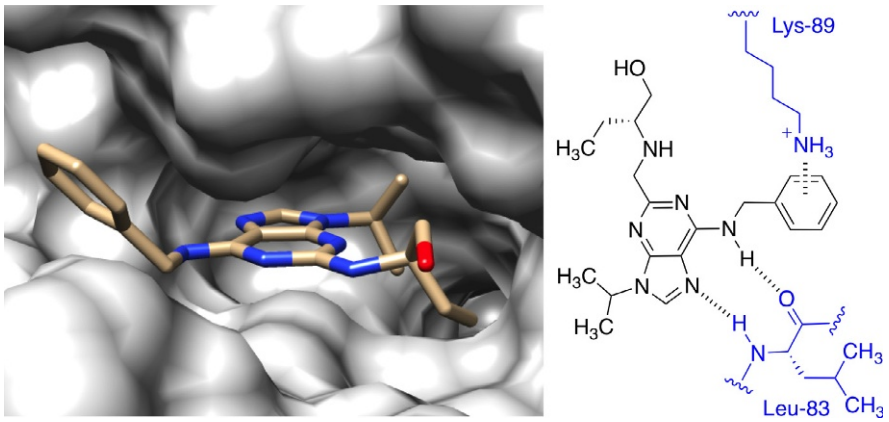


FIGURE 10.23

Binding of seleciclib to CDK2. The three-dimensional structure was generated from Protein Data Bank reference 2A4L and displayed with Chimera 1.81.

group in this compound by a *tert*-butyl, in order to enhance hydrophobic interactions with the enzyme, and introduction of a piperidine moiety to improve the pharmacokinetic properties led to SNS-032 (Figure 10.24).[135] This inhibitor entered clinical trials,[136] although its absorption is limited because it is a substrate of the P-glycoprotein efflux pump.[137]

X-ray crystallographic studies showed that SNS-032 binds to the active site of CDK2 by two hydrogen bonds involving Leu-83 and the aminothiazole moiety and also through hydrophobic interactions of the thiomethylene and *tert*-butyl groups with two hydrophobic pockets (Figure 10.25).[134]

The use of fragment-based design[138] and high-throughput X-ray crystallography[139] led to the identification of AT7519,[140] an inhibitor of CDK1, -2, -4, and -5. Starting with 500 fragments, crystallography allowed researchers to identify more than 30 fragments that bound to the ATP-binding site of these kinases through at least one hydrogen bond. Later, 3 of these fragments were optimized using structure-based design, as shown in Figure 10.26 for the optimization of 1H-indazole. Replacement of the indazole moiety by a pyrazole did not notably reduce the ligand efficiency, and the introduction of a piperidine moiety instead of the fluorobenzene ring led to AT7519, with improved solubility.

AT-7519 inhibits phosphorylation for a range of CDK substrates and induces apoptosis in multiple myeloma via GSK-3β activation and RNA polymerase II inhibition.[141] Based on its potent antitumor activity in preclinical models, a first-in-human clinical trial of refractory solid tumors investigated its safety, tolerability, pharmacokinetics, and pharmacodynamics,[142] and other phase I/II studies to determine its effectiveness in multiple myeloma are ongoing.

The sulfonamide indisulam (E-7070) has a complex mechanism of action, partially involving interaction with CDKs. This compound decreases the expression of several cell cycle proteins (cyclins A and B1, CDK2, and CDC2) and also suppresses the CDK2 catalytic activity with induction of p53 and p21 proteins in lung cancer cells, disturbing the cell cycle at multiple points including both the

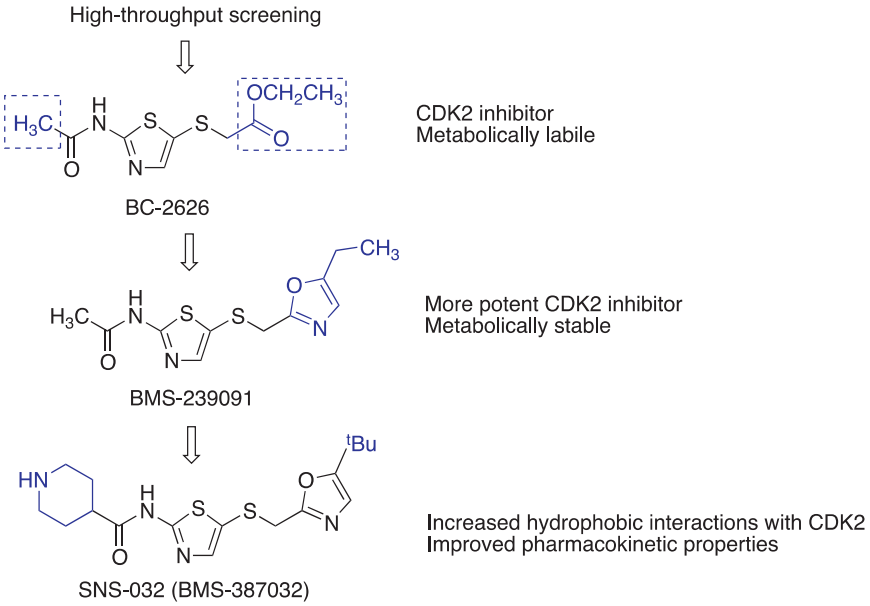


FIGURE 10.24

Design of SNS-032.

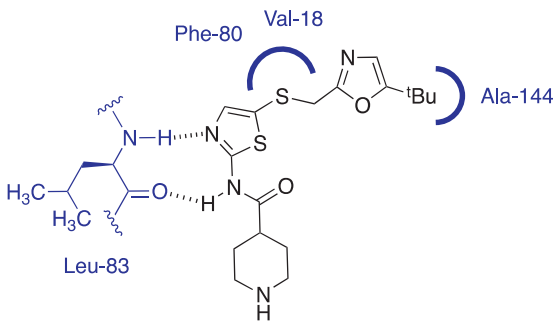


FIGURE 10.25

Binding of SNS-032 to CDK2.

G_1/S and the G_2/M transition.[143] Indisulam is also a potent carbonic anhydrase IX inhibitor (for the relevance of this enzyme in cancer, see Section 6 of Chapter 14).[144] Subsequent research has located other potential targets for this drug, such as of cytosolic malate dehydrogenase, which is inhibited by preventing the binding of its cofactor nicotinamide adenine diphosphate,[145] and leucine

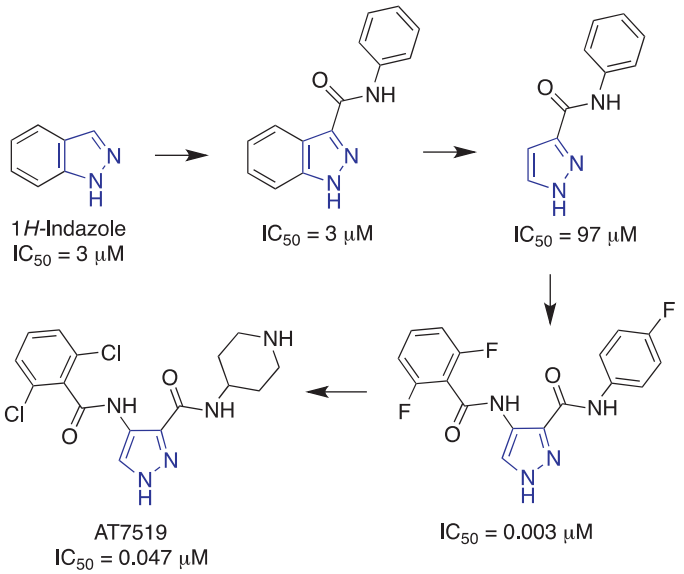


FIGURE 10.26

Stages in the design of AT-7519.

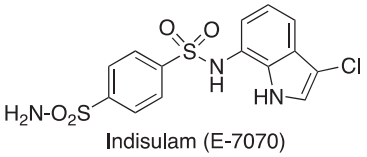
and uracil transporters.[146] Indisulam has reached phase II studies in patients with metastatic melanoma[147] and other solid tumors.[148]

Although preclinical studies showed that indisulam is effective for the treatment of human breast cancer, its efficacy for this indication in clinical trials did not meet expectations due to dose-limiting toxicities. One of the reasons for the hematological toxicity of the aqueous formulations tested in clinical trials is that indisulam binds to plasma proteins (albumin) and to erythrocytes (carbonic anhydrase) in a saturable manner.[149] For this reason, a micellar formulation in which the distribution and toxicity to normal tissues are very much reduced, and the drug is accumulated by passive targeting at the tumor sites by extravasations due to leaky vasculature, has been proposed as a novel targeted nanomedicine (see Chapter 13).[150]

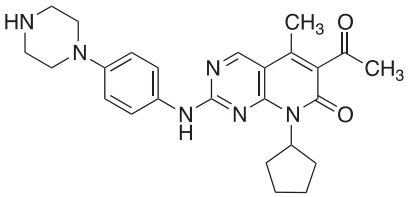
Palbociclib (PD-0332991, Ibrance®) is an orally available pyridopyrimidine derivative that inhibits CDK4/cyclin D1 kinase and the subsequent phosphorylation of the protein retinoblastome (pRb) by binding to the CDK ATP site. This prevents Rb-positive tumor cells from entering the S phase of the cell cycle (arrest in the G_1 phase), resulting in suppression of DNA replication and decrease of tumor cell proliferation. Palbociclib has been the subject of multiple phase I and phase II studies for the first-line treatment of estrogen receptor-positive HER2-negative advanced breast cancer.[151] In early 2015, it received FDA approval for this indication. Palbociclib is also being evaluated for other tumor types. LEE011 is a selective inhibitor of CDK4/6 kinases that induces complete dephosphorylation of Rb and G_1 arrest in cancer cells and is active in cancers harboring

mutations that increase CDK4/6 activity. A phase I/II study of LEE011 in patients with solid tumors and lymphoma is currently ongoing.

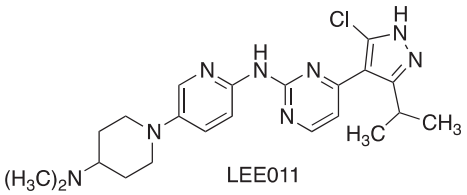
Dinaciclib is another CDK inhibitor with nanomolar IC_{50} values for several kinases of the CDK family (CDK1, CDK2, CDK5, and CDK9).[152] In preclinical work, this compound proved to be superior to flavopiridol in terms of both higher activity and an improved therapeutic index, and it is being evaluated in clinical trials for a variety of malignancies. SB-1357 (TG-02), which has an unusual macrocyclic structure, inhibits CDK1, -2, and -9 along with other kinase targets (JAK2, FLT3, and ERK5), and it has entered phase I clinical trials for advanced/refractory hematological malignancies.



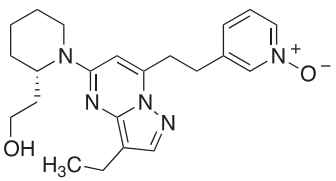
Indisulam (E-7070)



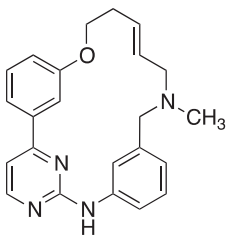
Palbociclib (PD 0332991)



LEE011

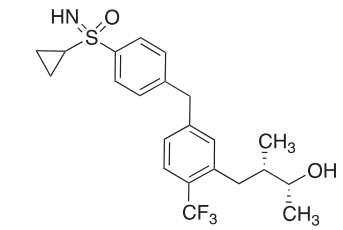


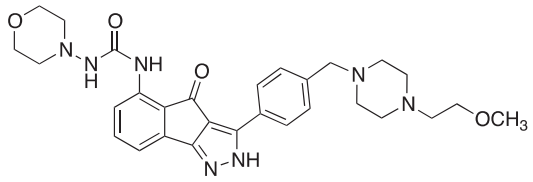
Dinaciclib (SCH-727965)

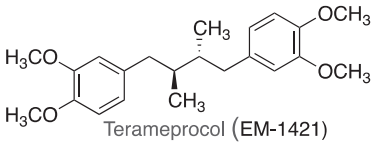


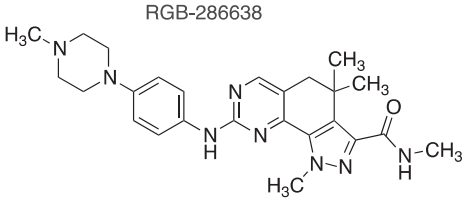
SB-1317 (TG-02)

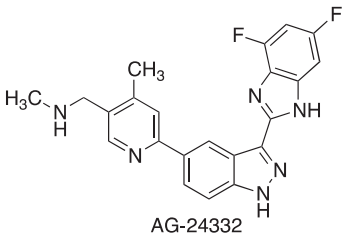
BAY-1000394 is an orally bioavailable pan-CDK inhibitor that acts primarily by inhibiting the activity of the CDK1/cyclin B, CDK2/cyclin E, CDK4/cyclin D1, and CDK9/cyclin T1 kinases, thereby inducing cell cycle arrest at the G_1/S transition. This compound is under phase I/II clinical evaluation for small cell lung cancer. RGB-286638 is another multitargeted kinase inhibitor with anti-multiple myeloma activity triggered through inhibition of transcriptional CDKs[153] and is in phase II clinical evaluation. Terameprocol (EM-1421) is a semisynthetic tetra-*O*-methyl derivative of the previously mentioned nordihydroguaiaretic acid (INSM-18, NDGA), an inhibitor of IGFR-1 (see Section 4.5). Terameprocol inhibits CDK1 and survivin, and it has been clinically studied (phase I) in leukemia patients. Milciclib (PHA-848125) is another pan-CDK inhibitor that is under evaluation in phase II clinical trials. Additional CDK inhibitors that have at some point been in clinical trials, which have subsequently been terminated, include R-547, ZK-304709, AZD-5438, and AG-24322.

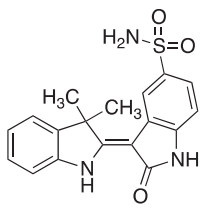

BAY-1000394

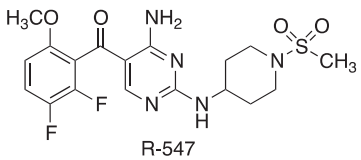

RGB-286638

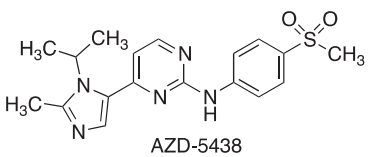

Terameprocol (EM-1421)


Milciclib (PHA-848125)


AG-24332

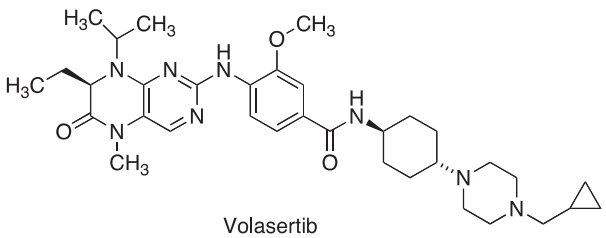

ZK-304709


R-547


AZD-5438

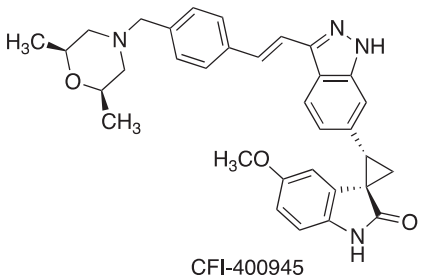
5.2 POLO-LIKE KINASES (PLKs)

The serine/threonine protein kinase known as polo-like kinase 1 (PLK1) is an early trigger for G_2/M transition. Among other activities, PLK1 phosphorylates and activates the phosphatase cdc25C, which dephosphorylates and activates the cyclin B/cdc2 complex and activates components of the anaphase-promoting complex (APC/C). It is considered a proto-oncogene, and its overexpression is often observed in tumor cells. The loss of PLK1 expression can induce pro-apoptotic pathways and inhibition of cell growth. In cancer cells, PLK1 inhibition results in G_2/M cell cycle arrest followed by programmed cell death, whereas in normal cells this inhibition only causes reversible G_1 and G_2 arrest without programmed cell death. The PLK1 inhibitor volasertib (BI6727) blocks cell division by competitively binding to the ATP binding pocket of this kinase and is being developed to treat AML. The FDA has granted this drug a breakthrough therapy designation.[154]



Volasertib

Centrioles are the main microtubule-organizing centers. An accurate control of the number of centrosomes is critical for the maintenance of genome integrity, and abnormalities in this number can promote errors in spindle formation that lead to subsequent chromosome missegregation. Centriole duplication during the cell cycle is regulated by the enzyme polo-like kinase 4 (PLK4),[155] whose activity limits centrosome overduplication. Overexpression of this enzyme correlates with aggressive tumor growth, high levels of hypoxia, and metastasis. Therefore, PLK4 is an interesting molecular target for drug development, especially for pancreatic cancer patients.[156]CFI-400945 is a potent and selective PLK4 inhibitor for which phase I clinical trials have been recently initiated, and it is particularly efficient in solid tumors deficient in the phosphatase and tensin homolog gene (*PTEN*), one of the most frequently inactivated tumor suppressor genes, because PTEN-deficient cancer cells depend on PLK4 for survival.[157]



CFI-400945

5.3 CHECKPOINT KINASES (CHKs)

In response to DNA damage such as strand breaks or stalled replication forks, multiple checkpoints are activated to stop the cell cycle and activate DNA repair mechanisms. Once the repair has been performed, cell duplication continues; if reparation is not possible, the cell is directed to apoptosis. Checkpoint kinases CHK1 and CHK2 are serine–threonine kinases that have a key role in this process because they transmit the signals received from DNA damage-sensing proteins such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) proteins to downstream effectors responsible for cell cycle arrest and DNA repair. These processes act as resistance mechanisms against DNA-damaging therapy, including chemotherapy with cytotoxic agents and radiotherapy.

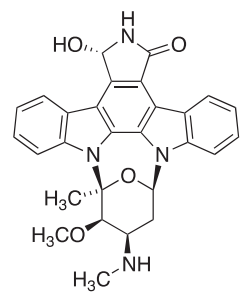
Many cancer cells have mutated or inactivated p53 and therefore have defects in the early, p53-dependent, G_1/S checkpoint. They are thus dependent on later checkpoints, including those that are controlled by CHK1 in the S and G_2/M phases. This affords a therapeutic opportunity to selectively target cancer cells bearing defects in the p53-dependent checkpoint by combining inhibition of CHK1 with classical DNA-damaging cytotoxic drugs or radiotherapy.[158]

Most CHK1 inhibitors compete with ATP and bind to the hinge peptide region existing between the N- and C-terminal lobes of the kinase domain, although there are also some allosteric CHK1 inhibitors. A brief discussion of the inhibitors that have been clinically studied is given here.

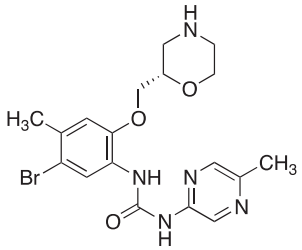
UCN-01 is a multitargeted analog of the natural product staurosporine, which, in addition to inhibiting checkpoint-regulated kinase CHK1 and perhaps CHK2,[159] is a potent inhibitor of phosphatidylinositide-dependent protein kinase 1 (PDPK1; see Section 5.4.2) and PKC (see Section 5.6). Due to the complexity of its effects, the interpretation of SAR data for this compound is difficult.[160]

UCN-01 has entered clinical development,[161] being in phase II studies for T-cell lymphomas and metastatic melanoma, among others. For the latter type of tumors, this compound was shown to be well tolerated but without sufficient clinical activity as a single agent to warrant further study.[162]

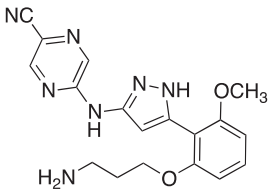
In addition to UCN-01, the urea derivative LY 2603618 seems to be the clinically most advanced CHK1 inhibitor, having undergone several phase II clinical studies in combination with gemcitabine, pemetrexed, or pemetrexed + cisplatin in several solid tumors. The less advanced LY 2606368 shows little selectivity between CHK1 and CHK2, and a study of its combination with cisplatin or cetuximab in patients with advanced cancer is underway. Other CHK inhibitors that are in early clinical stages are PF-00477736, which was studied in advanced solid tumors in combination with gemcitabine but whose development has been discontinued; SCH900776 (in combination with cytarabine for relapsed and/or refractory acute leukemias in adults); and the dual CHK1/CHK2 inhibitor AZD-7762, which has shown a good potential in combination with radiotherapy in pancreatic and metastatic lung cancers. Some additional CHK inhibitors under clinical trials whose structures have not been disclosed include XL-844 (EXEL-9844), GDC-0575 (ARRY-575), and GDC-0425.



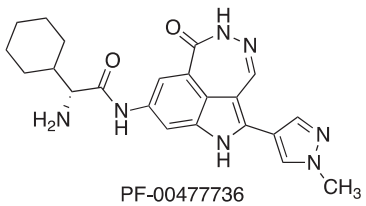
UCN-01



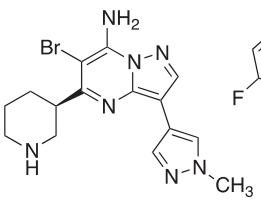
LY 2603618



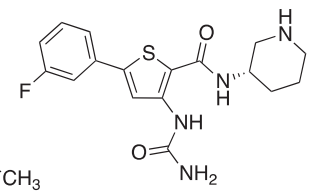
LY 2606368



PF-00477736



SCH-900776



AZD-7762

5.4 PI3K/PDPK1/AKT/mTOR KINASES PATHWAY

The PI3K/PDPK1/AKT/mTOR pathway controls many cellular processes that are important for the formation and progression of cancer, including apoptosis, transcription, translation, metabolism, angiogenesis, and cell cycle progression. Genetic alterations and biochemical activation of this pathway are frequent events in pre-neoplastic lesions and advanced cancers and often portend a poor prognosis. Thus, its inhibition is an attractive concept for cancer prevention and/or therapy.[163,164] The sequence of events in this pathway requires the activation of the serine–threonine kinase PDPK1 and starts when PI3K is activated in response to growth factors, cytokines, or insulin. This activation induces the phosphorylation of the membrane phospholipid PIP2 [phosphatidylinositol(4,5)-diphosphate] to PIP3 [phosphatidylinositol(3–5)-trisphosphate]. PIP3 binds to the PH domain of AKT, another serine–threonine kinase that is also known as protein kinase B (PKB), and causes its translocation to the membrane where it contacts PDPK1, which is responsible for at least one of the two phosphorylations necessary to activate AKT—namely the phosphorylation of Thr-308 in its T-loop. Phosphorylation of AKT at Ser-473, which is required for maximal activation, is mediated by mTORC2, one of the two molecular complexes related to the factor known as mTOR (mammalian target of rapamycin). This serine–threonine protein kinase can be viewed as the catalytic subunit of the mTORC1 and mTORC2 complexes.[165] Formation of the mTORC1 complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress, and it functions as a nutrient/energy/redox sensor that controls protein synthesis. As previously mentioned, elaboration of cyclin D is followed by activation of CDKs (Figure 10.27). PI3K and PDK1 also activate the serum–glucocorticoid-regulated kinase 1 (SGK1). Mutations in the PI3Kα isoform have been observed in many types of cancers and may lead to increased activity of this pathway, which is often associated with resistance to cancer therapies. Accordingly, development of novel molecules that effectively and specifically block the PI3K pathway may inhibit the proliferation and growth of tumor cells and sensitize them to apoptosis.[166] For instance, targeting PI3K overcomes *in vivo* resistance to everolimus (see Section 5.4.4) in estrogen receptor (ER^+) breast cancer.

AKT is overexpressed in several cancers due to mutations of PTEN, a phosphatidylinositol-3,4,5-triphosphate 3-phosphatase that negatively regulates intracellular levels of PIP3 and functions as a tumor suppressor by negatively regulating the AKT signaling pathway.

5.4.1 PI3K Inhibitors

Enzymes of the PI3K family share many structural motifs with other protein kinases, including an overall two-lobe architecture, a DFG (Asp–Phe–Gly) loop for magnesium ion coordination, and similar motifs for the process of phosphate transfer from ATP. In common with the vast majority of the small-molecule inhibitors of protein kinases, PI3K inhibitors target the ATP site, and the crystal structures of PI3K inhibitor complexes show the existence of an H bonding to the Val-882 residue. PI3K inhibitors are structurally diverse, although some major structural classes targeting the different isoforms of PI3K can be distinguished.

A range of PI3K inhibitors are being investigated for the treatment of different types of cancer.[167] Among them, NVP-BEZ235 (BEZ235), a dual ATP-competitive PI3K and mTOR inhibitor, the PI3K inhibitor buparlisib (BKM-120), and the selective PI3Kα inhibitor alpelisib (BYL-719) are in phase I/II clinical trials for solid tumors.[168]

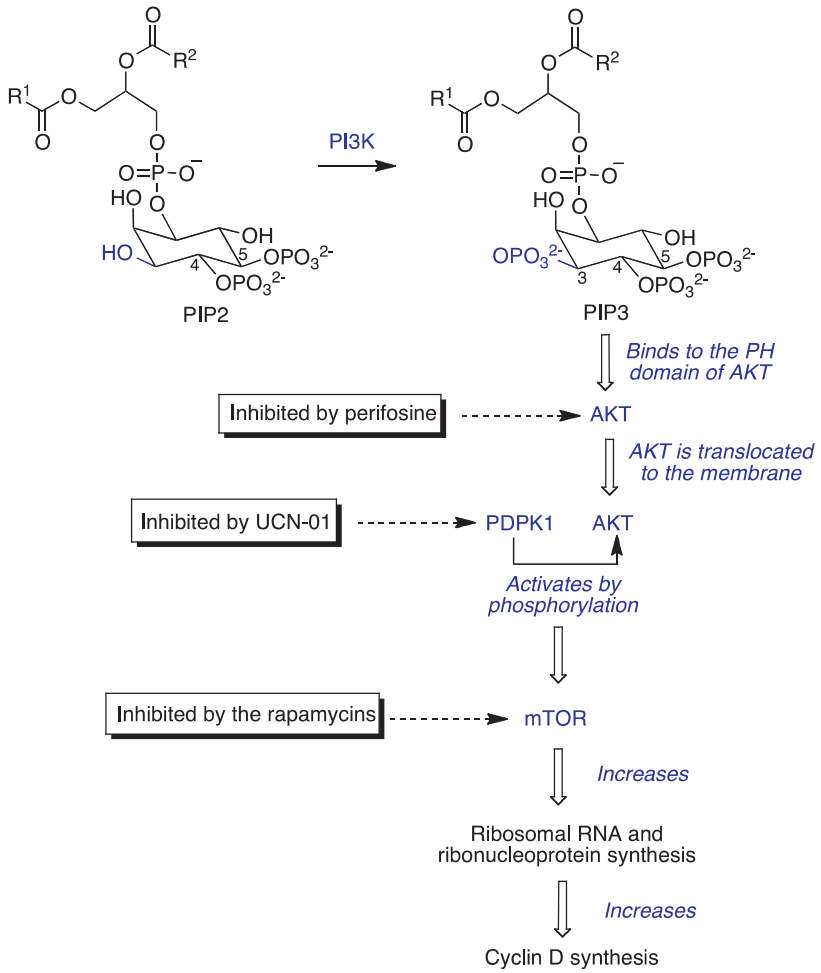
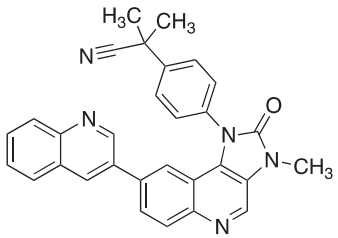


FIGURE 10.27

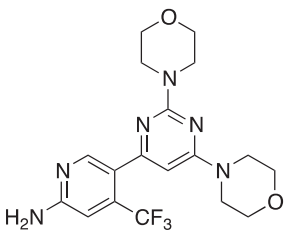
Activation of cyclin D synthesis involving PI3K, PDK1, AKT, and mTOR kinases.

NVP-BEZ235 was originated from a series of imidazo[4,5-*c*]quinoline-based inhibitors of PDK1. A study of NVP-BEZ235 docked into a homology model of one of the PI3K isoforms suggested, along with van der Waals contacts with conserved hydrophobic residues in the ATP site, the existence of a H bond between the core quinoline nitrogen and the backbone Val-851, as well as other H-bonded interactions between the cyano group and Ser-774 and the peripheral quinoline N and Asp-933.[169]

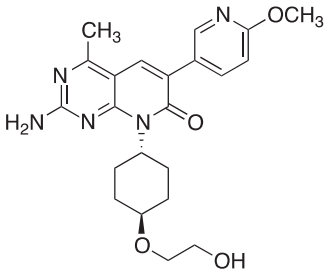
A high-throughput screen initiated the development of the pyrido[2,3-*d*]pyrimidinone PF-04691502, a potent and selective oral inhibitor of PI3K and mTOR kinases that entered clinical trials.[170] XL-765 (SAR245409), which has the same 4-methylpyrido[2,3-*d*]pyrimidinone scaffold, is a dual PI3K–mTOR inhibitor for which results have been reported in phase II trials for breast cancer.



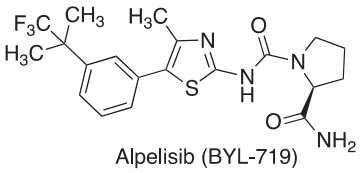
NVP-BEZ235 (BEZ235)



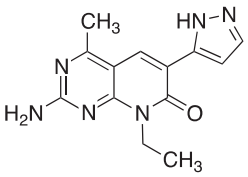
Buparlisib (BKM-120)



PF-04691502



Alpelisib (BYL-719)



XL-765 (SAR-245409)

A co-crystal structure of PF-04691502 with the PI3K isoform p110γ showed H bonds between Val-882 and the 2-amino and 3-ring nitrogens of the aminopyrimidine, with the ring nitrogen on the methoxypyridine forming a key H bond with a conserved water molecule in the selectivity pocket and an additional H bond between the hydroxy end of the chain at C-4 of the cyclohexyl ring and Lys-890 (Figure 10.28).[171]

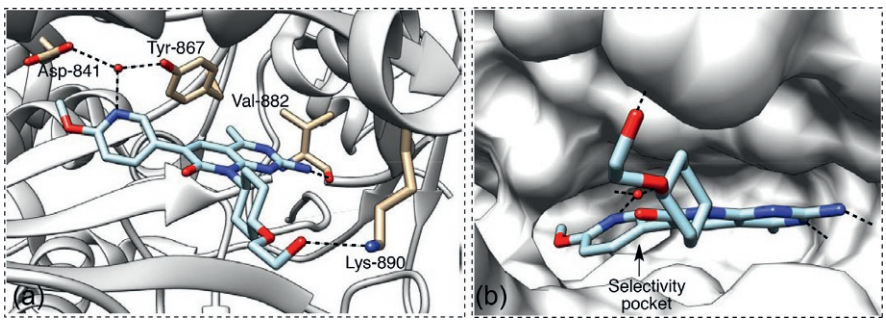
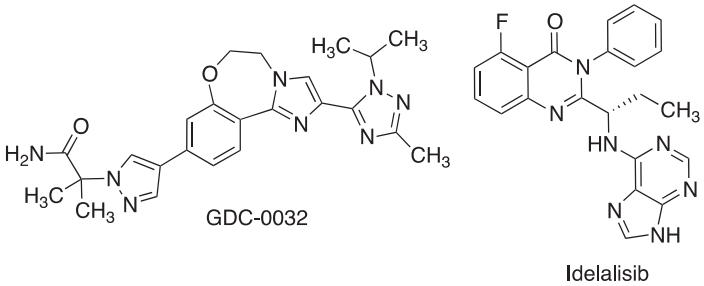


FIGURE 10.28

Binding of PF-04691502, a PI3K/mTOR dual inhibitor, to the PI3K catalytic subunit γ isoform. The structure was generated from Protein Data Bank reference 3ML9 and displayed with Chimera 1.8.1.

Idelalisib (GS-1101, Zydelig®) is another PI3K inhibitor that was approved by the FDA in 2014 for the treatment of patients with relapsed small lymphocytic lymphoma who have received at least two prior systemic therapies and patients with relapsed follicular B-cell non-Hodgkin's lymphoma. In combination with rituximab, it is also indicated for patients with relapsed chronic lymphocytic leukemia.[172] GDC-0032 is another PI3K inhibitor with high affinity for mutated PI3Kα and reduced inhibitory activity against PI3Kβ. It is is being evaluated, alone and in combination with other therapies, in patients with advanced cancer, especially for ER^+ breast cancer treatment.



Wortmannin (Wtmn), an oxa-steroid metabolite of some *Penicillium* species, is a nonselective inhibitor of some PI3K isoforms. As shown in Figure 10.29, the mechanism of this inhibition relies on the reactivity of Wtmn as a Michael acceptor and involves the irreversible alkylation of a Lys-802 residue that resides in the enzyme active site and is critical for the phosphate transfer, forming an enamine that is stable at physiological pH.

Wortmannin failed in its clinical translation because of its poor water solubility and high toxicity, although a nanoparticle formulation has recently established its potential.[173] Wortmannin prodrugs, in which the furan ring is masked in an open form by generation of a β-enaminone, are less toxic and more stable. PX-866, the most stable of these semisynthetic analogs, has entered clinical trials.[174]

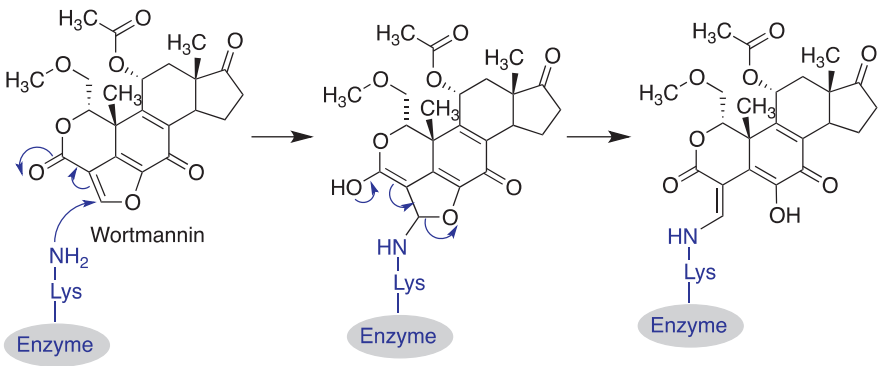
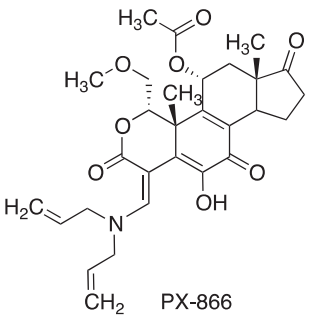


FIGURE 10.29

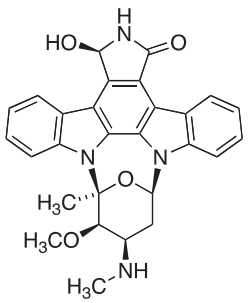
Inactivation of kinase PI3K by wortmannin.



PX-866

5.4.2 Phosphatidylinositide-Dependent Protein Kinase 1 (PDPK1) Inhibitors

UCN-01 is a natural staurosporine derivative that was originally described as a selective inhibitor of PKC, but further research showed that it is also a potent inhibitor of CDKs and CHK1. Its antitumor activity seems to be related mainly to CHK1 inhibition and to disruption of the PI3 kinase/AKT pathway through inhibition of PDPK1.[175]



UCN-01

The binding of UCN-01 to the active site of PDPK1 has been studied by X-ray crystallography and compared to that of staurosporine, indicating the importance of the hydroxy group in the former.[176] The inhibitor is located in the ATP-binding site, and the heterocyclic moiety is sandwiched with hydrophobic residues Leu-88, Val-96, Ala-109, and Leu-98 of the N-terminal lobe and with Thr-222 and Leu-212 of the C-terminal lobe. The lactam group mimics the adenine interactions in ATP and shows two H bonds with the backbone Ser-160 and Ala-162 residues. The key hydroxyl group interacts with side chains of Gln-220 and Thr-222, the latter with the intermediacy of a molecule of water. An additional H bond, similar to the one formed by the ATP ribose, is formed between the methylamino group and Glu-166. A second H bond of the methylamino group involves Glu-211 (Figure 10.30).

5.4.3 AKT Inhibitors

AKT exists in three isoforms called AKT-1, -2, and -3. The kinase domain is highly conserved among these isoforms, but the PH domain, where PIP3 binds, provides a target for allosteric AKT inhibitors with potential isoform selectivity. Accordingly, two types of AKT inhibitors are known, namely

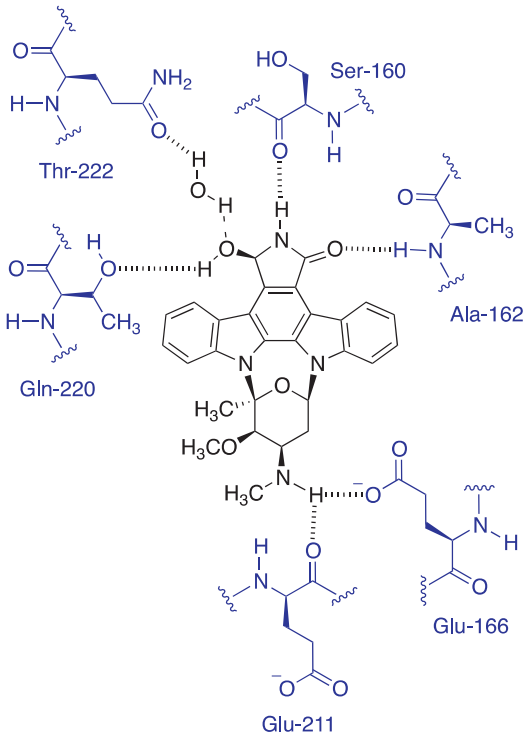
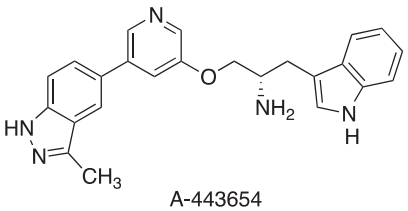


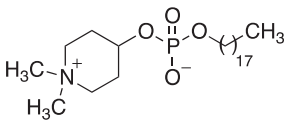
FIGURE 10.30

Binding of UCN-01 to PDPK1.

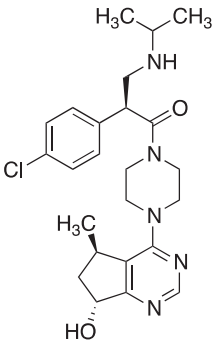
ATP-competitive and -allosteric inhibitors.[177] The first group is exemplified by A-443654, a pan-AKT inhibitor with particular activity on AKT-1. *In vivo*, it slows the progression of tumors when used as monotherapy or in combination with paclitaxel or rapamycin. Tumor growth inhibition was observed during the dosing interval, and the tumors regrew when compound administration was ceased.[178] Among allosteric inhibitors, perifosine (KRX-0401) is a lipophilic choline analog, which is the prototype of a new group of anticancer drugs referred to as alkylphosphocholines. Perifosine disrupts AKT membrane localization and activation, possibly by interference with the interaction of natural phosphatidylinositol phosphate groups with the PH domain of AKT. This compound shows selectivity for other kinases of the same pathway and was proven to be very efficient in phase II clinical trials against metastatic colon cancer and in phase III studies against multiple myeloma. It has also shown promising results against hormone-sensitive recurrent prostate cancer.[179,180] In 2010; the FDA approved fast track designation for perifosine in the treatment of refractory advanced colorectal cancer. Ipatasertib (GDC-0068) is a pan-AKT inhibitor[181] that is under clinical evaluation for a variety of cancers.[182]



A-443654



Perifosine



Ipatasertib (GDC-0068)

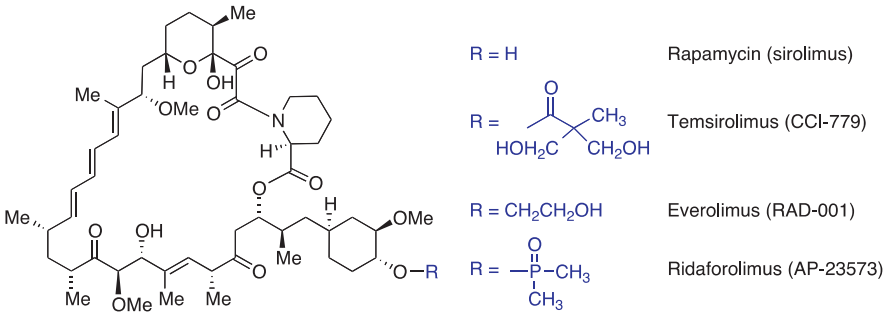
The association of these inhibitors with compounds targeting AKT-associated kinases, such as casein kinase 1, γ3 (CSNK1G3), and/or inositol polyphosphate multikinase (IPMK), has been suggested as a way to achieve increased efficacy and an improved therapeutic index.[183,184] Inhibition of heat shock protein 90 (HSP90), which is discussed in Chapter 11, provides a third, indirect way to achieve AKT inhibition.

Inhibition of AKT by 9-methoxy-*N*-methylellipticinium acetate (see Chapter 7, Section 2) was described in 2005.[185] This compound selectively induced apoptosis in ovarian cancer cell lines with overactivation of AKT, showing minimal effect on normal cells. Growth of NSCLC epithelial cells A549 was also inhibited by ellipticine, whose induced cytotoxicity was proposed to involve modulation of the signaling pathways and subcellular redistribution of AKT and p53.[186] On the other hand, some ellipticine derivatives significantly inhibit both wild-type and D816V mutated c-Kit kinase, occupying in part the ATP binding pocket of this kinase according to docking studies[187] and molecular mechanics simulations.[188]

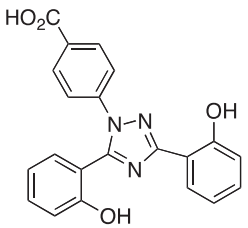
5.4.4 mTOR Inhibitors

The previously mentioned downstream serine–threonine kinase known as the "mammalian target of rapamycin" (mTOR) is another cancer target related to the PI3K/PDPK1/AKT/mTOR pathway that acts as a regulator of the translation of specific mRNA subpopulations that are important for cell proliferation and survival.[189–191] Inhibition of mTOR results in the suppression of growth and proliferation of lymphocytes and certain tumor cell lines. The parent inhibitor of this kinase was the macrolide rapamycin (sirolimus), a natural product isolated from a *Stremtomyces hygroscopicus* that was approved as an immunosuppressor for the prevention of rejection following organ transplantation. Rapamycin and its derivatives do not bind directly to mTOR but, rather, to an immunophilin of the FK-506 family called FKBP-12, and this complex then interacts with mTOR at a region adjacent to its kinase domain, thereby preventing the interaction of mTOR with its kinase substrates. FKBP-12 also has an inhibitory effect on TGF-β receptors (see Section 7) through its binding to the GS domain of these receptors.

The more important rapamycin derivatives[192] clinically assayed or approved as antitumor agents are the water-soluble rapamycin ester prodrug temsirolimus (CCI-779, Torisel®), the *O*-hydroxyethyl derivative everolimus (RAD-001, Votubia®, Afinitor®), and ridaforolimus (AP-23573). Temsirolimus was approved in 2007 for the treatment of advanced renal cell carcinoma and later for relapsed and/or refractory mantle cell lymphoma. Everolimus is used as an immunosuppressant and was approved in 2009, with the trade name Afinitor®, for advanced kidney cancer and later for other tumors, including breast cancer in postmenopausal women with advanced hormone receptor-positive and HER-2-positive type cancer, in conjunction with exemestane, for tumors that have developed resistance to trastuzumab. Ridaforolimus was tested in phase I/II clinical trials and showed promising results in several tumor types, including sarcoma.[193] Based on the results of a phase III study for the latter indication, it was submitted in 2011 for approval to the FDA, although it was rejected in 2012.



Deferasirox (DBO-1609, Exjade®) is an orally effective iron chelator currently used for the treatment of iron (Fe) overload disease, whose anticancer activity is due not only to its ability to deplete cancer cells of Fe but also to other mechanisms such as mTOR inhibition.[194] It is in phase III trials in non-transfusion-dependent thalassemia for hemotologic tumors.

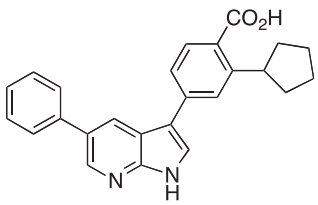


Deferasirox

5.4.5 Inhibitors of SGK1

The serum–glucocorticoid-regulated kinase 1 (SGK1) is another serine–threonine protein kinase whose expression is regulated by glucocorticoids and serum, in addition to various other types of signals. Its enzymatic activity is regulated by specific phosphorylation events initiated by PI3K activation. Thus, it is first phosphorylated at Ser-422 by an unidentified kinase known as the phosphoinositide-dependent protein kinase (PDPK)-2, followed by a second PDPK1-catalyzed phosphorylation of Thr-256 within the activation loop of the SGK1 catalytic domain.[195]

In recent years, SGK1 has become a therapeutic target in prostate cancers that mostly rely on androgens for growth and survival. Patients with advanced prostate cancers generally undergo androgen deprivation therapy (see Chapter 3) with chemical and/or surgical castration as a primary intervention, but most of them eventually experience a relapse of the disease, which is then considered to be hormone refractory because it is no longer responsive to androgen deprivation therapy even though the androgen receptor (AR) signaling pathways remain active. Thus, in addition to androgen ablation, inhibition of pathways downstream of AR may have therapeutic utility in prostate cancer. It was already mentioned that many cancers, as is the case for prostate cancer, rely on the PI3K signaling pathway for growth and survival. Together with AKT, SGK1 is involved in the regulation of cell growth and survival downstream of PI3K activation, and inhibition of its expression or activity antagonizes androgen-induced growth of prostate cancer. Thus, the competitive SGK1 inhibitor GSK-650394 completely abrogated androgen-mediated growth of LNCaP cells, which are PTEN-null and therefore exhibit constitutive activation of PI3K.[196] However, this approach to prostate cancer treatment has not been clinically studied.



GSK-650394

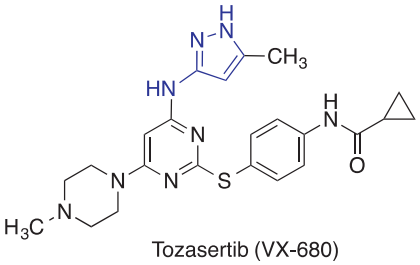
Interestingly, SGK1 is also a negative regulator of the tumor suppressor factor p53, and this effect is mediated by glucocorticoids. This observation explains why the elevation of glucocorticoid levels in chronic restraint stress, which causes anxiety- and depression-like behaviors, attenuates p53 function and promotes tumorigenesis.[197]

5.5 AURORA KINASES

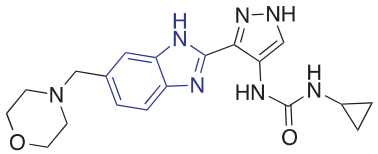
Aurore kinases are a small family formed by three serine–threonine kinases (Aurora A, B, and C) that play a crucial role in mitosis because they are important for centrosome maturation, chromosome segregation, and cytokinesis. Aurora kinases are involved in the onset and progression of many human cancers by dysregulating the phosphorylation of histone H3 and the tumor suppressor p53, being overexpressed in a wide range of human tumors including 50% of colorectal, ovarian, and gastric cancers. This overexpression transforms microblasts into cells containing multiple centrosomes and multipolar spindles, and the resulting genetic instability contributes to tumorigenesis.[198] For these reasons, Aurora kinases are an emerging target in cancer chemotherapy.[199,200] The main difference between their inhibitors and other antimitotic drugs is that they push the cells through aberrant and irreversible rounds of the cell cycle, resulting in a delayed but sustained response in animal models. So far, the clinical activity of the Aurora kinase inhibitors as single agents in patients with solid tumors has been rather disappointing, but they seem to have a future in patients with leukemias expressing Bcr-Abl or in patients with fast-growing tumors and Aurora kinase overexpression.

The few known Aurora kinase inhibitors are ATP-competitive inhibitors.[201] Among these, the pan-Aurora kinase inhibitor tozasertib (VX-680, MK-0457) was designed using the 4-aminopyrimidine template on the basis of the crystal structures of the ATP binding sites of the three Aurora kinases.[202] Although this compound was the first Aurora kinase inhibitor tested in clinical trials, its observed activity in patients with T315I Abl-mutated CML or Philadelphia chromosome-positive acute lymphocytic leukemia is caused by the binding of the drug to the active conformation of the ABL kinase domain. This mechanism explains the effectiveness of tozasertib against imatinib- and dasatinib-resistant forms of ABL. Its use in combination therapies has been proposed as first-line treatment for Philadelphia chromosome-positive acute lymphoblastic leukemias.[203] Preclinical studies have shown the antimyeloma activity of the multitargeted kinase inhibitor AT-9283, which inhibits Aurora kinases and STAT3.[204]

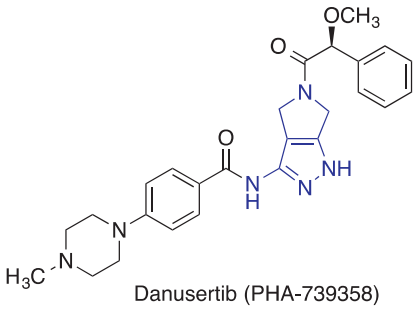
Other compounds that have entered phase I/II clinical studies in patients with acute myeloid leukemia are danusertib (PHA-739358), which is active against all known Aurora kinases and also against other cancer-relevant kinases such as the Bcr-Abl tyrosine kinase, being very effective against Bcr-Abl-positive leukemia cell lines, including the imatinib-resistant cell lines harboring the T315I mutation,[205] and barasertib (AZD-1152), which was designed by manipulation of the well-known 4-aminoquinazoline privileged structure.



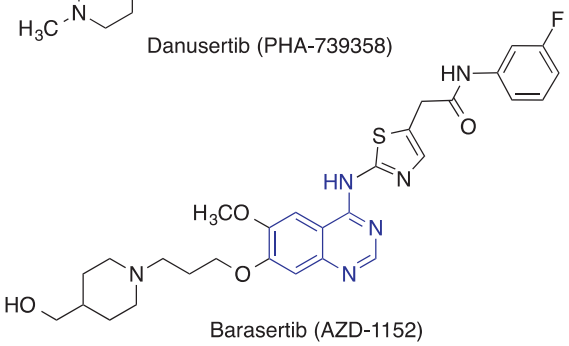
Tozasertib (VX-680)



AT-9283



Danusertib (PHA-739358)



Barasertib (AZD-1152)

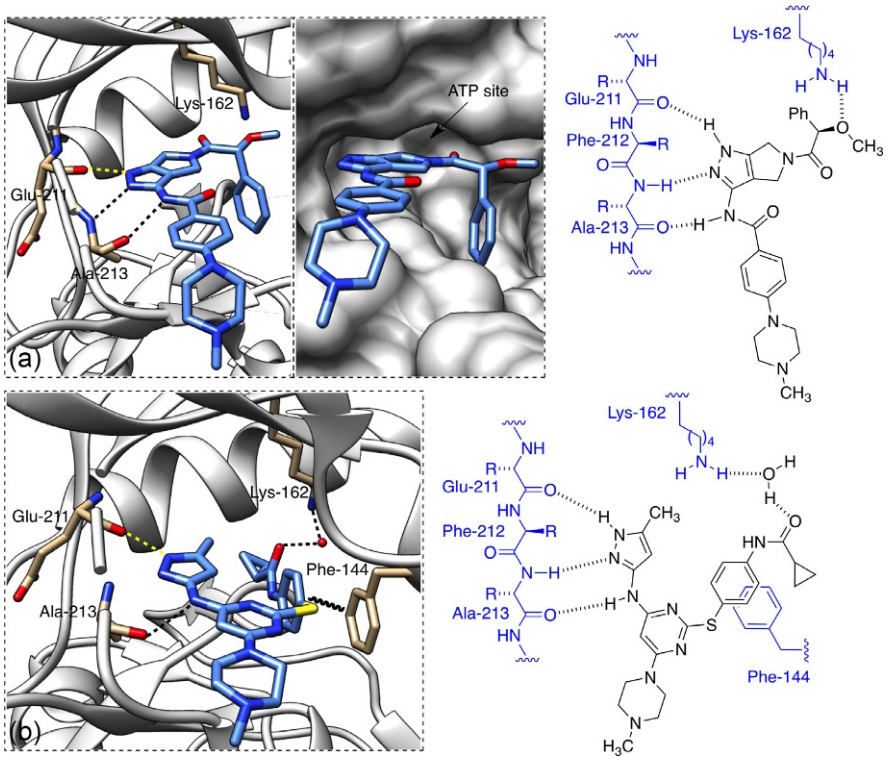


FIGURE 10.31

Recognition of danusertib (a) and tozasertib (b) by the ATP binding site of Aurora kinase A, which in the latter case was also bound to TPX2, a protein cofactor. The three-dimensional structures were generated from Protein Data Bank references 2 J50 (danusertib) and 3E5A (tozasertib) and displayed with Chimera 1.8.1.

These compounds were designed as adenine mimics at the ATP site of Aurore kinases, as shown in Figure 10.31 for two representative examples. In the case of danusertib, the aminotetrahydropyrrolo [3,4-*c*]pyrazole framework is responsible for the recognition of this site by H bonding to the peptidic framework at the Glu-111 and Ala-213 residues, whereas the methoxy substituent establishes an additional H bond with Lys-162 (Figure 10.31a).[206] In the case of tozasertib, the aminopyrazole fragment establishes similar H bonds with Glu-211 and Ala-213, and the carbonyl group of the cyclopropylamide moiety interacts with Lys-162. In this particular case, one of the loops in the kinase adopts a unique bent conformation that allows a $\pi-\pi$ interaction of the side chain of its Phe-144 residue with the phenyl group of the drug (Figure 10.31b).[207]

5.6 PROTEIN KINASE C (PKC) MODULATORS

PKC is a family of closely related serine–threonine kinases that can be activated by G protein-coupled receptors containing seven transmembrane domains. Activation of these receptors produces the activation of phospholipase C (PLC), which catalyzes the hydrolysis of the phosphatidylinositol

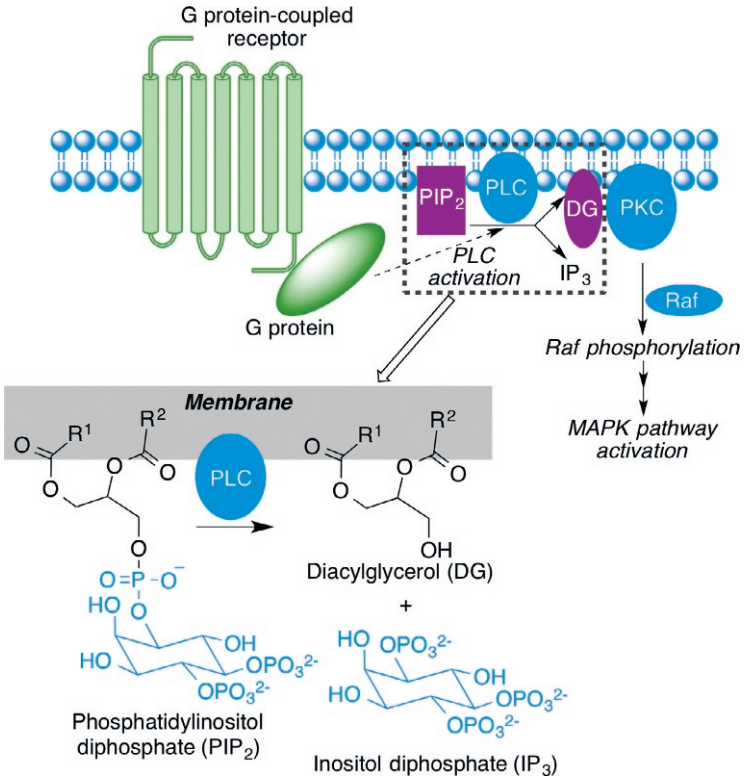


FIGURE 10.32

PKC-mediated activation of Raf.

diphosphate (PIP_2) integrated into the membrane. This hydrolysis generates two secondary messengers, namely inositol triphosphate (IP_3) and diacylglycerol (DG). The latter compound is lipophilic and remains in the cell membrane, where it activates PKC. Once back in the cytoplasm, the activated PKC produces the activation of Raf by phosphorylation of serine–threonine residues, thereby providing input into the MAPK pathway (Figure 10.32). In addition, various oncogenes increase the levels of lipidic second messengers, which may lead to constitutive activation of PKC and neoplastic transformation. PKC has therefore been suggested as a target for the development of anticancer drugs,[208] and its inhibition offered a novel approach to the chemotherapy of B-cell malignancies.[209]

Some analogs of the natural kinase inhibitor staurosporine have been developed as PKC inhibitors with anticancer activity. They include the previously mentioned multikinase inhibitor UCN-01 and also midostaurin (PKC-412),[210] ruboxistaurin, and enzastaurin (Figure 10.33). Midostaurin (*N*-benzoylstaurosporine, PKC-412, formerly known as CGP-41251) is the less potent though more selective inhibitor of the PKC enzyme family, preferentially inhibiting the calcium- and DAG-dependent PKC subtype. It is safe and well tolerated, and it reduces the tumor load in chronic B-cell malignancies, having also been evaluated as a multidrug resistance reversal agent.[211] Ruboxistaurin (Arxxant®, LY-333531) and enzastaurin (LY-317615) are specific inhibitors of PKC-β, an enzyme involved in

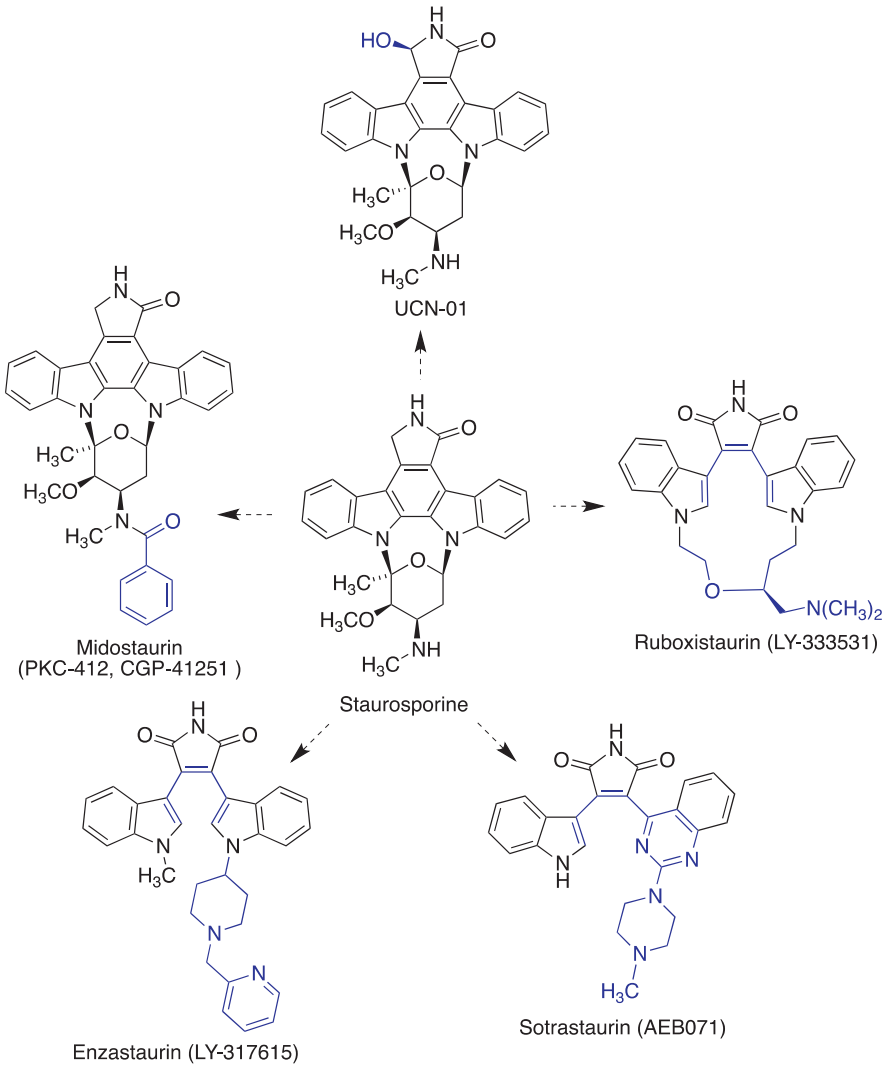
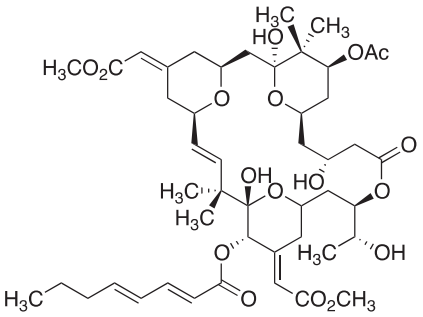


FIGURE 10.33

Staurosporine-related PKC modulators in clinical trials.

the induction of VEGF-stimulated neo-angiogenesis. Ruboxistaurin is under clinical study for the treatment of microvascular complications of diabetes,[212] and enzastaurin underwent a phase III study for recurrent malignant glioblastomas. This trial concluded that although it is well tolerated and has a good hematologic toxicity profile, its efficacy is not superior to that of lomustine,[213] and its development as an anticancer agent was halted in 2013. Sotrastaurin (AEB071), an immunosuppressant that blocks early T-lymphocyte (T-cell) activation via PKC inhibition,[214] may be a therapeutic option for psoriasis and has entered phase I trials for metastatic uveal melanoma.

Bryostatin 1, a cyclic macrolide isolated from the marine bryozoan *Bugula neritina*, is in clinical development as an antileukemic agent and in phase II clinical trials against melanomas, lymphomas, and renal cancer.[215] Although the mechanism of activity of the bryostatin family is not completely understood, it may be related to their ability to modulate the PKC receptor. Human clinical trials have been less promising than *in vitro* studies, but they suggest that bryostatins have a synergistic action with other chemotherapeutic agents such as paclitaxel.



Bryostatin 1

ISIS-3521 (LY-900003, Affinitac®) is a phosphorothioate antisense oligonucleotide that hybridizes to PKC mRNA and has undergone clinical trials, among others, in patients with locally advanced or metastatic colorectal cancer[216] and recurrent epithelial ovarian cancer. For a more detailed treatment of the chemistry of antisense oligonucleotides, see Section 6 of Chapter 12.

5.7 INHIBITORS OF PIM KINASES

The serine–threonine–protein Pim kinases phosphorylate different targets involved in cell cycle progression or apoptosis whose expression is induced by a number of cytokines relevant in the immune system. Considered as promoters for the immune response, Pims are implicated in multiple human cancers, including prostate cancer, AML, and other hematopoietic malignancies.[217] Among other studied compounds, LGH447 is a selective pan-Pim kinase inhibitor that is currently under phase I development for the treatment of hematologic malignancies and solid tumors.

6 INHIBITORS OF THE RAS/RAF/MEK SIGNALING PATHWAY

6.1 INTRODUCTION TO RAS SIGNALING

The Ras proteins are a large family of GTP-binding proteins (GTPases), which were among the first proteins identified as cell growth regulators. In normal cells, the Ras activity is controlled by the GTP/GDP ratio. Approximately 25% of human tumors, including nearly all pancreatic cancers and at least 30% of colon, thyroid, and lung tumors, have undergone an activating mutation in one of the *RAS* genes that leads to proteins remaining locked in an active state, especially those corresponding to three members of the family known as H-Ras, K-Ras, and N-Ras. Because of the large percentage of human tumors containing *RAS* mutants and their key role in maintaining the malignant phenotype, interruption

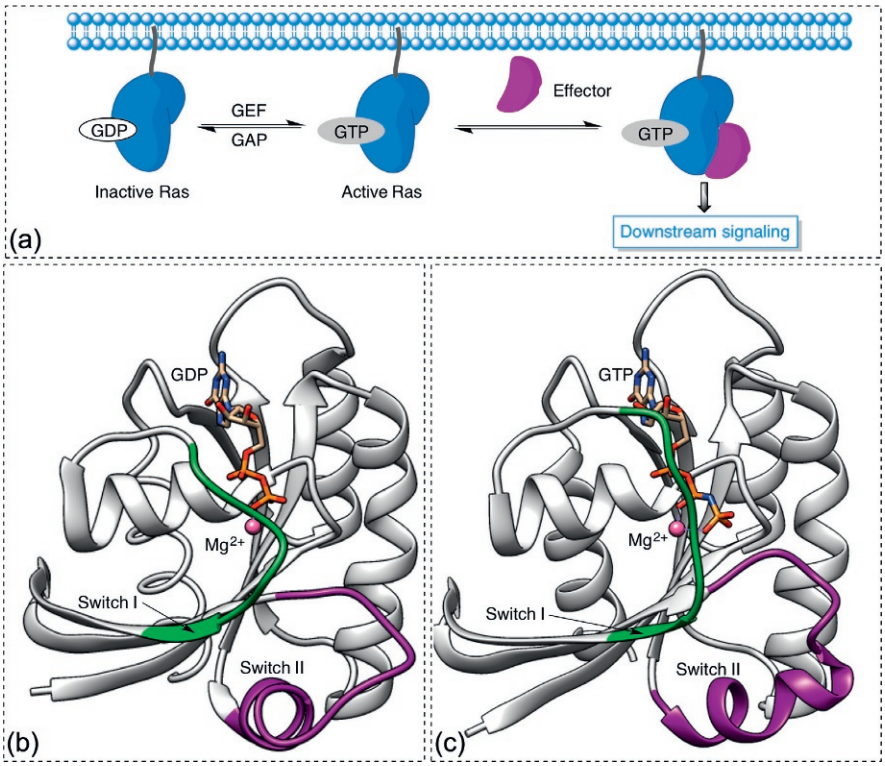


FIGURE 10.34

(a) Schematic summary of Ras activation and subsequent downstream signaling. (b) Ras in its "off" state, bound to GDP; PDB entry 5P21. (c) Ras in its "on" state, bound to GTP; PDB entry 4Q21. The structures in panels b and c displayed with Chimera 1.8.1.

of the Ras signaling pathway is an important focus of anticancer drug development[218] and has resulted in a large number of new antitumor agents in clinical trials. There are several indirect ways to modulate Ras signaling, as discussed in the following sections.

Ras signaling is initiated at the membrane and requires the activation of Ras by its binding to GTP, allowing the subsequent recruitment of effectors (Figure 10.34a). Thus, membrane-bound Ras cycles between the quiescent GDP-bound and the activated GTP-bound forms, which are interconverted via a conformational change that alters two loops of the protein (*switches*) near the nucleotide (Figures 10.34b and 10.34c). The equilibrium between these two forms of Ras is regulated by proteins belonging to the guanine nucleotide exchange factor (GEF) family, which promotes the activation of Ras, and GTPase-activating proteins (GAPs), which hydrolyze GTP.

Ras has a high affinity with GDP, and therefore the exchange of nucleotides requires its alteration by binding to an effector protein. This process is initiated by the adaptor molecule *growth factor receptor bound* (Grb), which binds to phosphorylated tyrosine receptors to recruit the effector *Son of Sevenless* (SOS), belonging to the guanine nucleotide exchange factor (GEF) family of proteins.

The SOS catalytic subunit contains a helical hairpin motif belonging to the CDC25 homology core domain, which becomes inserted between the Ras switch I and II regions. As a consequence, the nucleotide-binding pocket is opened and GDP is released, allowing Ras to bind to GTP. This active form of Ras then recruits several Raf family kinases that, in turn, activate mitogen-activated protein kinase kinases (MAPKKs or MEKs) to phosphorylate mitogen-activated protein kinases (MAPKs; also known as ERKs, "extracellular signals regulated kinases"), which finally influence gene expression (Figure 10.35). Mutations associated with components of the Ras and Raf upstream pathway contribute to the oncogenic phenotype through activation of MEKs and then ERKs.

Whereas the previously discussed activation mechanism promotes GTP binding to Ras, a competing process that involves GTPase activating proteins (GAPs) prevents it by promoting GTP hydrolysis ("Ras switch"; Figure 10.36). A single amino acid change at codons 12 (the most common in human cancer), 13, or 61 results in mutant Ras proteins that are not sensitive to control by GAPs, and hence, Ras is maintained in an active, GTP-bound ("on") state.

In the presence of water, the third phosphate of the three groups belonging to GTP can be hydrolyzed spontaneously, but this process is very slow. This hydrolysis is accelerated by Ras by a magnitude

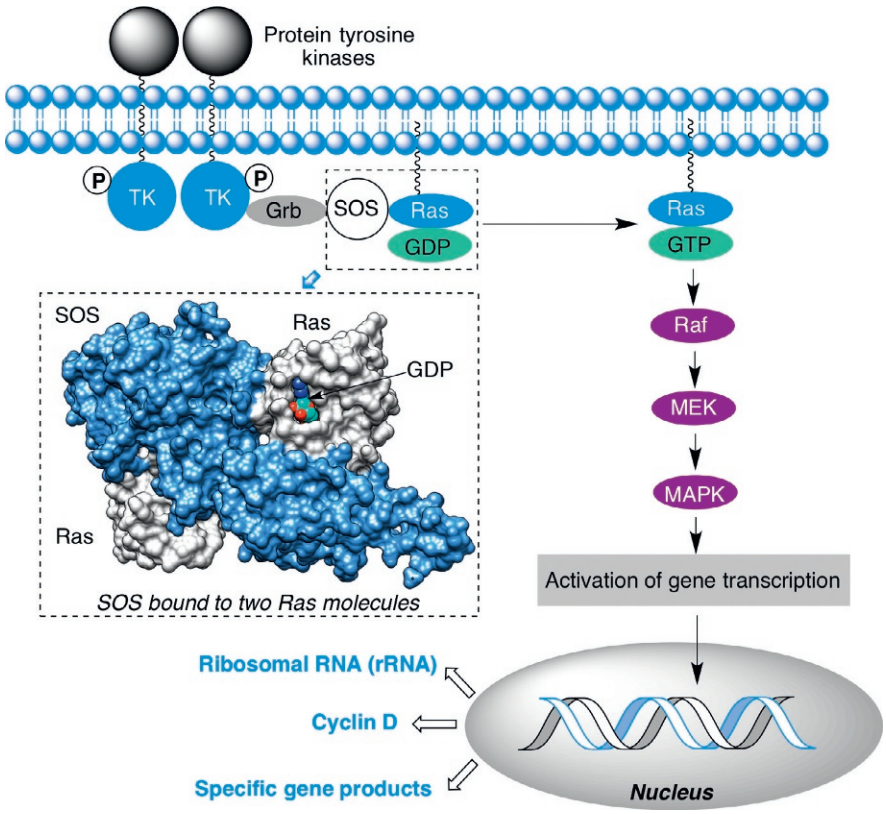


FIGURE 10.35

Activation of Ras and its consequences. The structure of the complex between Ras and SOS shown in the inset was generated from protein Data Bank reference 1NVW and displayed with Chimera 1.8.1.

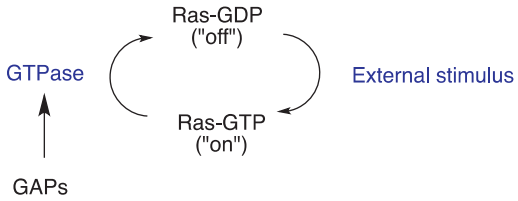


FIGURE 10.36

Control of Ras activation by GTPase activating proteins.

of 10^5 and by GAP by an additional factor of 10^5. The molecular mechanism by which the cleavage of GTP is accelerated has been described by using a combination of infrared spectroscopy and computer simulations,[219] a finding that could help the development of small molecules that restore the Ras proteins to the correct degree of activity. In this mechanism, GTP is destabilized by Ras because of a conformational change of the Ras–GTP–Mg^{2+} complex. In this complex, magnesium is coordinated to Thr35 and Ser17 residues of Ras and two oxygen atoms of the γ- and β-phosphate groups of GTP (Figure 10.37).

The staggered position of the nonbridging oxygen atoms of γ- and β-phosphate present in the GTP–Mg^{2+} complex in water becomes eclipsed in Ras–GTP–Mg^{2+}, whereas in the Ras–GTP–Mg^{2+}–GAP complex the Arg-789 finger of GAP further leads to an eclipsed position of the γ-, β-, and α-phosphates, and this tension facilitates the hydrolysis (Figure 10.38).

The main approaches that have so far yielded clinically useful compounds acting at the Ras pathway can be classified as follows:

1. Inhibition of Ras protein expression
2. Inhibition of Ras processing by farnesyltranferase
3. Inhibition of the attachment of farnesyl–Ras to the cell membrane
4. Inhibition of downstream effectors of Ras function

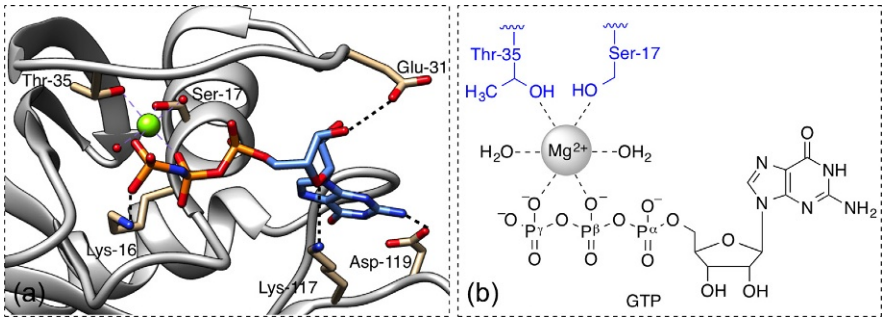


FIGURE 10.37

Structure of the Ras active site. The three-dimensional structure was generated from Protein Data Bank reference 5P21 and displayed with Chimera 1.8.1.

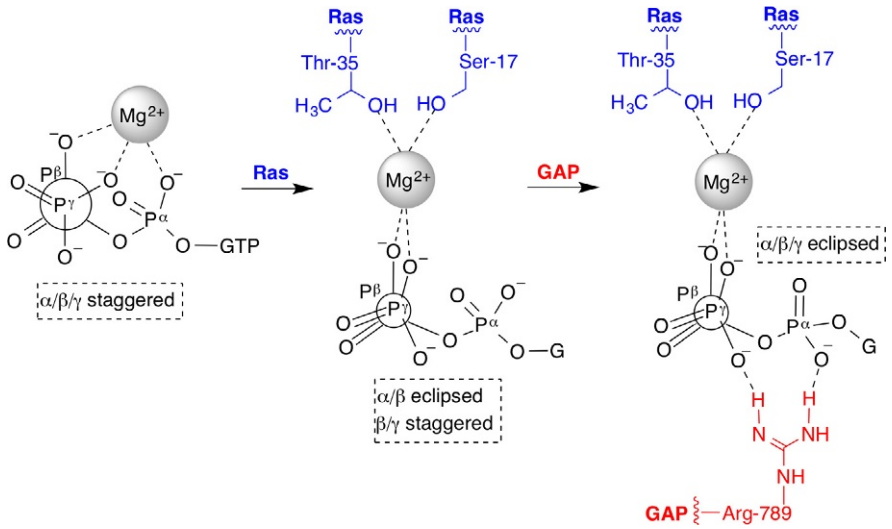


FIGURE 10.38

Conformational effects in the Ras-catalyzed hydrolysis.

6.2 INHIBITORS OF RAS PROTEIN EXPRESSION

Antisense oligonucleotides targeted at H-Ras mRNA have been developed for this purpose. The most relevant is the phosphorothioate oligodeoxynucleotide ISIS-2503, which contains 20 nucleotides (5′-TCCGTCATCGCTCCTCAGGG-3′) and has entered clinical assays for pancreatic carcinoma and other cancers.[220]

6.3 INHIBITORS OF RAS PROCESSING BY FARNESYLTRANFERASE

Newly synthesized Ras are cytoplasmatic proteins that require a post-translational structural modification to render them sufficiently lipophilic to allow their anchoring in the membrane in order to be able to recruit their target enzymes. This is achieved by the incorporation of lipidic chains at the C-terminus of the Ras protein via the following steps (Figures 10.39 and 10.40):

1. Prenylation by farnesyltransferase (FTase), an enzyme that recognizes a terminal CAAX sequence of Ras. In this sequence, C represents cysteine; A an aliphatic amino acid (Leu, Ileu, or Val); and X is Met, Ser, Leu, or Gln. This reaction attaches the 15-carbon farnesyl group ($C_{15}H_{25}$) to the Cys residue. Depending on the X residue, some Ras proteins may be modified through addition of the 20-carbon geranylgeranyl group ($C_{20}H_{33}$), which is catalyzed by geranylgeranyl transferases (GGTases).
2. Proteolysis by an endoprotease (RCE-1) that removes the last three amino acids of this modified C-terminus.

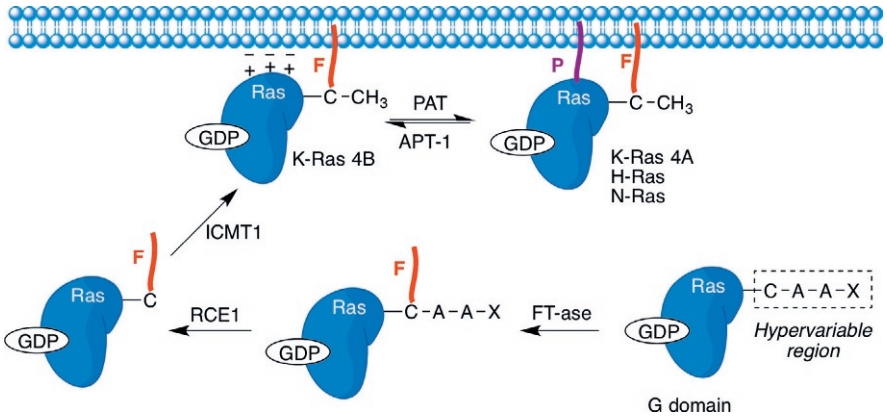


FIGURE 10.39

Steps involved in the anchoring of Ras to the cell membrane.

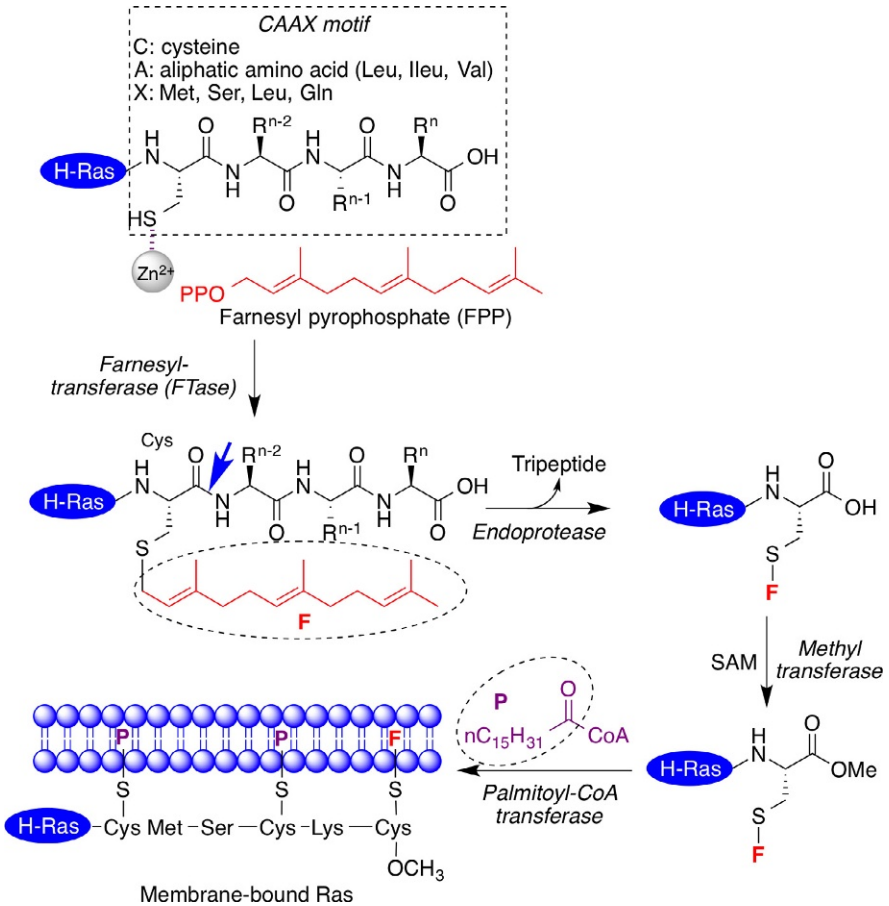


FIGURE 10.40

Chemical details of the processes involved in the anchoring of Ras proteins to the cell membrane.

3. Esterification of the new C- terminus by a methyltransferase (ICMT-1).
4. Introduction of palmitoyl groups ($C_{15}H_{21}CO$) by acylation of the thiol groups of two Cys residues by a palmitoyl–CoA transferase. This reaction does not take place in the case of K-Ras, whose interaction with the plasma membrane is aided by electrostatic bonding between a group of charged lysine residues and charged phospholipid head groups.

Thus, farnesylation is critical for Ras function and is therefore an important target for drug development.[221,222] FTase is a heterodimeric zinc metalloprotein formed by α and β subunits that binds to the "CAAX box" of the Ras protein after adoption by Ras of an extended conformation with the cysteine sulfur coordinated to the zinc ion in the enzyme active site. This coordination apparently lowers the pK_a of the thiol, increasing the local concentration of thiolate anion and facilitating its farnesylation with farnesyl pyrophosphate (FPP). This is illustrated in Figure 10.41 with the binding to FTase of a CVFM tetrapeptide.[223]

The design of farnesyltransferase inhibitors (FTIs) has been achieved using three approaches:[224–226]

1. Analogs that compete with FPP
2. Peptide or non-peptide peptidomimetic compounds targeted at the terminal CAAX sequence of Ras
3. Bisubstrate analogs that combine both structural features

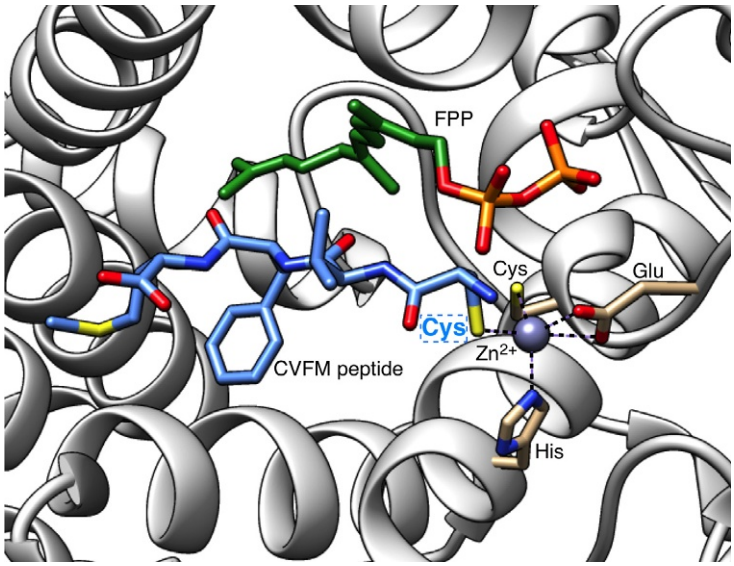
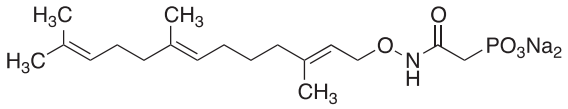


FIGURE 10.41

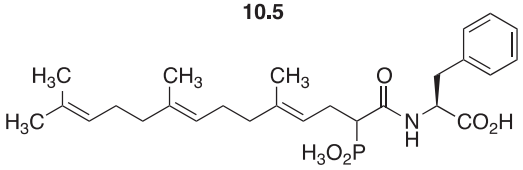
Complex formed by rat farnesyltransferase and the nonsubstrate tetrapeptide inhibitor CVFM. Generated from Protein Data Bank reference 1JCR and displayed with Chimera 1.8.1.

6.3.1 Farnesyl Pyrophosphate Mimics

This class of inhibitors has not attracted much interest because of their potential lack of selectivity due to the fact that FPP is a substrate for other enzymes such as squalene synthase. Some of these compounds (e.g., **10.5** and **10.6**) are potent inhibitors of the enzyme but have failed to show *in vivo* activity.



10.5



10.6

6.3.2 Peptides and Peptidomimetics That Mimic the CAAX Motif

Initial reports about the FT-inhibitory activity of CAAX tetrapeptides led to the identification of Cys-Val-Phe-Met as a lead for systematic structural modification. Most of these analogs were aimed at achieving suitable pharmacokinetic properties while retaining the thiol group, important for coordination to zinc. Some of the changes consisted of replacing the labile peptide bonds with stable methylenamino or methylenoxy links (e.g., L-739750) or the use of non-proteinogenic amino acids such as aminobenzoic acid derivatives (e.g., FTI-276). L-739750 and FTI-276 were normally employed as ester prodrugs (L-744832 and FTI-277, respectively) in order to enhance their absorption (Figure 10.42). Despite the encouraging *in vivo* data obtained for these peptidomimetics, there were reservations regarding their clinical use because of their potential thiol-related toxicity; nevertheless, L-744832 has reached clinical trials.[227] A combination of the modifications used for the design of L-739750 and FTI-276, with the additional replacement of the reduced cysteine moiety by a mercaptoproline and the thiol and the carboxylic groups masked as esters, has led to the design of the double-prodrug AZD-3409, which has reached clinical trials.[228]

The non-peptide peptidomimetic FTIs are heterocyclic compounds that have normally been discovered through screening approaches. BMS-214662, tipifarnib (R-115777, Zarnestra®), L-778123, lonafarnib (SCH-66336), and SCH-226374 are representative examples. BMS-214662 is a benzodiazepine derivative that may reverse the malignant phenotype of H-Ras-transformed cells and has been shown to be active against tumor cells with and without *RAS* mutations. Tipifarnib (initially developed as an antifungal agent) and L-778123 contain imidazole rings that are able to coordinate the catalytic zinc cation competing with the cysteine unit at the CAAX motif in Ras. Lonafarnib, which was discovered through a library screening, does not have a group able to act as a zinc ligand, which led to the design of its imidazole-bearing analog SCH-226374.

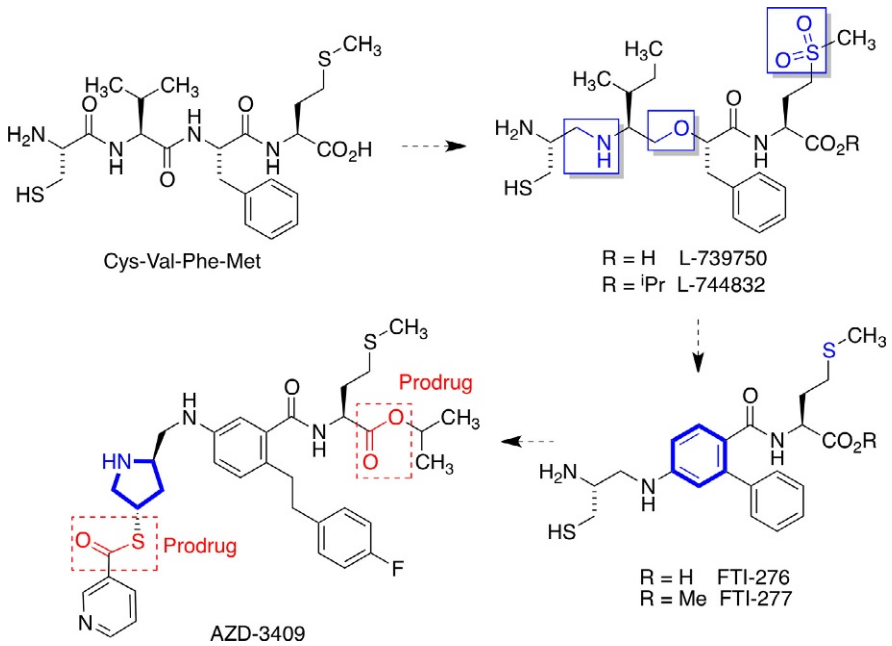
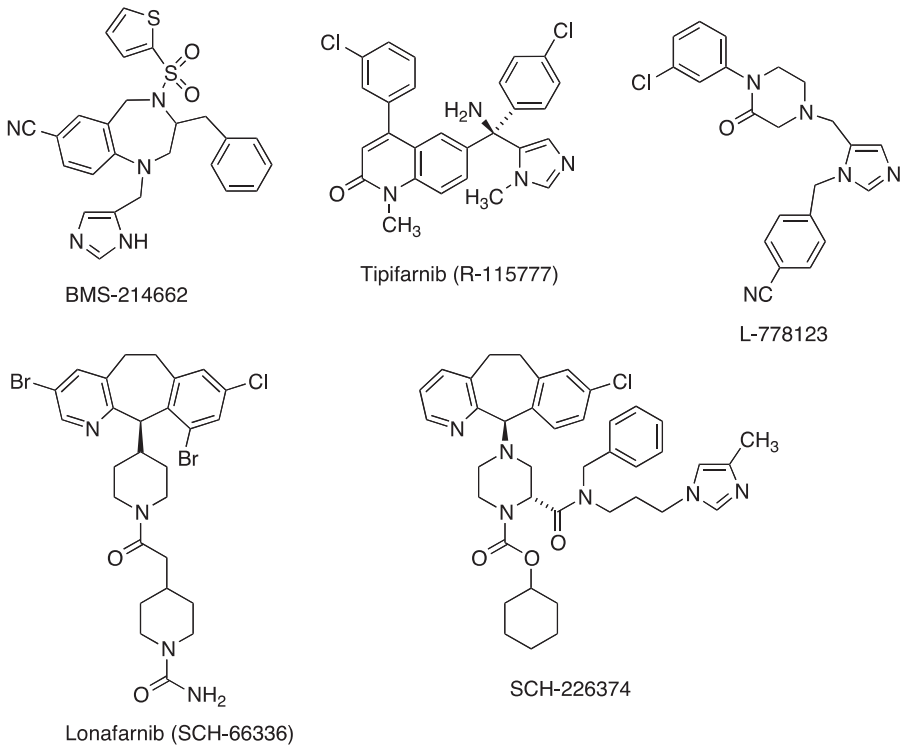


FIGURE 10.42

Peptide-like compounds that mimic the CAAX motif in FTase.



X-ray diffraction studies of BMS-214662 and tipifarnib complexed with FTase show that they bind to a hydrophobic cleft formed at the interface of the α and β subunits, forming a ternary complex with the FPP substrate and the enzyme, binding the catalytic zinc cation at the rim of the active site. Therefore, they act by a peptide-competitive mechanism.[229] This interaction is exemplified in Figure 10.43 for the case of tipifarnib, which adopts a U shape stabilized by π-stacking interactions between the two chlorophenyl rings. The imidazole nitrogen coordinates with the zinc cofactor at the catalytic center, and water-mediated H bonds are established between the quinolone carbonyl oxygen and the Phe-360 at the protein backbone, as well as between the amino group and the FPP α-phosphate moiety.

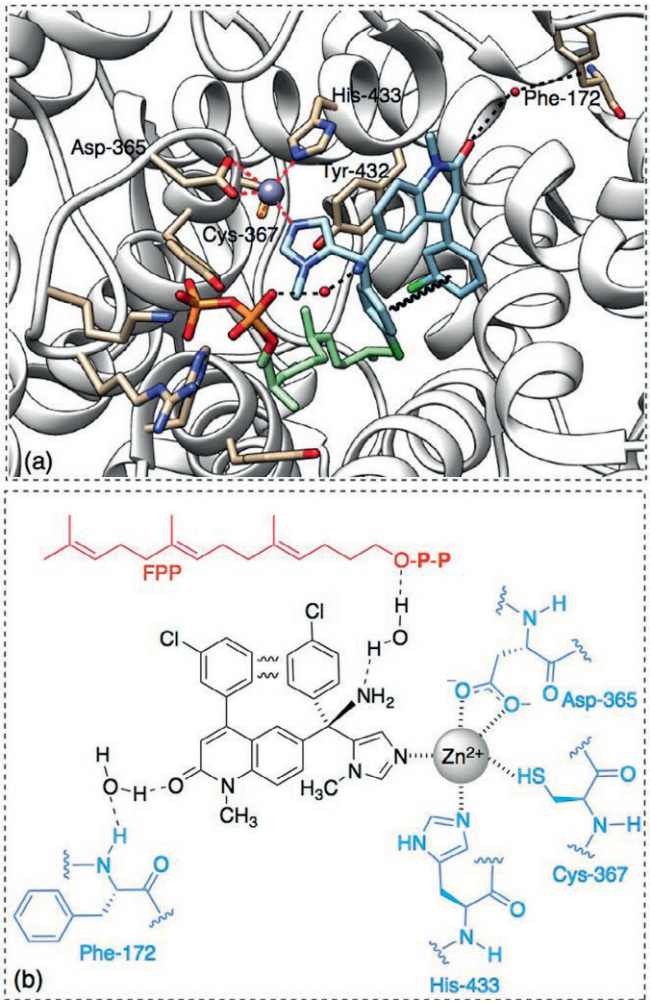


FIGURE 10.43

Structure of the ternary complex formed by tipifarnib, FPP, and FTase. The three-dimensional structure was generated from Protein Data Bank reference 4LNG and displayed with Chimera 1.8.1.

Similarly, the imidazole ring in BMS-214662 binds to the zinc cation in the active site, and the union is stabilized by several π-stacking interactions.[223]

L-778123 was designed to selectively compete with the binding of the CAAX fragment of Ras in FTase, but *in vivo* studies showed that it also inhibited GGTase I in the presence of anions such as sulfate and phosphate by unexpectedly competing with the geranylgeranyl diphosphate (GGPP) substrate rather than with the peptide. The inhibitor adopts a U shape by Van der Waals stacking between the cyanophenyl and piperazine units, with the imidazole unit occupying the apex of the structure and coordinating with the zinc cation. In FTase, FPP binds adjacently at the corresponding site, with the pyrophosphate group occupying a positively charged pocket (Figure 10.44a). However, in GGTase I, the inhibitor does not form a ternary complex with geranylgeraniol pyrophosphate; instead, it occupies the lipid substrate binding pocket and a portion of the peptide substrate binding pocket. The cationic site is occupied by a sulfate anion, which is placed where the pyrophosphate of GGPP normally binds (Figure 10.44b).[230]

Many of these FTIs have entered clinical trials for several cancers,[231] but their antitumor activity has been far lower than anticipated. Tipifarnib was the first FTI to be clinically tested,[232,233] and its most promising activity was reported in patients with untreated poor-risk AML or myelodysplastic syndrome. Both tipifarnib and lonafarnib[234] are orally bioavailable, whereas BMS-214662 and L-7781123

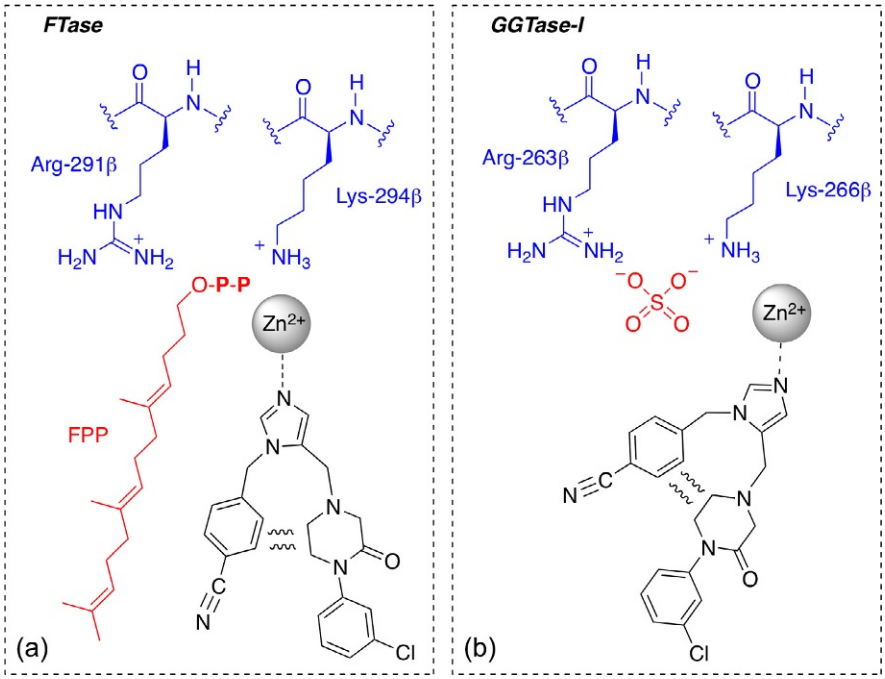


FIGURE 10.44

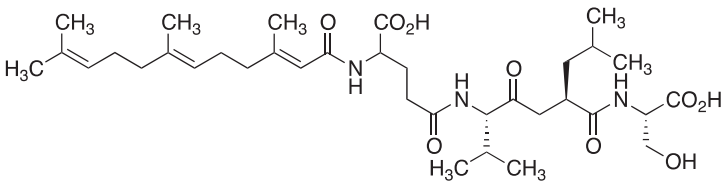
Binding of L-778123 to FTase (a) and GGTase-I (b).

have been studied as intravenous formulations.[235] Many trials focus on combining FTIs with classic chemotherapeutic drugs or radiotherapy.

The relatively low clinical efficacy of FTIs as anticancer agents has been explained by the observation that Ras geranylgeranylation may compensate for the inhibition of their farnesylation. Nevertheless, geranylgeranyl transferase inhibitors or dual inhibitors also did not show clinical efficacy. On the other hand, the inhibition of the farnesylation of several other proteins may also be relevant in cancer therapy.

6.3.3 Bisubstrate Analogs

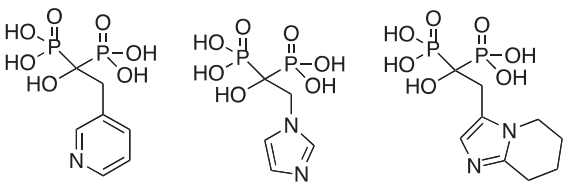
Some FTIs incorporate structural motifs from both farnesyl pyrophosphate and the CAAX sequence. One example is compound **10.7**, in which the thiol moiety of CAAX was substituted by a carboxylic group and the farnesyl chain was covalently attached to the peptide through an amide linkage.



10.7

6.3.4 Inhibitors of Farnesyl Diphosphate Synthase and Geranylgeranyl Diphosphate Synthase

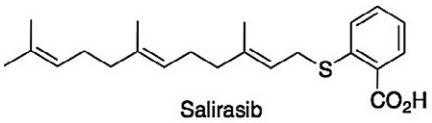
In addition to Ras, there are other small GTPases, such as Rho, Rac, cdc42, and Rab, that need to be prenylated by transfer of farnesyl or geranylgeranyl units onto a Cys residue in order to be anchored to cell membranes and to be able to affect protein–protein interactions. Nitrogen-containing biphosphonates (N-BPs) are normally used in therapeutics for the treatment of degenerative bone diseases such as osteoporosis, but third-generation BPs that contain a nitrogen heterocycle, such as risendronate, zolendronate, and minodronate, have shown a dual anti-bone resorption and antitumor cell proliferation activity and are undergoing preclinical and clinical studies for several cancers, including breast, prostate, lung, renal, osteosarcoma, and chondreosarcoma. The antitumor activity of these phosphonates is due to the inhibition of FPP and GGPP, and hence of the farnesylation or geranylgeranylation of small GTPases.



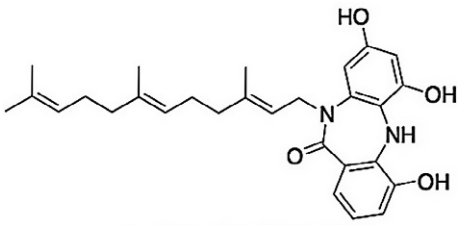
Risedronate Zoledronate Minodronate

6.4 INHIBITORS OF THE ATTACHMENT OF FARNESYL–RAS TO THE CELL MEMBRANE

Another line of work in this area has been the design of farnesyl cysteine mimics. These compounds have been designed to compete with the binding of Ras with farnesyl-binding proteins at the plasma membrane. One of these compounds, salirasib (*S*-farnesylthiosalicylic acid), has undergone a number of phase I and II clinical trials for lung and pancreatic cancer, with varying degrees of success.[236] Diazepinomicin (TLN-46019) has also undergone phase II clinical trials.



Salirasib



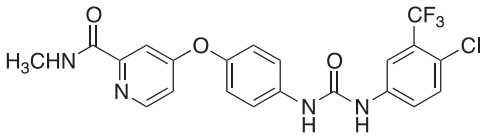
Diazepinomicin (TLN-4601)

6.5 INHIBITORS OF DOWNSTREAM EFFECTORS OF THE RAS FUNCTION

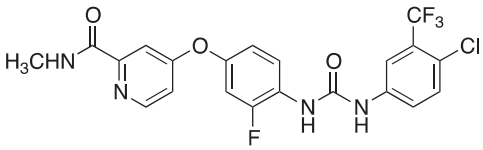
Among the multiple Ras effectors, the best known is the Raf kinase–MEK–MAPK (ERK) pathway. The activity of this cascade is increased in approximately one-third of all human cancers, and inhibition of their components represents an important antitumor strategy. However, only inhibition of mutant *B-RAF* has been found to be therapeutically active.[237] MAPK activation induces the expression of genes that regulate the inflammatory response; therefore, MAPK pathways are also targets in the treatment of autoimmune and inflammatory diseases.[238]

6.5.1 Raf Inhibitors

Three Raf proteins are known, namely c-Raf (Raf-1), b-Raf, and a-Raf. *B-RAF*, the human gene that encodes b-Raf, is mutated in several human cancers. Sorafenib (BAY43-9006, Nexavar®) is a multitargeted tyrosine kinase inhibitor that acts on c-Raf/b-Raf as well as on VEGFR-2 and PDGFRβ by binding to their ATP sites.[239] Among other mechanisms, it induces apoptotic cell death in human NSCLC cells by down-regulating mTOR-dependent surviving expression, events that are associated with sensitization to TRAIL-induced apoptotic cell death (see Chapter 11).[240] Based on its activity against renal cell[241] and hepatocellular carcinomas, sorafenib was approved by the FDA for the first and second indications in 2005[242] and 2007, respectively, and in 2013 for the treatment of differentiated thyroid carcinoma refractory to radioactive iodine treatment. Its fluoro analog regorafenib (BAY73-4506, Stivarga®) increased the overall survival of patients with metastatic colorectal cancer and was approved by the FDA in 2012. Regorafenib is an analog of sorafenib containing an additional fluoro substituent, which increased the overall survival of patients with metastatic colorectal cancer and was approved by the FDA in 2012.



Sorafenib



Regorafenib

The initial hit discovery eventually leading to sorafenib took place in 1995, 10 years before its approval. A phenyl-urea thiophene ester **10.8** was identified from a high-throughput screening of small-molecule chemical libraries against the c-Raf–MEK–ERK kinase cascade. This hit had only moderate activity against c-Raf, but some improvement was observed in its methyl derivative **10.9**. A library of bis-aryl urea analogs of the lead compound was then constructed to further explore the SARs of the series, which identified the 3-amino-isoxazole **10.10** and its analog **10.11**. Compound **10.11** possessed oral bioavailability and inhibited the growth of HCT116 xenografts *in vivo*, providing proof of principle for this new kinase inhibitor class. Further SAR studies finally led to the discovery of sorafenib (Figure 10.45).[243]

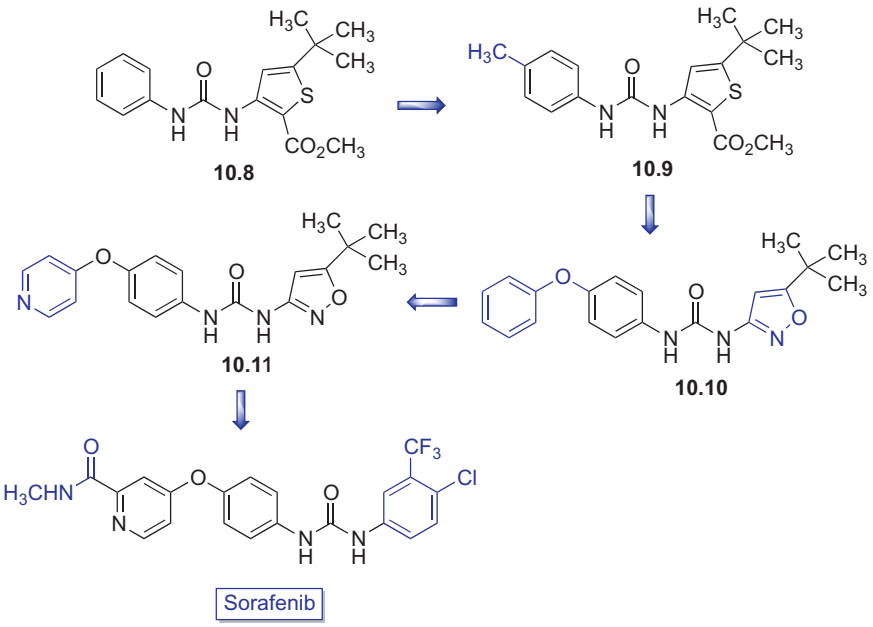


FIGURE 10.45

Main stages in the discovery of sorafenib.

Vemurafenib (PLX-4032, RG-7204, Zelboraf®) is a b-Raf inhibitor that works in melanoma patients in which this enzyme has the V600E mutation (the normal valine amino acid at position 600 is replaced by glutamic acid). Following rapid clinical development, it was approved in 2011 by the FDA and in 2012 by the EMA for this indication.[244,245] The discovery of vemurafenib started with the screening of a library of 20,000 small molecules with favorable chemical properties (i.e., low molecular weight, a low number of hydrogen bond donors and acceptors and rotatable bonds, and good solubilities) against five different kinases. More than 200 of these compounds inhibited a minimum of three kinases and were selected for co-crystallography in at least one of these kinases. Among the PIM1 kinase co-structures, 3-anilino-7-azaindole (**10.12**) showed a single binding mode, making two hydrogen bond contacts with the kinase hinge. Further optimization coupled with additional co-crystallography, this time with FGFR1, identified 3-(*m*-methoxybenzyl)-7-azaindole (**10.13**), with a substantial potency increase likely due to an additional hydrogen bond interaction between the methoxy oxygen and the protein. Libraries of mono- and disubstituted analogs built around the 7-azaindole core were then prepared and screened, leading to the identification of the difluorophenylsulfonamide substructural motif that gave excellent b-Raf potency. These compounds were then co-crystallized in engineered forms of b-RafV600E and wild-type b-Raf, which allowed for an additional iteration of optimization and resulted in identification of PLX4720 and vemurafenib, first prepared in early 2005 (Figure 10.46). Vemurafenib and PLX4720 were chosen to progress over analogs with similar *in vitro* and *in vivo* activities due to their consistent pharmacokinetics in rodents. For further drug development, vemurafenib was eventually selected in preference to PLX4720 because its pharmacokinetic properties, studied in dogs and monkeys, were considered more favorable.[246]

Dabrafenib (GSK-2118436, Tafinlar®), another selective b-Raf inhibitor, was approved by the FDA in 2013 for the treatment of melanoma patients who have a b-Raf V600E mutation. This compound was originated in a program aiming to develop a follow-up drug to the dual EGFR–erbB2 kinase inhibitor

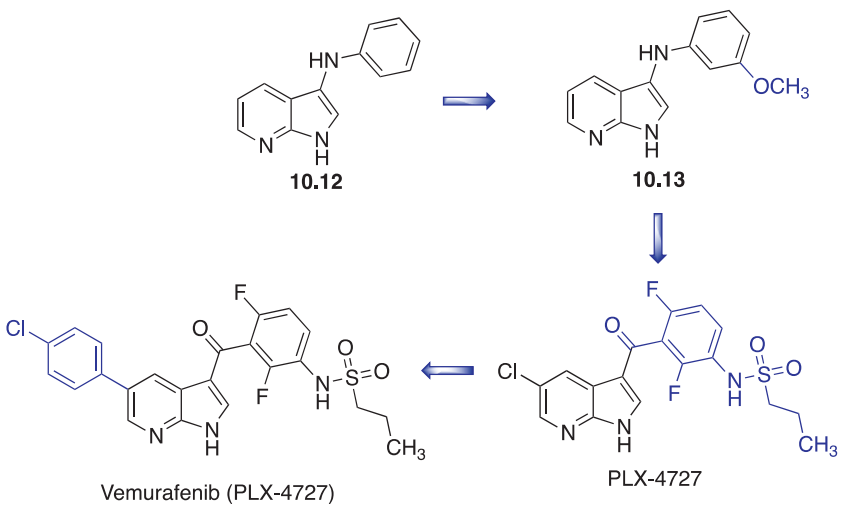


FIGURE 10.46

Key steps leading to the discovery of vemurafenib.

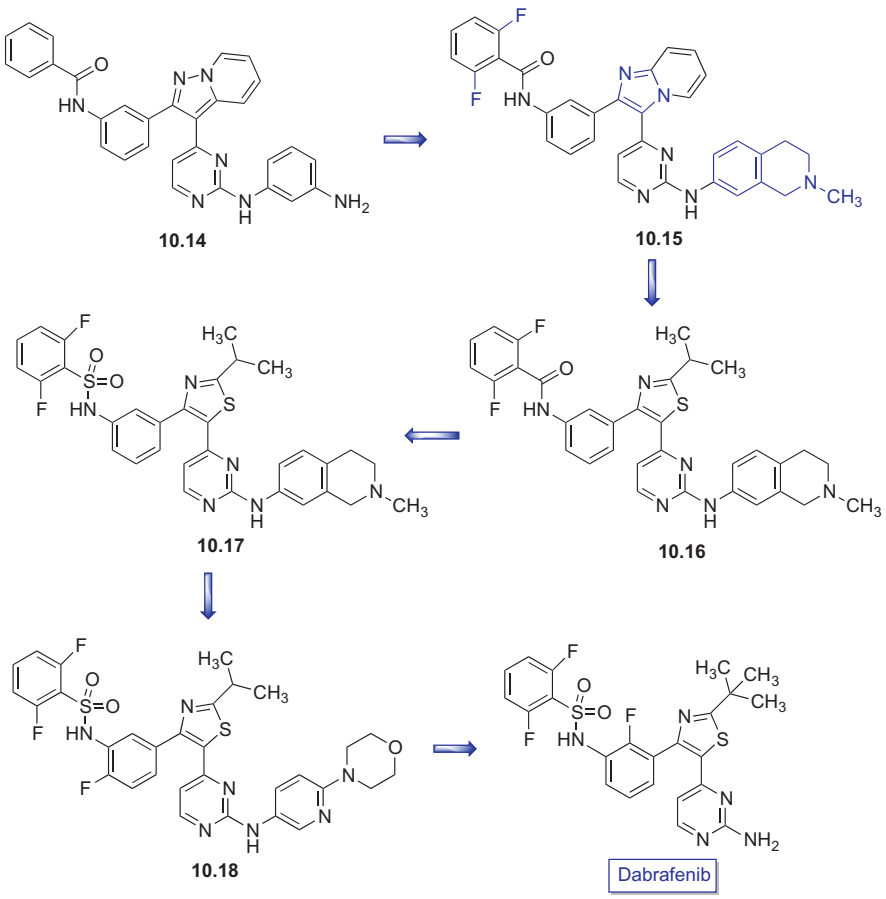


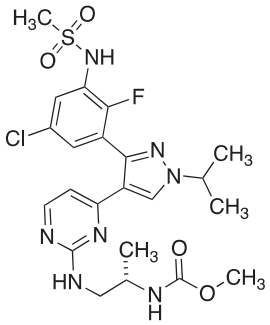
FIGURE 10.47

Milestones leading to the discovery of dabrafenib.

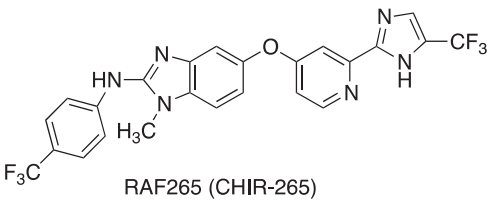
lapatinib (Tykerb®), which in the early 2000s was progressing through clinical trials (Figure 10.47). The goal of the program at that time was to identify an inhibitor with activity against both EGFR–erbB2 and additional kinases, such as b-Raf and IGF1R, that were thought to be involved in tumors that had acquired resistance to lapatinib. Screening of a kinase inhibitor collection led to identification of the pyrazolopyridine **10.14**, which had excellent EGFR–erbB2 inhibition plus modest activity against both b-Raf and IGF1R. A significant increase in b-Raf potency was observed by switching the heterocycle to an imidazopyridine core and attachment of the *N*-methyl-tetrahydroisoquinoline to the pyrimidine amine to yield **10.15**. Further lead optimization by using alternative heterocycle cores led to the identification of the thiazole **10.16**. At this time, the aim of the project was to search for a b-Raf alone inhibitor. Several replacements for the aryl amide linker revealed that the arylsulfonamide thiazole **10.17** showed a substantial improvement in cellular potency and metabolic stability and was thus used for further SAR exploration in other regions of the molecule. Fluorination of the benzene ring in the position *para* to

the thiazole as shown in **10.18** produced an increased metabolic stability, by blocking a major metabolic site, and oral absorption, perhaps by affording an intramolecular H-bonding interaction with the sulfonamide NH, thus masking one H bond donor. Because metabolite identification studies conducted in dog and monkey liver microsomes identified several major metabolites clustered in the isopropylthiazole core and 6-(4-morpholinyl)-3-pyridinamine regions of **10.18**, the isopropyl group attached to the thiazole was replaced with a *t*-butyl group and the pyrimidine moiety was truncated to a free 2-amino-pyrimidine. Finally, relocation of the fluorine *ortho* to both the thiazole and the sulfonamide yielded dabrafenib.[247]

Recent initial clinical trials with the b-Raf kinase inhibitor encorafenib (LGX818), either alone or in combination, to treat melanoma with a V600E mutation have provided encouraging results.[248] RAF265 (CHIR-265) is an orally bioavailable, selective inhibitor of b-Raf, c-Raf, and mutant b-Raf,[249] which also shows antiangiogenic activity through inhibition of VEGFR-2. It is currently being investigated in phase I clinical trials in patients with advanced malignant melanoma.[250]



Encorafenib (LGX818)



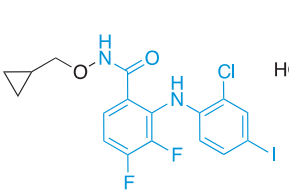
RAF265 (CHIR-265)

ISIS-5132 is a 20-mer phosphothiorate antisense oligonucleotide that is complementary to c-Raf kinase mRNA and hence downregulates its expression. It has entered phase II clinical trials for colorectal[251] and recurrent epithelial ovarian[252] cancers.

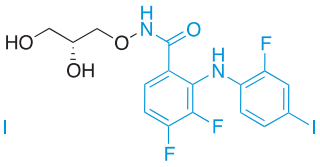
6.5.2 MEK Inhibitors

MEKs, also known as MAP kinase kinases (MKKs), are dual-specificity enzymes that phosphorylate threonine and tyrosine residues within the activation loop of their substrates.[253] Inhibitors of MEKs have been shown to effectively block upregulated ERK/MAPK signaling in a range of cancer cell lines. In particular, several MEK1/2 inhibitors have been tested clinically or are currently undergoing clinical trial evaluation as single agents or in combination with PI3K inhibitors. PD-184352 (CI-1040) was studied in patients with advanced non-small cell lung, breast, colon, and pancreatic cancer; it is an orally active, potent, and selective inhibitor of MEK that targets a non-ATP site of the kinase.[254] Its analog, PD-0325901, is a second-generation MEK1/2 inhibitor with significantly improved pharmaceutical properties, but after a phase I/II study it was associated with more severe toxicity and its development was discontinued in 2008. Cobimetinib (GDC-0973, XL-518) is another related, highly selective MEK inhibitor that is in early stage clinical trials.[255] ARRY-424704 (AZD-8330) is another noncompetitive inhibitor that has been considered for clinical trials. The somewhat related trametinib

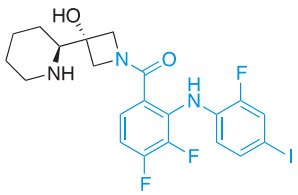
(GSK1120212, Mekinist®) is a potent, orally available, and highly selective MEK1/2 inhibitor[256] that was approved in 2013 for the treatment of patients with metastatic NSCLC whose tumors have EGFR exon 19 deletions or exon 21 (L858R) substitution mutations. The benzimidazole derivatives selumetinib (ARRY-142886, AZD-6244)[257] and binimetinib (MEK162, ARRY-162)[258] are potent inhibitors of MEK1/MEK2 that have reached phase II or III evaluation for several cancers. RO4927350, which has a novel chemical structure derived from an hydantoin core and a unique mechanism of action, is an orally active, potent, and highly selective MEK1/2 inhibitor.[259] Finally, GDC-0973 (XL-518) is another potent, highly selective MEK inhibitor that is in early stage clinical trials.[260]



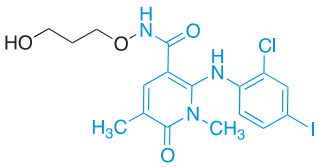
PD-184352 (CI-1040)



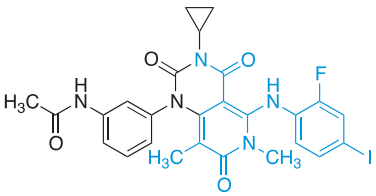
PD-0325901



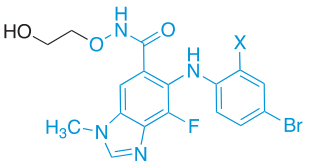
Cobimetinib (GDC-0973, XL-518)



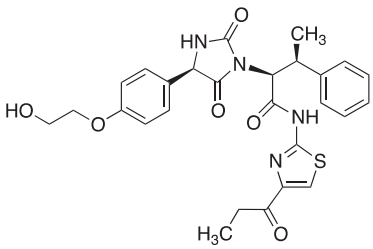
ARRY-424704, AZD-8330



Trametinib (GSK1120212)



X= Cl, Selumetinib
(ARRY-142886, AZD-6244)
X = F, Binimetinib
(MEK162, ARRY-162)



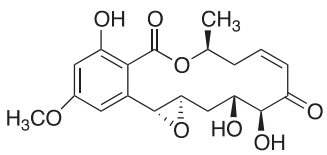
RO4927350

6.5.3 The MAPK (ERK) Pathway

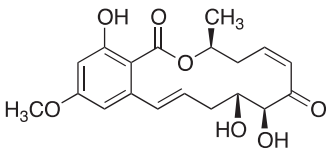
The ERK_1 and ERK_2 MAPKs are the most abundant ERKs in mammalian cells. Their activation is mediated by MEK1 and MEK2, which catalyze their phosphorylation at Tyr-204/187 and then Thr-202/185 residues. Whereas the Raf kinase and MEK families have narrow substrate specificity, ERK1/2 are proline-directed kinases that catalyze the phosphorylation of hundreds of cytoplasmic and nuclear substrates. The dephosphorylation of ERK1/2, mediated by several types of phosphatases, makes the overall process reversible.

After their translocation into the nucleus by active and passive processes involving nuclear pores, ERKs catalyze phosphorylation of nuclear transcription factors such as Ets, Elk, and c-Fos, which

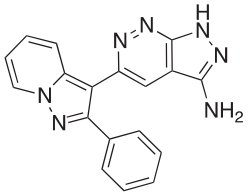
participate in the immediate-early gene response.[230] After the failure of many ERK1/2 inhibitors such as hypothemycin or compounds FR148083 and FR180204,[261,262] the first member of this class to enter clinical trials was GDC-0994, which is currently advancing in a phase I trial in patients with solid tumors.



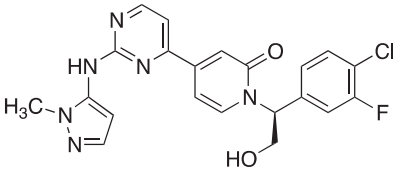
Hypothemycin



FR148083



FR180204



GDC-0994

The sustained inhibition of ERK1 and/or ERK2 using short hairpin RNAs (shRNAs), which are sequences of RNA that can be used to silence target gene expression via RNA interference (RNAi), increases the clinical activity of the previously mentioned bRaf inhibitor vemurafenib (PLX-4032, Zelboraf®).[263] On the other hand, the use of MEK1/2 inhibitors or transfected shERK1 and shERK2 cell lines in combination with chemotherapeutic drugs may be beneficial in the treatment of malignant mesotheliomas and other tumors because both kinases play critical roles in multidrug resistance and survival.[264]

6.5.4 Jun Kinases and p38 MAPK Pathways

The Jun N-terminal kinase (JNK) and p38 MAPK pathways are activated by environmental and genotoxic stresses and have key roles in inflammation and in tissue homeostasis because they control cell proliferation, differentiation, survival, and the migration of specific cell types. Certain cells use these signaling pathways to antagonize cell proliferation and morphological transformation, whereas cancer cells can subvert these pathways to facilitate proliferation, survival, and invasion. JNKs are essential for both cell proliferation and apoptosis, depending on the stimuli and the cell type involved in their activation.[265]

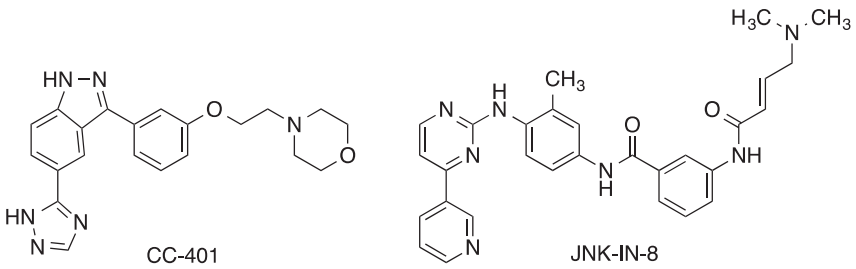
The oncogenic functions of JNKs are mostly based on their ability to phosphorylate c-Jun, a component of the activator protein 1 (AP-1), a transcription factor that regulates gene expression in response to environmental stress, radiation, and growth factors, all of which are stimuli that activate JNKs. The JNK/JUN pathway regulates a plethora of target genes that contain AP1 binding sites, including genes that control the cell cycle, survival and apoptosis, metalloproteinases, and nuclear hormone receptors.[266] The role of JNKs in prostate cancer development is of particular interest.

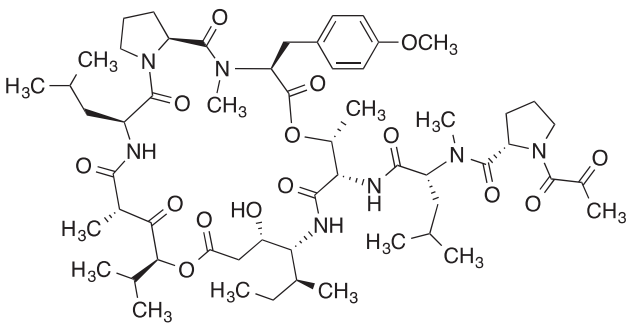
P38 mitogen-activated protein kinase (p38 MAPK) phosphorylates a number of substrates and regulates a variety of cytokines produced in the tumor microenvironment. It is activated and highly

expressed in several human cancers, and it may play a role in tumor growth, invasion, and metastasis. TGF-α can also activate various MAPK signaling pathways, most prominently the MKK4–JNK and MMK3–p38 pathways, and the interplay between the Smad and JNK or p38 pathways could underlie diverse forms of integration and reciprocal regulation between TGF-α signaling and other pathways in the cell (see later).

Despite the interest in inhibitors of JNKs and p38 MAPKs for the treatment of cancer, their development is in its infancy. A large number of small molecules have been reported to act as selective ATP-competitive JNK inhibitors, but most of them exhibit poor kinase selectivity. For this reason, the development of the JNK inhibitor CC-401, which reached phase I/II stage of clinical study for the treatment of myelogenous leukemia, was discontinued.[267] Structure-based drug design to develop ATP site-directed covalent inhibitors of JNK kinases by using the phenylaminopyrimidine core of imatinib as a scaffold has led to the potent and selective covalent inhibitor JNK-IN-8.[268]

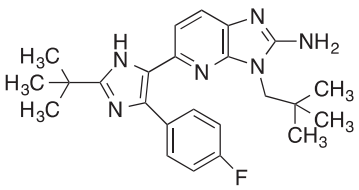
The marine natural product aplidine (dihydrodidemnin B, plitidepsin, PM01183, Aplidin®), a cyclodepsipeptide isolated from the Mediterranean ascidian *Aplidium albicans*, has a complex mechanism of action that involves cell cycle arrest, inhibition of protein synthesis, and apoptosis induction. The latter process is due mainly to activation of JNK and seems to be one of the primary mechanisms.[269] Aplidine has been granted orphan drug status for the treatment of acute lymphoblastic leukemia and multiple myeloma, and it is under clinical studies for a number of tumors.



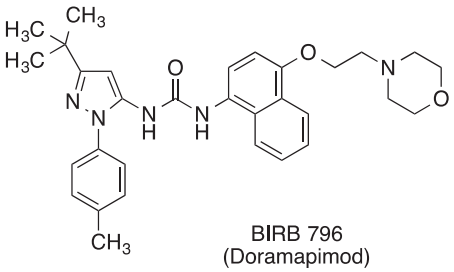


Aplidine (dihydrodidemnin B, Aplidin®)

Among p38 MAPK inhibitors, LY228820[270] is in phase I/II trials for recurrent ovarian cancer, and BIRB-796 (doramapimod) had a great promise for its affinity and selective profile.[271] Unfortunately, its clinical trials were discontinued because of lack of efficacy for the primary indications and the development of liver function abnormalities.[272]



LY2228820



BIRB 796
(Doramapimod)

7 TRANSFORMING GROWTH FACTOR-β–SMAD SIGNALING

The TGF-β superfamily of ligands are active cytokines and potent regulators of tumorigenesis with two different behaviors. Thus, at an early stage of the disease, they act as tumor suppressors, but at a later stage they behave as tumor promoters because cancer cells become able to use them to exacerbate their own proliferative, invasive, and metastatic behavior.[273]

The TGF-β–Smad signaling pathway involves receptor serine–threonine kinases, called TβRs, at the cell surface and Smad proteins, which are intracellular proteins that transduce extracellular signals from TGF-β ligands to the cell nucleus. TGF-β binding to the TβR-II receptor leads to the formation of a complex with another receptor known as TβR-I (also called activin-like kinase, ALK5). This allows TβR-II to phosphorylate TβR-I. The Smad proteins (Smad2 and Smad3) are then activated by TαR-I via phosphorylation and hence form a complex with Smad4. This complex is imported into the nucleus, where it regulates the expression of hundreds of genes with the subsequent activation and repression responses (Figure 10.48). Activation of TGF-β signaling may also be achieved through Smad-independent pathways that were discussed previously, such as those mediated by the Ras–Raf–Erk MAP kinase, PI3 kinase–AKT, JNK, and p38 MAP kinase pathways, through mechanisms that are involved in the pro-oncogenic responses to TGF-β. Through these Smad-independent pathways, TGF-β can regulate the expression of a wide range of genes inducing other signaling cascades.

When this signaling is corrupted in tumor cells, it can overcome the functional immune response through the suppression of T cell function, allowing these cells to escape the cytotoxic T lymphocyte (CTL)-mediated clearance. In this situation, the administration of inhibitors of the pathway may increase the recognition and destruction of tumor cells by the immune system. In addition to this immunosuppressive effect, cells become more invasive and undergo epithelial–mesenchymal transdifferentiation in response to TGF-β through a combination of Smad-dependent transcriptional events and Smad-independent effects on cell junction complexes.[274,275] Once the tumor has converted to a mesenchymal phenotype, TGF-β promotes tumor progression and metastasis. TGF-β can also induce angiogenesis through upregulation of VEGF, enhance the adherence of tumor cells to the endothelium and facilitate their extravasation, stimulate the expression of genes such as the osteoclast differentiation factor interleukin-11 and the angiogenic connective tissue growth factor that promote osteolytic bone metastasis.[276] For these reasons, drugs targeting the TGF-β pathway are interesting as metastasis inhibitors.

Three approaches to inhibit the TGF-β signaling pathway have been investigated to date. The more advanced TGF-β signaling antagonists are large molecules (antisense oligonucleotides and monoclonal

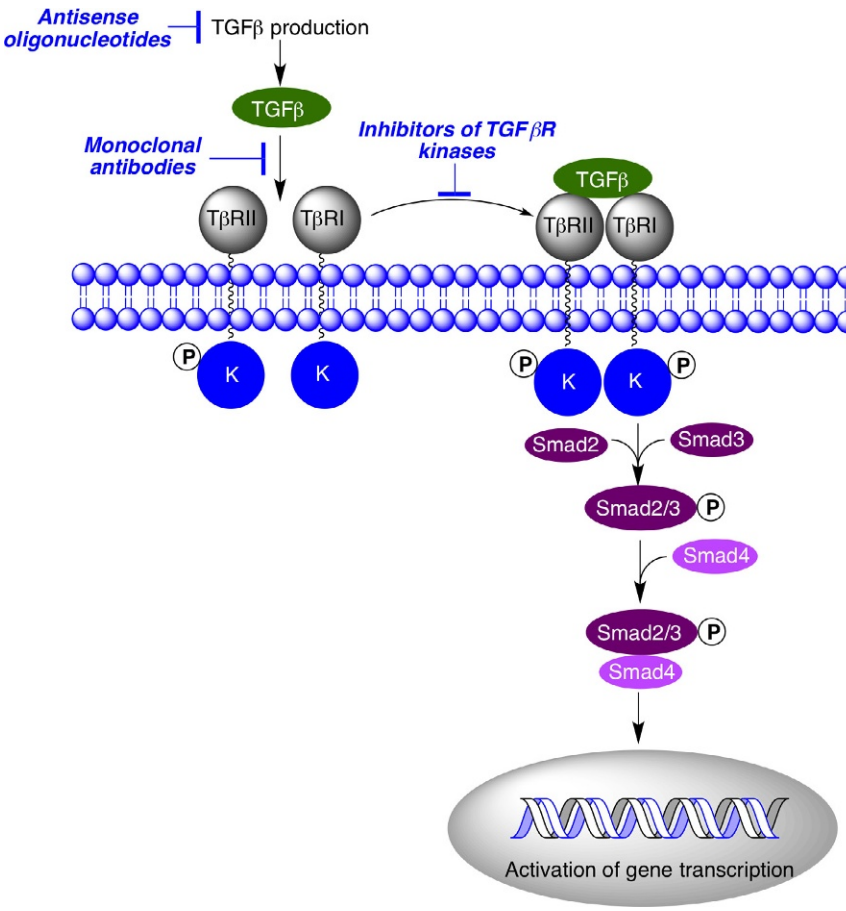


FIGURE 10.48

TGF-β–Smad signaling.

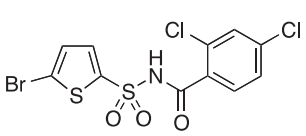
antibodies), most of which are being developed for treatment of fibrotic disorders. Some compounds have already been shown to be efficacious in limiting tumor invasion and metastasis *in vivo*. The dual role of TGF-β as a tumor suppressor and a tumor promoter has been a major concern in deciding if an inhibitor of TGF-β and/or its downstream signaling pathway would be beneficial in the treatment of cancer. Therefore, the main challenge in these approaches is to identify the group of patients in whom targeted tumors are not only refractory to TGF-β-induced tumor suppressor functions but also responsive to the tumor-promoting effects of TGF-β.[277]

Antisense oligonucleotides (ASOs) can induce direct or indirect inhibition of TGF-β secretion. Trabedersen (AP-12009) is a specific phosphorothioate ASO directed against the mRNA of TGF-β_2 that entered phase III studies for several advanced cancers,[278] and AP-11014 is a TGF-β_1-specific ASO in advanced preclinical development aiming at the treatment of NSCLC, colorectal, and prostate carcinomas (where TGF-β_1 rather than TGF-β_2, is overexpressed).[279] TGF-β_2 antisense-modified allogenic

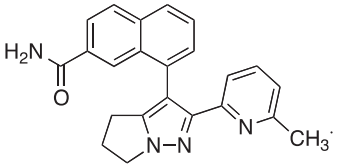
tumor cell vaccines that enhance tumor antigen recognition, such as Glionix® and Lucanix®, have also been studied.[280,281]

Monoclonal antibodies (mAbs) are particularly effective for inhibiting the TGF-β/receptor binding. Lerdelimumab (CAT-152, Trabio®)[282] and metelimumab (CAT-192) are IgG4 mAbs directed against TGF-β_2 and TGF-β_1 that have entered phases III and II, respectively. Both mAbs were awarded orphan drug status in Europe for the prevention of postoperative scarring following glaucoma surgery (lerdelimumab) and for the treatment of scleroderma (metelimumab). However, the development of lerdelimumab was stopped in 2005, and metelimumab was dropped from further development in favor of fresolimumab (GC-1008), a human pan-TGF-β monoclonal antibody directed against all three isoforms of TGF-β in clinical investigation for advanced metastatic melanoma or renal cell carcinoma. Another pan-TGF-β monoclonal antibody is 2G7.[283]

Small-molecule inhibitors of TGF-β kinases,[284] the best-studied compounds, target the kinase domain of TβR-I (ALK5), which differs considerably from that of TβR-II.[285] The molecular scaffolds of known inhibitors contain H-bond acceptors that form specific bonds with His-283, Lys-232, Glu-245, and Tyr-249, in some cases with the intermediacy of a water molecule.[286] Among a large number of compounds that have been preclinically evaluated, tasisulam (LY573636) was clinically studied in patients with malignant melanoma, soft tissue sarcoma, NSCLC, and ovarian cancer, but its development for metastatic melanoma was suspended.[287] The dihydropyrrolopyrazole derivative LY2157299[288] has entered phase I/II trials for advanced/metastatic cancers.



Tasisulam (LY573636)



LY2157299

8 GLUCOSE METABOLISM AND CANCER: INHIBITORS OF KINASES INVOLVED IN ANAEROBIC GLYCOLYSIS

Cancer is commonly related to impaired mitochondrial respiration, activation of key oncogenes, and hypoxic tumor microenvironment induced by activation of the transcriptional activator hypoxia-inducible factor-1 (HIF-1). In normal cells, the uptake of nutrients from the environment is controlled by fine-tuned mechanisms regulated by growth factor signals that bind and stimulate receptor tyrosine kinases (RTKs), which can activate both PI3K–Akt and Ras–ERK signaling pathways.[289] Cancer cells overcome this growth factor dependence by acquiring genetic mutations that reprogram their metabolism. This rewiring for growth makes tumors more vulnerable to nutrient deprivation, and cancer-promoting mutations result in addiction to nutrients, particularly glucose. Nutrients also modify the epigenome through metabolic intermediates such as acetyl-CoA, *S*-adenosylmethionine, nicotinamide adenine dinucleotide (NAD^+), and α-ketoglutarate (α-KG). Caloric restriction inhibits tumorigenesis possibly through reduced IGF-1 levels, although this inhibition may be related to AMPK activation. An understanding of tumor metabolism could give place to a new class of drugs that target altered metabolism in cancer cells.[290]

In mitochondrial glucose oxidation (oxidative phosphorylation, OXPHOS), the main pathway for energy metabolism in normal cells, electrons are transferred from electron donors to electron acceptors such as oxygen in redox reactions that release energy used to form ATP. It is a highly efficient way of releasing energy, which results in the complete oxidation of glucose to CO_2 and water, compared to alternative fermentation processes such as anaerobic glycolysis, which takes place in the cytoplasm and does not require the presence of oxygen. Glycolysis is a metabolic pathway that generates the energy required to form ATP and NADH by the degradation of glucose into pyruvate. One alternative to glycolysis is the pentose phosphate pathway, which converts glucose-6-phosphate into NADPH and ribose-5-phosphate that can be used in the synthesis of nucleotides and amino acids, whereas glutaminolysis is the conversion of glutamine to glutamate and ammonium catalyzed by the enzyme glutaminase (GLS1), followed by conversion of glutamate to α-KG (Figure 10.49).

In the mitochondrial matrix, the enzyme pyruvate dehydrogenase kinase (PDK) regulates the activity of the pyruvate dehydrogenase complex in aerobic conditions. This complex converts pyruvate into acetyl-CoA, which is then oxidized in the mitochondria to carbon dioxide and water through the citric acid cycle and the respiratory chain, ultimately generating up to 36 ATP molecules. Under anaerobic conditions, pyruvate is converted to lactate and regenerates oxidized NAD^+.

In addition to energy production, the glycolysis process provides vitally important precursors for the synthesis of amino acids, fatty acids, and nucleotides, which are required for macromolecular synthesis and, ultimately, cell proliferation. Because of these advantages, cancer cells depend more on glycolysis than on oxidative phosphorylation. For this reason, inhibition of glycolysis is an effective strategy to kill cancer cells and overcome drug resistance associated with mitochondrial defects and hypoxic conditions. The glycolytic enzymes hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) catalyze the three irreversible reactions of glycolysis (Figure 10.50).

The HIF-1 factor, aided by the *c-myc*, *c-ras*, and *c-src* oncogenes, induces gene expression of glucose transporter 1 and 3 (GLUT1/3), hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 and 4 (PFKFB3/4), pyruvate kinase M2 (PKM2), and lactate dehydrogenase (LDH). Expression of these proteins leads to the so-called Warburg phenotype, first documented and theorized by Otto Warburg: Due to a mitochondrial respiratory defect, cancer cells have a much higher rate of glycolysis. Despite the lower net energy yield of aerobic glycolysis compared with oxidative phosphorylation, the energy production is much faster because the tricarboxylic acid cycle produces reactive oxygen species. A decreased reliance on oxygen for energy generation also ensures cancer cell survival and growth under oxygen-limited conditions, and an accelerated glycolysis provides the necessary precursors for the biosynthesis of nucleotides and lipids that are essential for cell division and proliferation. Another advantage for the tumor is that the enhanced lactate production lowers intracellular and extracellular pH levels of tumor tissues, causing the apoptosis of neighboring normal cells, tumor invasion enhancement, and resistance against the immune system and cancer drugs.

The Warburg effect has a clinical application in the diagnosis and monitoring of cancers by positron emission tomography using the radioactive glucose analog [2-^{18}F]-2-deoxyglucose as the probe, which allows for locating sites of high glucose uptake. However, significant therapeutics have begun to be developed only recently. 2-Deoxy-D-glucose (2-DG) has the 2-hydroxy group of glucose replaced by hydrogen so that it cannot undergo further glycolysis and its accumulation within the cells results in glycolysis inhibition. 2-DG entered I/II clinical trials in combination with docetaxel in advanced solid tumors, but its further development is compromised by the fact that its tolerated dose produces cardiac side effects through the prolongation of the Q-T interval.[291]

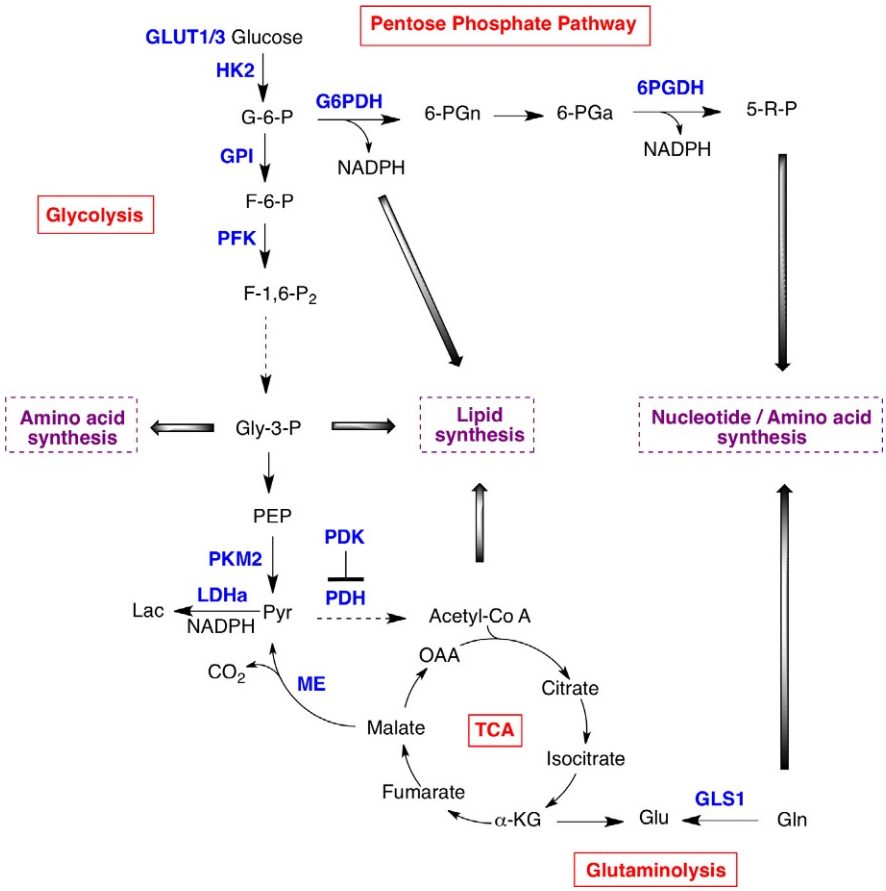


FIGURE 10.49

Some biochemical pathways involved in cancer cell metabolism. **Glycolysis**: GLUT, glucose transporter; HK2, hexokinase 2; G-6-P, glucose-6-phosphate; GPI, phosphoglucose isomerase; F-6-P, fructose-6-phosphate; PFK, phosphofructokinase; F-1,6-P_2, fructose-1,6-diphosphate; Gly-3-P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PKM2, pyruvate kinase M2; Pyr, pyruvate; LDHa, lactate dehydrogenase; Lac, lactate; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase. **Pentose phosphate pathway**: G-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; 6-PGn, 6-phosphogluconolactone; 6-PGa, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase; R-5-P, ribose-5-phosphate. **TCA cycle** (tricarboxylic acid cycle or citric acid cycle): α-KG, α-ketoglutarate; OAA, oxalacetate.

Lonidamine is a derivative of indazole-3-carboxilic acid that suppresses glycolysis in cancer cells, leading to a decrease in cellular ATP. It is in clinical trials in combination with other anticancer agents for the treatment of different types of cancer.[292] It probably acts through the inhibition of the mitochondrially bound hexokinase HKII, leading to a decrease in cellular ATP. Another potent inhibitor of HKII that triggers cell death, supposedly through depletion of cellular ATP, is 3-bromopyruvate.

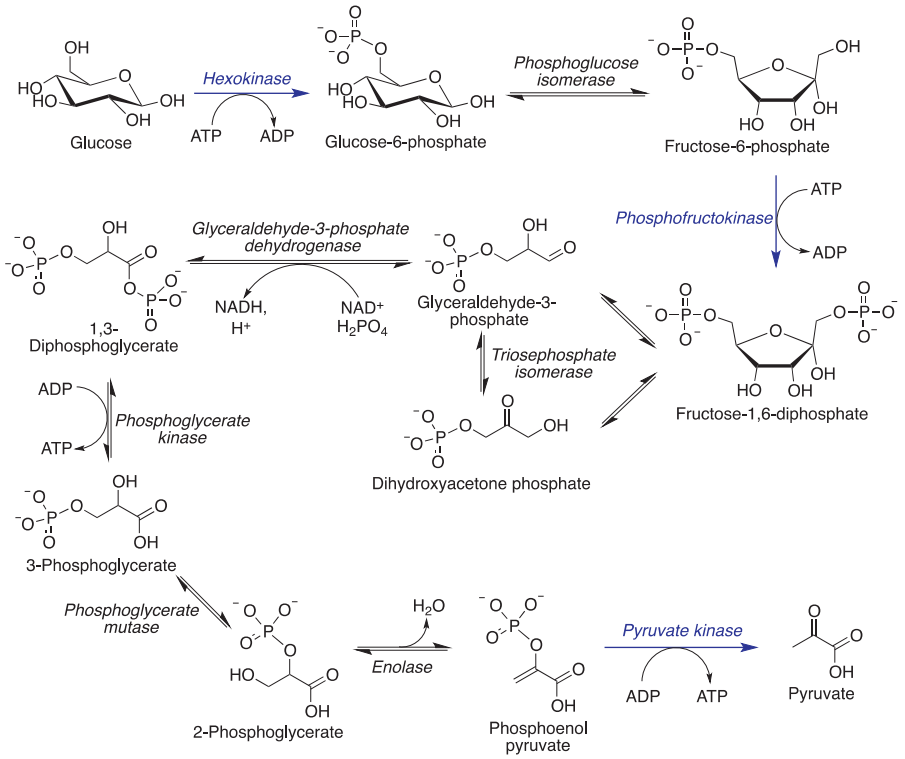


FIGURE 10.50

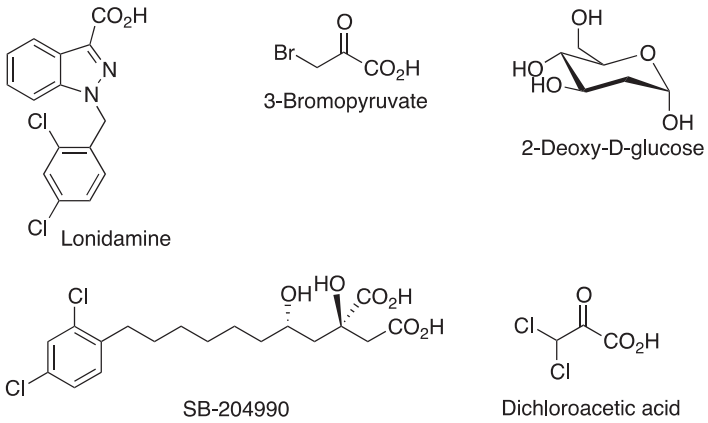
The anaerobic glycolytic pathway.

This compound causes a covalent modification of the sulfhydryl group of cysteine residues of the mentioned enzyme, triggering its dissociation from mitochondria. The subsequent release of apoptosis-inducing factor from the mitochondria to cytosol induces cell death.[293] 3-Bromopyruvate and its propyl ester also inhibit the enzyme glyceraldehyde-3-phosphate dehydrogenase,[294] thereby increasing its potential as an anticancer agent.

PFK is extremely sensitive to small changes in pH, increasing its activity at high pH values. For this reason, inhibitors of the Na^+/H^+ exchange reducing the intracellular pH, such as amiloride or 5,5-dimethylamiloride, may produce anticancer effects.

Pyruvate is required for glucose oxidation. PDK inhibits the flow of pyruvate into the mitochondria and forces the cell to resort to glycolysis, even if oxygen is available. On the other hand, if PDK is inactive, pyruvate is transported into the mitochondria. For this reason, inhibitors of PDK, such as dichloro acetate (DCA), may inhibit glycolysis and produce anticancer effects.[295] The anticancer activity of DCA has been studied in several cancers,[296] including multiple myeloma,[297] but its utility is uncertain because it is a potential carcinogen and may also induce peripheral neuropathy.[298]

ATP citrate lyase (ACL), which catalyzes the conversion of citrate to cytosolic acetyl-CoA, is a key enzyme linking glucose metabolism to lipid synthesis. SB-204990, an inhibitor of this enzyme, limits proliferation and survival of tumor cells displaying aerobic glycolysis.[299]



An integrated approach between cancer metabolism and associated epigenetic modifications is expected to make a valuable contribution to the understanding of cancer and to the development of effective therapeutics in the near future. Altered metabolism in cancer cells is suspected to cause changes in patterns of epigenetic modifications because some metabolic intermediates, such as acetyl-CoA, *S*-adenosylmethionine, NAD^+, and α-KG, can cause epigenetic changes.

REFERENCES

1 Hanahan D, Weinberg RA. *Cell* 2000;**100**:57.
2 Malumbres M, Barbacid M. *Nat Rev Cancer* 2002;**3**:7.
3 Ljungman M, Lane DP. *Nature Rev Cancer* 2004;**4**:727.
4 (a) Ciriello G, Cerami E, Sander C, Schultz N. *Genome Res* 2012;**22**:398; (b) Vogelstein B, Papadopoulos N, Velculescu VI, Zhou S, Díaz LA, Kinzler KW. *Science* 2013;**339**:1546.
5 Dancey J, Sausville EA. *Nature Rev Drug Discov* 2003;**2**:296.
6 Collins I, Workman P. *Curr Signal Transduct Ther* 2006;**1**:13.
7 Kéri G, Örfi L, Erös D, Hegymegi-Barakonyi B, Szántai-Kis C, Horváth Z, et al. *Curr Signal Transduct Ther* 2006;**1**:67.
8 Guillemard V, Saragovi HU. *Curr Cancer Drug Targets* 2004;**4**:313.
9 Williams SP, Kuyper LF, Pearce KH. *Curr Opin Chem Biol* 2005;**9**:371.
10 Fabbro D, McCormick F, editors. *Protein tyrosine kinases. From inhibitors to useful drugs*. Totowa, NJ: Humana; 2006.
11 Klein S, Levitzki A. *Curr Signal Transduct Ther* 2006;**1**:1.
12 Lainey E, Thépot S, Bouteloup C, Sébert M, Adès L, Tailler M, et al. *Biochem Pharmacol* 2011;**82**:1457.
13 Hause RJ, Leung KK, Barkinge JL, Ciaccio MF, Chuu CP, Jones RB. *PLoS One* 2012;**14**:486.
14 Tiseo M, Loprevite M, Ardizzoni A. *Curr Med Chem Anticancer Agents* 2004;**4**:139.
15 Pozo N, Zahonero C, Fernández P, Liñares JM, Ayuso A, Hagiwara M, et al. *J Clin Invest* 2013;**123**:2475.
16 Becker W, Sippl W. *FEBS J* 2011;**278**:246.
17 Barker AJ, Gibson KH, Grundy W, Godfrey AA, Barlow JJ, Healy MP, et al. *Bioorg Med Chem Lett* 2001;**11**:1911.
18 Barker AJ. In: Campbell MM, Blagbrough IS, editors. *Medicinal chemistry into the millennium*. London: Royal Society of Chemistry; 2001. p. 140.

19 Herbst RS, Fukuoka M, Baselga J. *Nature Rev Cancer* 2004;**4**:956.
20 Muhsin M, Graham J, Kirkpatrick P. *Nature Rev Drug Discov* 2003;**2**:515.
21 Hedge S, Schmidt M. *Annu Rep Med Chem* 2005;**40**:443.
22 Dowell J, Minna JD, Kirkpatrick P. *Nature Rev Drug Discov* 2005;**4**:13.
23 Rosell R, Ichinose Y, Taron M, Sarries C, Queralt C, Méndez P, et al. *Lung Cancer* 2005;**50**:25.
24 Camidge DR. *Lancet Oncol* 2013;**14**:913.
25 Rusnack DW, Affleck K, Cockerill SG, Stubberfield C, Harris R, Page M, et al. *Cancer Res* 2001;**61**:7196.
26 Stamos J, Sliwkowski MX, Eigenbrot C. *J Biol Chem* 2002;**277**:46265.
27 Clark J, Cools J, Gilliland DG. *PLoS Med* 2005;**2**:195.
28 Barf T, Kaptein A. *J Med Chem* 2012;**55**:6243.
29 Nyati MK, Maheshwari D, Hanasoge S, Sreekumar A, Rynkiewicz SD, Chinnaiyan AM, et al. *Clin Cancer Res* 2004;**10**:691.
30 Erlichman Ch, Hidalgo M, Boni JP, Martins P, Quinn SE, Zacharchuk Ch, et al. *J Clin Oncol* 2006;**24**:2252.
31 Wissner A, Mansour TS. *Arch Pharm Chem Life Sci* 2008;**341**:465.
32 Minkovsky N, Berezov A. *Curr Opin Invest Drugs* 2008;**9**:1336.
33 Tsou HR, Mamuya N, Johnson BD, Reich MF, Gruber BC, Ye F, et al. *J Med Chem* 2001;**44**:2719.
34 Walter AO, Sjin RTT, Haringsma HJ, Ohashi K, Sun J, Lee K, et al. *Cancer Discov* 2013;**3**:1404.
35 Cross DAE, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. *Cancer Discov* 2014;**4**:1046.
36 Lu H, Li X, Luo Z, Liu J, Fan Z. *Mol Cancer Ther* 2013;**12**:2187.
37 Rowinsky EK, Schwartz GH, Gollob JA, Thompson JA, Vogelzang NJ, Figlin R, et al. *J Clin Oncol* 2004;**22**:3003.
38 Fury MG, Lipton A, Smith KM, Winston CB, Pfister DG. *Cancer Immunol Immunother* 2008;**57**:155.
39 Babu KG, Viswanath L, Reddy BK, Shenoy K, Shenoy A, Naveen T, et al. *J Clin Oncol* 2010;**28**:15s.
40 Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. *Semin Oncol* 1999;**26**:60.
41 Han S-W, Cha Y, Paquet A, Huang W, Weidler J, Lie Y, et al. *PLoS One* 2012;**7**:e39943.
42 Aurisicchio L, Marra E, Roscilli G, Mancini R, Ciliberto G. *Oncotarget* 2012;**3**:744.
43 Göstring L, Malm M, Höidén-Guthenberg I, Frejd FY, Stahl S, Löblom J, et al. *PLoS One* 2012;**7**:e40023.
44 Zumsteg A, Caviezel Ch, Pisarky L, Striimatter K, García-Echeverría C, Hofmann F, et al. *Mol Cancer Res* 2012;**10**:800.
45 Nahta R, Yuan LXH, Zhang B, Kobayashi R, Esteva FJ. *Cancer Res* 2005;**65**:11118.
46 Negi A, Ramarao P, Kumar R. *Mini Rev Med Chem* 2013;**13**:653.
47 Wolf S, Lorenz J, Mössner J, Wiedmann M. *World J Gastroenterol* 2010;**16**:156.
48 Haisa M. *J Int Med Res* 2013;**41**:253.
49 Allison M. *Nature Biotechnol* 2012;**30**:906.
50 Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. *Clin Cancer Res* 2009;**15**:2207.
51 (a) Liu X, Wang Q, Yang G, Marando C, Koblish HK, Hall LM, et al. *Clin Cancer Res* 2011;**17**:7127; (b) Keir ST, Roskoski MA, Wagner S, Tiedt R, Bigner DD, Friedman HS. *Cancer Res* 2013;**73**:2079.
52 For reviews of small-molecule inhibitors of VEGFR signaling, see. (a) Rakesh KJ, Duda DG, Clark JW, Loeffler JS. *Nature Clin Practice Oncol* 2006;**3**:24; (b) Ivy SP, Wick JY, Kaufman BN. *Nature Rev Clin Oncol* 2009;**6**:569.
53 Mendel DB, Laird AD, Smolich BD, Blake RA, Liang C, Hannah AL, et al. *Anticancer Drug Des* 2000;**15**:29.
54 Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, et al. *Cancer Res* 2000;**60**:4152.
55 Abrams TJ, Murray LJ, Pesenti E, Holway VW, Colombo T, Lee LB, et al. *Mol Cancer Ther* 2003;**2**:1011.
56 Akin C. *J Mol Diagn* 2006;**8**:412.
57 Miettinen M, Lasota J. *Appl Immunohistochem Mol Morphol* 2005;**13**:205.
58 Blay JY, Reichardt P. *Expert Rev Anticancer Ther* 2009;**9**:831.
59 Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, et al. *Cancer Res* 2000;**60**:2178.
60 Scott EN, Meinhardt G, Jacques Ch, Laurent D, Thomas AL. *Expert Opin Invest Drugs* 2007;**16**:367.
61 Joensuu H, De Braud F, Grignagni G, De Pas T, Spitalien G, Coco P, et al. *Br J Cancer* 2011;**104**:1686.

62 Kubota K, Ichinose Y, Scagliotti G, Spigel D, Kim JH, Shinkai T, et al. *Ann Oncol* 2014;**25**:529.
63 Manley PW, Bold G, Fendrich G, Furet P, Mestan J, Meyer T, et al. *Cell Mol Biol Lett* 2003;**8**:532.
64 Wells SA, Robinson BG, Gagel RF, Dralle H, Fagin JA, Santoro M, et al. *J Clin Oncol* 2012;**30**:134.
65 Hennequin LF, Stokes ES, Thomas AP, Johnstone C, Ple PA, Ogilvie DJ, et al. *J Med Chem* 2002;**45**:1300.
66 Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, et al. *Cancer Res* 2005;**65**:4389.
67 Hedgethorne K, Huang PH. *Drugs Future* 2010;**35**:893.
68 https://clinicaltrials.gov/ct2/results?term=Foretinib+.
69 Cainap C, Qin S, Huang W-T, Chung I-J, Pan H, Cheng Y, et al. *J Clin Oncol* 2013;**31**(Suppl. 4), abstr 249.
70 Sleijfer S, Ray-Coquard I, Papai Z, Le Cesne A, Scurr M, Schöffski P, et al. *J Clin Oncol* 2009;**27**:3126.
71 Rini B, Campbell SC, Rathmell WK. *Curr Opin Oncol* 2006;**18**:289.
72 Rugo HS, Herbst RS, Liu G, Park JW, Kies MS, Steinfeldt HM, et al. *J Clin Oncol* 2005;**23**:5474.
73 Gori B, Ricciardi S, Fulvi A, Intagliata S, Del Signore E, de Marinis F. *Ther Clin Risk Manag* 2011;**7**:429.
74 Gingrich DE, Reddy DR, Iqbal MA, Singh J, Aimone LD, Angeles TS, et al. *J Med Chem* 2003;**46**:5375.
75 Ferrara N, Hillari KJ, Gerber H-P, Novotny W. *Nature Rev Drug Discov* 2004;**3**:391.
76 Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, et al. *N Engl J Med* 2011;**365**:2473.
77 Wang TF, Lockhart AC. *Clin Med Insights Oncol* 2012;**6**:19.
78 Weng DE, Usman N. *Curr Oncol Rep* 2001;**3**:141.
79 Morrow PK, Murthy RK, Ensor JD, Gordon GS, Margolin KA, Elias AD, et al. *Cancer* 2012;**118**:4098.
80 https://www.clinicaltrials.gov/ct/gui/show/NCT00066768?order=2.
81 Ahles TA, Herndon JE, Small EJ, Vogelzang NJ, Kornblith AB, Ratain MJ, et al. *Cancer* 2004;**15**:2202.
82 André F, Bachelot T, Campone M, Dalenc F, Pérez-García JM, Hurvitz SA, et al. *Clin Cancer Res* 2013;**19**:3963.
83 (a) Wesche J, Haglund K, Haugsten EM. *Biochem J* 2011;**437**:199; (b) Guagnano V, Kauffmann A, Wöhrle S, Stamm C, Ito M, Barys L, et al. *Cancer Discov* 2012;**2**:1118.
84 Trudel S, Stewart AK, Rom E, Wei E, Li ZH, Kotzer S, et al. *Blood* 2006;**107**:4039.
85 Qing J, Du X, Chen Y, Chan P, Li H, Wu P, et al. *J Clin Invest* 2009;**119**:1216.
86 Kelly LM, Yu JC, Boulton CL, Apatira M, Li J, Sullivan CM, et al. *Cancer Cell* 2002;**1**:421.
87 Cheng Y, Paz K. *IDrugs* 2008;**11**:46.
88 Shepard DR, Cooney MM, Elson P, Bukowski RM, Dreicer R, Rini BI, et al. *Invest New Drugs* 2012;**30**:364.
89 Smith BD, Levis M, Beran M, Giles F, Kantarjian H, Berg K, et al. *Blood* 2004;**103**:3669.
90 Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, et al. *Blood* 2005;**105**:54.
91 Díaz T, Navarro A, Ferrer G, Gel B, Gaya A, Artells R, et al. *PLoS One* 2011;**6**:e18856.
92 Manley PW, Cowan-Jacob SW, Mestan J. *Biochim Biophys Acta* 2005;**1754**:3.
93 Capdeville R, Buchdunger E, Zimmermann J, Matter A. *Nature Rev Drug Discov* 2002;**1**:493.
94 Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon NB. *Bioorg Med Chem Lett* 1997;**7**:187.
95 Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. *Science* 2000;**289**:1938.
96 (a) Nagar B, Bornmann W, Pellicena P, Schindler T, Veach DR, Miller WT, et al. *Cancer Res* 2002;**62**:4236; (b) Cowan-Jacob SW, Fendrich G, Floersheimer A, Furet P, Liebetanz J, Rummel G, et al. *Acta Crystall D Biol Crystall* 2007;**D63**:80.
97 Nowakowski J, Sridhar V, Thompson DA, Cronin CN, Vaughn DE, Gangloff AR, et al. *Cell Mol Biol Lett* 2003;**8**:556.
98 Pricl S, Fermeglia M, Ferrone M, Tamborini E. *Mol Cancer Ther* 2005;**4**:1167.
99 McLean SR, Gana-Weisz M, Hartzoulakis B, Frow R, Whelan J, Selwood D, et al. *Mol Cancer Ther* 2005;**4**:2008.
100 Weisberg E, Manley P, Mestan J, Cowan-Jacob S, Ray A, Griffin JD. *Br J Cancer* 2006;**94**:1765.
101 Von Bubnoff N, Manley PW, Mestan J, Sanger J, Peschel C, Duyster J. *Blood* 2006;**108**:1328.
102 Huang WS, Metcalf CA, Sundaramoorthi R, Wang Y, Zou D, Thomas RM, et al. *J Med Chem* 2010;**53**:4701.
103 Chandra J, Tracy J, Loegering D, Flatten K, Verstovsek S, Beran M, et al. *Blood* 2006;**107**:2501.

104 Gumireddy K, Baker SJ, Cosenza SC, John P, Kang AD, Robell KA, et al. *Proc Natl Acad Sci U S A* 2005;**102**:1992.
105 Gambacorti-Passerini C, Gasser M, Ahmed S, Assouline S, Scapozza L. *Leukemia* 2005;**19**:1267.
106 Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. *Science* 2004;**305**:399.
107 Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, et al. *N Engl J Med* 2010;**362**:2260.
108 Cortes JE, Kim DW, Kantarjian HM, Brümmendorf TH, Dyagil I, Griskevicus L, et al. *J Clin Oncol* 2011;**38**:7522.
109 Summy JM, Gallick GE. *Clin Cancer Res* 2006;**12**:1398.
110 Gucalp A, Sparano JA, Caravelli J, Santamauro J, Patil S, Abbruzzi A, et al. *Clin Breast Cancer* 2011;**11**:306.
111 Cortés J, Lipton JH, Rea D, Digumarti R, Chuah C, Nanda N, et al. *Blood* 2012;**120**:2573.
112 For a review, see Puig de la Bellacasa R, Karachaliou N, Estrada-Tejedor R, Teixidó J, Costa C, Borrell JI. *Transl Lung Cancer Res* 2013;**2**:72.
113 For reviews, see; (a) Kinoshita K, Oikawa N, Tsukuda T. *Annu Rep Med Chem* 2012;**47**:281; (b) Awad MM, Shaw AT. *Clin Adv Hematol Oncol* 2014;**12**:429
114 Cui JJ, Tran-Dubé M, Shen H, Nambu M, Kung PP, Pairish M, et al. *J Med Chem* 2011;**54**:6342.
115 For a review of the design and discovery of ALK inhibitors, see Wang W-C, Shiao H-Y, Lee C-C, Funga K-S, Hsieh H-P. *Med Chem Commun* 2014;**5**:1266.
116 Shaw AT, Mehra R, Kim DW, Felip E, Chow LQM, Camidge DR, et al. *J Clin Oncol* 2013;**31**(Suppl.), abstr 8010.
117 Kodama T, Tsukaguchi T, Yoshida M, Kondoh O, Sakamoto H. *Cancer Lett* 2014;**351**:215.
118 Johnson TW, Richardson PF, Bailey S, Brooun A, Burke BJ, Collins MR, et al. *J Med Chem* 2014;**57**:4720.
119 For reviews of the role of STAT inhibitors in cancer therapy, see. (a) Fagard R, Metelev V, Souissi I, Baran-Marszak F. *JAK-STAT* 2013;**2**:e22882; (b) Siveen KS, Sikka S, Surana R, Dai X, Zhang J, Kumar AP, et al. *Biochim Biophys Acta* 2014;**1845**:136.
120 Mesa RA, Yasothan U, Kirkpatrick P. *Nature Rev Drug Discov* 2012;**11**:103.
121 Wei CC, Ball S, Lin L, Liu A, Fuchs JR, Li PK, et al. *Int J Oncol* 2011;**38**:279.
122 Damiano J, Rendahl K, Karim Ch, Jeffry U, Wasserman E, Holash J, et al. *Cancer Res* 2011;**71**:doi:http://dx.doi.org/10.1158/1538-7445. AM2011-DDT02-02.
123 Pan Z, Scheerens H, Li SJ, Schultz BE, Sprengeler PA, Burrill LC, et al. *ChemMedChem* 2007;**2**:58.
124 Elledge SJ. *Science* 1996;**274**:1664.
125 Skladanowski A, Bozko P, Sabisz M. *Chem Rev* 2009;**109**:2951.
126 For reviews, see; (a) Senderowicz AM. *Oncogene* 2003;**22**:6609; (b) Galons H, Oumata N, Meijer L. *Expert Opin Ther Pat* 2010;**20**:377; (c) Bose P, Simmons PGL, Grant S. *Expert Opin Invest Drugs* 2013;**22**:723; (d) Galons H, Oumata N, Gloulou O, Meijer L. *Expert Opin Ther Pat* 2013;**23**:945.
127 Schang LM. *Curr Drug Targets Infect Dis* 2005;**5**:29.
128 Prèvel C, Kurzawa L, Van TNN, Morris MC. *Eur J Med Chem* 2014;**88**:74.
129 Blagosklonny MV. *Cell Cycle* 2004;**3**:1537.
130 Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. *N Engl J Med* 2002;**346**:92.
131 http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=13289797.
132 Honma T, Hayashi K, Aoyama T, Hashimoto NT, Fukasawa K, Iwama T, et al. *J Med Chem* 2001;**44**:4615.
133 Raje N, Kumar S, Hideshima T, Roccaro A, Ishitsuka K, Yasui H, et al. *Blood* 2005;**106**:1042.
134 Bach S, Knockaert M, Reinhardt J, Lozach O, Schmitt S, Baratte B, et al. *J Biol Chem* 2005;**280**:31208.
135 Borman S. *Chem Eng News* 2003;**81**:29.
136 Heath EI, Bible K, Martell RE, Adelman DC, LoRusso PM. *Invest New Drugs* 2008;**26**:59.
137 Kamath AV, Chong S, Chang M, Marathe PH. *Cancer Chemother Pharmacol* 2005;**55**:110.
138 Rees DC, Congreve M, Murray CW, Carr R. *Nature Rev Drug Discov* 2004;**3**:660.
139 Blundell TL, Patel S. *Curr Opin Pharmacol* 2004;**4**:490.
140 Congreve M, Chessari G, Tisi D, Woodhead AJ. *J Med Chem* 2008;**51**:3661.
141 Santo L, Vallet S, Hideshima T, Cirstea D, Ikeda H, Pozzi S, et al. *Oncogene* 2010;**29**:2325.

142 Mahadevan D, Plummer R, Squires MS, Rensvold D, Kurtin S, Pretzinger C, et al. *Ann Oncol* 2011;**22**:2137.
143 Fukuoka K, Usuda J, Iwamoto Y, Fukumoto H, Nakamura T, Yoneda T, et al. *Invest New Drugs* 2001;**19**:219.
144 Thiry A, Dogné J-M, Mesereel B, Supuran CT. *Trends Pharmacol Sci* 2006;**27**:566.
145 Dittrich C, Dumez H, Calvert H, Hanauske A, Faber M, Wanders J, et al. *Clin Cancer Res* 2003;**9**:5195.
146 Tsukahara K, Watanabe T, Hata-Sugi N, Yoshimatsu K, Okayama H, Nagasu T. *Mol Pharmacol* 2001;**60**:1254.
147 Smyth JF, Aamdal S, Awada A, Dittrich C, Caponigro F, Schöffski P, et al. *Ann Oncol* 2005;**16**:158.
148 Supuran CT. *Expert Opin Invest Drugs* 2003;**12**:283.
149 Zandvliet AS, Copalu W, Schellens JH, Beijnen JH, Huitema AD. *Drug Metab Dispos* 2006;**34**:1041.
150 Cesur H, Rubinstein I, Pai A, Önyüksel H. *Nanomedicine* 2009;**5**:178.
151 https://www.clinicaltrials.gov/ct2/show/NCT00721409?term=PD+0332991&rank=10.
152 Parry D, Guzi T, Shanahan F, Davis N, Prabhavalkar D, Wiswell D, et al. *Mol Cancer Ther* 2010;**9**:2344.
153 Cirstea D, Hideshima T, Santo L, Eda H, Mishima Y, Nemani N, et al. *Leukemia* 2013;**27**:2366.
154 Rudolph D, Steegmaier M, Hoffmann M, Grauert M, Baum A, Quant J, et al. *Clin Cancer Res* 2009;**15**:3094.
155 Holland AJ, Lan W, Niessen S, Hoover H, Cleveland DW. *J Cell Biol* 2010;**188**:191; (b) Holland AJ, Cleveland DW. *Cancer Cell* 2014;**26**:151.
156 Lohse I, Cao P, Do T, Ibrahimov E, Tsao M-S, Hedley DW. *Mol Cancer Ther* 2013;**12**:B282.
157 Mason JM, Lin DCC, Wei X, Che Y, Yao Y, Kiarash R, et al. *Cancer Cell* 2014;**26**:163.
158 For a review of checkpoint kinase inhibitors as potential anticancer therapies, see Matthews TP, Jones AM, Collins I. *Expert Opin Drug Discov* 2013;**8**:621.
159 Graves PR, Yu LJ, Schwarz JK, Gales J, Sausville EA, O'Connor PM, et al. *J Biol Chem* 2000;**275**:5600.
160 For reviews, see; (a) Prudhomme M. *Anticancer Agents Nat Prod* 2005;**499**; (b) Venditto VJ, Simaneck EE. *Mol Pharmacol* 2010;**7**:307v.
161 Fuse E, Kuwabara T, Sparreboom A, Sausville EA, Figg WD. *J Clin Pharmacol* 2005;**45**:394.
162 Christensenm SD, Frankel PH, Margolin KA, Agarwala SS, Luu T, Mack PC, et al. *Invest New Drugs* 2012;**30**:741.
163 Fry MJ. *Breast Cancer Res* 2001;**3**:304.
164 (a) Wymann MP, Zvelebil M, Laffargue M. *Trends Pharmacol Sci* 2003;**24**:366; (b) Granville CA, Memmott RM, Gills JJ, Dennis PA. *Clin Cancer Res* 2006;**12**:679.
165 Franke TF, Kaplan DR, Cantley LC, Toker A. *Science* 1997;**275**:665.
166 Rodon J, Dienstmann R, Serra V, Tabernero J. *Nature Rev Clin Oncol* 2013;**10**:143.
167 Massacesi C, Tomaso E, Fretault N, Hirawat S. *Ann N Y Acad Sci* 2013;**1280**:19.
168 Mukherjee B, Tomimatsu N, Amancherla K, Camacho CV, Pichamoorthy N, Burma S. *Neoplasia* 2012;**14**:34.
169 Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, et al. *Mol Cancer Ther* 2008;**7**:1851.
170 Yuan J, Mehta PP, Yin M-J, Sun S, Zou A, Chen J, et al. *Mol Cancer Ther* 2011;**10**:2189.
171 Cheng H, Li C, Bailey S, Baxi SM, Goulet L, Guo L, et al. *ACS Med Chem Lett* 2013;**4**:91.
172 Wu M, Akinleye A, Zhu X. *J Hematol Oncol* 2013;**6**:36.
173 Karve S, Werner ME, Sukumar R, Cummings ND, Copp JA, Wang EC, et al. *Proc Natl Acad Sci U S A* 2012;**109**:8230.
174 Hong DS, Bowles DW, Falchook GS, Messersmith WA, George GC, O'Bryant CL, et al. *Clin Cancer Res* 2012;**18**:4173.
175 Sato S, Fujita N, Tsuruo T. *Oncogene* 2002;**21**:1727.
176 Komander D, Kular GS, Bain J, Elliott M, Alessi DR, van Aalten DMF. *Biochem J* 2003;**375**:255.
177 Machajewski T, Lin X, Jefferson AB, Gao Z. *Annu Rep Med Chem* 2005;**40**:263.
178 Luo Y, Shoemaker AR, Liu X, Woods KW, Thomas SA, de Jong R, et al. *Mol Cancer Ther* 2005;**4**:977.
179 Posadas EM, Gulley J, Arlen PM, Trout A, Parnes HL, Wright J, et al. *Cancer Biol Ther* 2005;**4**:1133.

180 Chee KG, Longmate J, Quinn DI, Chatta G, Pinski J, Twardowski P, et al. *Clin Genitourin Cancer* 2007;**5**:433.
181 Blake JF, Xu R, Bencsik JR, Xiao D, Kallan NC, Schlachter S, et al. *J Med Chem* 2012;**55**:8110.
182 http://www.clinicaltrials.gov/ct2/results?term=Ipatasertib+OR+GDC-0068&no_unk=Y.
183 Morgan-Lappe S, Woods KW, Li Q, Anderson MG, Schurdak ME, Luo Y, et al. *Oncogene* 2006;**25**:1340.
184 Gills J, Dennis PA. *Curr Oncol Rep* 2009;**11**:102.
185 Jin X, Gossett DR, Wang S, Yang D, Cao Y, Chen J, et al. *Br J Cancer* 2004;**91**:1808.
186 Fang K, Chen S-P, Lin C-W, Cheng W-C, Huang H-T. *Lung Cancer* 2009;**63**:227.
187 Vendome J, Letard S, Martin F, Svinarchuk F, Dubreuil P, Auclair C, et al. *J Med Chem* 2005;**48**:6194.
188 Thompson D, Miller C, McCarthy FO. *Biochemistry* 2008;**47**:10333.
189 Rao RD, Buckner JC, Sarkaria JN. *Curr Drug Targets* 2004;**4**:621.
190 Wullschleger S, Loewith R, Hall MN. *Cell* 2006;**124**:471.
191 Ory B, Moriceau G, Redini F, Heymann D. *Curr Med Chem* 2007;**14**:1381.
192 Vignot S, Faivre S, Aguirre D, Raymond E. *Ann Oncol* 2005;**16**:525.
193 Mita M, Sankhala K, Abdel-Karim I, Mit A, Giles F. *Expert Opin Invest Drugs* 2008;**14**:1947.
194 Ohyashiki JH, Kobayashi Ch, Hamamura R, Okabe S, Tauchi T, Ohyashiki K. *Cancer Sci* 2009;**100**:970.
195 Kobayashi T, Cohen P. *Biochem J* 1999;**339**:319.
196 Sherk AB, Frigo DE, Schnackenberg CG, Bray JD, Laping NJ, Trizna W, et al. *Cancer Res* 2008;**68**:7475.
197 Feng Z, Liu L, Zhang C, Zheng T, Wang J, Lin M, et al. *Proc Natl Acad Sci U S A* 2012;**109**:7013.
198 Andrews PD. *Oncogene* 2005;**24**:5005.
199 Montembault E, Prigent C. *Drugs Future* 2005;**30**:4.
200 Fancelli D, Moll J. *Expert Opin Ther Patents* 2005;**15**:1169.
201 Boss DS, Beijnen JH, Scheellens JHM. *Oncologist* 2009;**14**:780.
202 Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, et al. *Nature Med* 2004;**10**:262.
203 Fei F, Stoddart S, Groffen J, Heisterkamp N. *Mol Cancer Ther* 2010;**9**:1318.
204 Santo L, Hideshima T, Cirstea D, Bandi M, Nelson EA, Gorgun G, et al. *Clin Cancer Res* 2011;**17**:3259.
205 Gontarewicz A, Brümmendorf TH. *Recent Results Cancer Res* 2010;**184**:199.
206 Fancelli D, Berta D, Bindi S, Cameron A, Cappella P, Carpinelli P, et al. *J Med Chem* 2005;**48**:3080.
207 Zhao B, Smallwood A, Yang J, Koretke K, Nurse K, Calamari A, et al. *Prot Sci* 2008;**17**:1791.
208 Jirousek MR, Goekjian PG. *Expert Opin Invest Drugs* 2001;**10**:2117.
209 Virchis A, Ganeshaguru K, Hart S, Jones D, Fletcher L, Wright F, et al. *Hematol J* 2002;**3**:131.
210 Force T, Kuida K, Namchuk M. *Circulation* 2004;**109**:1196.
211 Utz I, Spitaler M, Rybczynska M, Ludescher C, Hilbe W, Regenass U, et al. *Int J Cancer* 1998;**77**:64.
212 PKC-DRS2 Group. *Ophthalmology* 2006;**113**:221.
213 Wick W, Puduvalli VK, Chamberlain MA, van den Bent MJ, Carpentier AF, Cher LM, et al. *J Clin Oncol* 2010;**28**:1168.
214 Evenou JP. *J Pharm Exp Ther* 2009;**330**:792.
215 Madhusudan S, Protheroe A, Propper D, Han C, Corrie P, Earl H, et al. *Br J Cancer* 2003;**89**:1418.
216 Cripps MC, Figueredo AT, Oza AM, Taylor MJ, Fields AL, Holmlund JT, et al. *Clin Cancer Res* 2002;**8**:2188.
217 Nawijn MC, Alendar A, Berns A. *Nature Rev Cancer* 2011;**11**:23.
218 For representative reviews, see. (a) Reuter CW, Morgan MA, Bergmann L. *Blood* 2000;**96**:1655; (b) Adjei AA. *Curr Pharm Des* 2001;**7**:1581; (c) Downward J. *Nature Rev Cancer* 2003;**3**:11; (d) Spiegel J, Cromm PM, Zimmermann G, Grossmann TN, Waldmann H. *Nature Chem Biol* 2014;**10**:613.
219 Rudack T, Xia F, Schlitter J, Köttinga C, Gerwert K. *Proc N Y Acad Sci* 2012;**109**:15295.
220 Morse MA. *Curr Opin Mol Ther* 2001;**3**:589.
221 For representative reviews, see. (a) Russo P, Loprevite M, Cesario A, Ardizzoni A. *Curr Med Chem Anticancer Agents* 2004;**4**:123; (b) Moorthy NS, Sousa SF, Ramos MJ, Fernandes PA. *Curr Med Chem* 2013;**20**:4888

222 Appels NMGM, Beijnena JH, Schellensb JHM. *Oncologist* 2005;**10**:565.
223 Long SB, Hancock PJ, Kral AM, Hellinga HW, Beese LS. *Proc Natl Acad Sci U S A* 2001;**98**:12948.
224 Leonard DM. *J Med Chem* 1997;**40**:2971.
225 Johnston SRD. *Lancet Oncol* 2001;**2**:18.
226 Bell IM. *J Med Chem* 2004;**47**:1869.
227 Le DT, Shannon KM. *Curr Opin Hematol* 2002;**9**:308.
228 Appels MMGM, Bolijn MJ, Chan K, Stephens TC, Hoctin-Boes G, et al. *Br J Cancer* 2008;**98**:1951.
229 Reid TS, Beese LS. *Biochemistry* 2004;**43**:6877.
230 Reid TS, Long SB, Beese LS. *Biochemistry* 2004;**43**:9000.
231 Caponigro F, Casale M, Bryce J. *Expert Opin Invest Drugs* 2004;**12**:943.
232 Santucci R, Mackley PA, Sebti S, Alsina M. *Cancer Control* 2003;**10**:384.
233 Rao S, Cunningham D, de Gramont A, Scheithauer W, Smakal M, Humblet Y, et al. *J Clin Oncol* 2004;**22**:3950.
234 Khuri FR, Glisson BS, Kim ES, Statkevich P, Thall PF, Meyers ML, et al. *Clin Cancer Res* 2004;**10**:2968.
235 Lobell RB, Liu D, Buser CA, Davide JP, DePuy E, Hamilton K, et al. *Mol Cancer Ther* 2002;**1**:747.
236 Laheru D, Shah P, Rajeshkumar NV, McAllister F, Taylor G, Goldsweig H, et al. *Invest New Drugs* 2012;**30**:2391.
237 For a review, see Roskoski R. *Pharmacol Res* 2012;**66**:105.
238 Arthur JSC, Ley SC. *Nature Rev Immunol* 2013;**13**:679.
239 Honma T, Yoshizumi T, Hashimoto N, Hayashi K, Kawanishi N, Fukasawa K, et al. *J Med Chem* 2001;**44**:4628.
240 Kim YS, Jin HO, Seo S-K, Woo SH, Choe T-B, An S, et al. *Biochem Pharmacol* 2011;**82**:216.
241 Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, et al. *N Engl J Med* 2007;**356**:125.
242 Keating GM, Santoro A. *Drugs* 2009;**69**:223.
243 Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, et al. *Nature Rev Drug Discov* 2006;**5**:835.
244 Bollag G, Tsai J, Zhang J, Zhang Ch, Ibrahim P, Nolop K, et al. *Nature Rev Drug Discov* 2012;**11**:873.
245 Swaika A, Crozier JA, Joseph RW. *Drug Des Dev Ther* 2014;**8**:775.
246 Bollag G, Tsai J, Zhang J, Zhang C, Ibrahim P, Nolop K, et al. *Nature Rev Drug Discov* 2012;**11**:873.
247 Rheault TR, Stellwagen JC, Adjabeng GM, Hornberger KR, Petrov KG, Waterson AG, et al. *ACS Med Chem Lett* 2013;**4**:358.
248 http://www.novartis.com/downloads/investors/event-calendar/2012/6-bridging-science-and-patients.pdf.
249 Amiri P, Aikawa ME, Dove J, Stuart DD, Poon D, Pick T, et al. *Proc Am Assoc Cancer Res* 2006;**47**, abstr 4855.
250 For a review of malignant melanoma treatment, seeWebster RM, Mentzer SE. *Nature Rev Drug Discov* 2014;**13**:491.
251 Cripps MC, Figueredo AT, Oza AM, Taylor MJ, Fields AL, Holmlund JT, et al. *Clin Cancer Res* 2002;**8**:2188.
252 Oza AM, Elit L, Swenerton K, Faught W, Ghatage P, Carey M, et al. *Gynecol Oncol* 2003;**89**:129.
253 Frémin Ch, Meloche S. *J Hematol Oncol* 2010;**3**:8.
254 (a) Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, et al. *J Clin Oncol* 2004;**22**:4456.
255 (a) Rice KD, Aay N, Anand NK, ChM Blazey, Bowles OJ, Bussenius J, et al. *ACS Med Chem Lett* 2012;**3**:416; (b) Hoeflich KP, Merchant M, Orr C, Chan J, Den Otter D, Berry L, et al. *Cancer Res* 2012;**72**:210.
256 Daouti S, Wang H, Li WH, Higgins B, Kolinsky K, Packman K, et al. *Cancer Res* 2009;**69**:1924.
257 Doyle MP, Yeh TC, Suzy B, Morrow M, Lee PA, Hughes AM, et al. *J Clin Oncol* 2005;**23**:3075.
258 Flaherty K, Arenberger P, Ascierto PA, De Groot JW, Hallmeyer S, Long GV, et al. *J Clin Oncol* 2014;**32** (Suppl. 5s), abstr TPS9102.
259 Daouti S, Wang H, Li WH, Higgins B, Kolinsky K, Packman K, et al. *Cancer Res* 2009;**69**:1924.
260 (a) Rice KD, Aay N, Anand NK, ChM Blazey, Bowles OJ, Bussenius J, et al. *ACS Med Chem Lett* 2012;**3**:416; (b) Hoeflich KP, Merchant M, Orr C, Chan J, Den Otter D, Berry L, et al. *Cancer Res* 2012;**72**:210.

261 Vakiani E, Solit DB. *J Pathol* 2011;**223**:219.
262 For a review, see Yap JL, Worlikar S, MacKerell AD, Shapiro P, Fletcher S. *ChemMedChem* 2011;**6**:38.
263 Qin J, Xin H, Nickoloff BJ. *J Transl Med* 2012;**10**:15.
264 Shukla A, Hillegass JM, MacPherson MB, Beuschel SL, Vacek PM, Pass HI, et al. *Mol Cancer* 2010;**9**:314.
265 For a review of the role of JNK signaling in apoptosis, see Dhanasekaran DN, Reddy EP. *Oncogene* 2008;**27**:6245.
266 Weston CR, Davis RJ. *Curr Opin Cell Biol* 2007;**19**:142.
267 Wagner EF, Nebreda AR. *Nature Rev Cancer* 2009;**9**:537.
268 Zhang T, Inesta-Vaquera F, Niepel M, Zhang J, Ficarro SB, Machleidt T, et al. *Chem Biol* 2012;**19**:140.
269 Cuadrado A, González L, Suárez Y, Martínez T, Muñoz A. *Oncogene* 2004;**23**:4673.
270 Tate CM, Blosser W, Wyss L, Evans G, Xue Q, Pan Y, et al. *J Biol Chem* 2013;**288**:6743.
271 Yasuil H, Hideshima T, Ikeda H, Jin J, Ocio EM, Kiziltepe T, et al. *Br J Haematol* 2006;**136**:414.
272 Iwano S, Asaoka Y, Akiyama H, Takizawa S, Nobumasa H, Hashimoto H, et al. *J Appl Toxicol* 2011;**31**:671.
273 Massagué J. *Annu Rev Biochem* 1998;**67**:753.
274 Massagué J. *Cell* 2008;**134**:215.
275 Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL, et al. *PLoS One* 2009;**4**:e4355.
276 Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. *Nature* 2005;**436**:518.
277 Nagaraj NS, Datta PK. *Expert Opin Invest Drugs* 2010;**19**:77.
278 Jaschinski F, Rothhammer T, Jachimczak P, Seitz C, Schneider A, Schlingensiepen KH. *Curr Pharm Biotechnol* 2011;**12**:2203.
279 Saunier EF, Akhurst RJ. *Curr Cancer Drug Targets* 2006;**6**:565.
280 Nemunaitis J, Nemunaitis M, Senzer N, Snitz P, Bedell C, Kumar P, et al. *Cancer Gene Ther* 2009;**16**:620.
281 http://www.cancer.gov/clinicaltrials/search/view?cdrid=613062&version=HealthProfessional.
282 Khaw P, Grehn F, Holló GP, Overton B, Wilson R, Vogel R, et al. *Ophthalmology* 2007;**114**:1822.
283 Muraoka-Cook RS, Dumont N, Arteaga CL. *Clin Cancer Res* 2005;**11**:937s.
284 Tsuchida K, Sunada Y, Noji S, Murakami T, Uezumi A, Nakatani M. *Mini Rev Med Chem* 2006;**6**:1255.
285 Sawyer TK. *Curr Med Chem Anticancer Agents* 2004;**4**:449.
286 Yingling JM, Blanchard KL, Sawyer JS. *Nature Rev Drug Discov* 2004;**3**:1011.
287 Calone I, Souchelnytskyi S. *Exp Oncol* 2012;**34**:9.
288 (a) Bueno L, de Alwis DP, Pitou C. *Eur J Cancer* 2008;**44**:142; (b) http://www.mdanderson.es/investigacion/ensayo-clinico/estudio-fase-1bii-con-gemcitabina-y-ly2157299-en-pacientes-con-cancer-m.
289 (a) Stoltzman CA, Peterson CW, Breen KT, Muoio DM, Billin AN, Ayer DE. *Proc Natl Acad Sci U S A* 2008;**105**:6912; (b) Havula E, Hietakangas V. *Semin Cell Dev Biol* 2012;**23**:640.
290 (a) Vander Heiden MG. *Nature Rev Drug Discov* 2011;**10**:671; (b) Jones NP, Schulze A. *Drug Discov Today* 2012;**17**:232.
291 Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, et al. *Cancer Chemother Pharmacol* 2013;**71**:523.
292 Pelicano H, Martin DS, Xu RH, Huang P. *Oncogene* 2006;**25**:4633.
293 Chen Z, Zhang H, Lu W, Huang P. *Biochim Biophys Acta* 2009;**1787**:553.
294 Cardaci S, Desideri E, Ciriolo MR. *J Bioenerg Biomembr* 2012;**125**:2115.
295 Sutendra G, Michelakis ED. *Front Oncol* 2013;**3**:7.
296 Vella S, Conti M, Tasso R, Cancedda R, Pagano A. *Int J Cancer* 2012;**130**:1484.
297 Sánchez WY, McGee SL, Connor T, Mottram B, Wilkinson A, Whitehead JP, et al. *Br J Cancer* 2013;**108**:1624.
298 (a) Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, et al. *Sci Transl Med* 2010;**2**:31; (b) Flavin DF. *J Oncol* 2010, article ID 414726.
299 Hatzivassiliou G, Zhao F, Bauer DE, Andreadis Ch, Shaw AN, Dhanak D, et al. *Cancer Cell* 2005;**8**:311.

CHAPTER

OTHER NONBIOLOGICAL APPROACHES TO TARGETED CANCER CHEMOTHERAPY

11

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00011-9

1 INTRODUCTION

This chapter discusses targeted anticancer drugs that interact with non-kinase targets such as hydrolytic proteins, adhesion molecules, and other mechanisms involved in angiogenesis and metastasis (Table 11.1). It also discusses Wnt, Notch, and Hedgehog signal pathways and oncogenic protein–protein interactions, including apoptotic signaling pathways, heat shock proteins, and other chaperones.

2 PROTEOLYTIC ENZYMES AS ANTICANCER TARGETS

Proteolytic enzymes, also called proteinases or proteases, break proteins into peptides and eventually into amino acids. These enzymes are essential for most physiological processes, but their overexpression or unregulated activity due to specific mutations in protease genes, protease precursors, protease folding and endogenous protease inhibitors, as well as cofactors, receptors, and transporters, are responsible for several human diseases. Clinically relevant inhibitors of HIV protease, thrombin, angiotensin-converting enzyme, and elastase may serve as examples of therapeutically useful protease inhibitors in other diseases. In the case of cancer, proteases are involved in the primary tumor growth by disruption of protein homeostasis and in the metastatic dissemination of tumor cells, which relies on the proteolytic degradation of extracellular matrix and basement membrane components. For these reasons, protease inhibitors are potential therapeutic tools to treat cancer.[1]

2.1 ANTICANCER DRUGS ACTING ON THE UBIQUITIN–PROTEASOME SYSTEM

Two types of cellular structures are in charge of protein degradation: proteasomes and lysosomes. Lysosomes exert their proteolytic function on transmembrane and extracellular proteins following endocytosis and phagocytosis mechanisms. In eukaryotic cells, protein homeostasis is controlled primarily through the ubiquitin–proteasome system (UPS), which plays an essential role in the degradation of proteins that are misfolded, oxidized, or otherwise damaged during processes such as the cell cycle, transcription, signal transduction, immunoresponses, and development. Many short-lived regulatory proteins are also degraded by the UPS, a process that is altered in some neurodegenerative diseases and especially in cancer.[2] The average human cell contains 20,000–30,000 proteasomes located in the cytoplasm and in the nucleus.

Proteins are targeted for degradation by UPS through a multistep process that involves the attachment of a polyubiquitin chain (ubiquitylation). The process starts by the activation of the carboxylic end of the 76 amino acid polypetide ubiquitin by reaction with ATP, followed by its attachment to a

Table 11.1 Selected Non-kinase Targeted Compounds on the Market or in Clinical Development

Mechanism	Type	Drugs
Inhibition of 26S proteasome	Small molecule	Bortezomib (PS-341, MLN-341, Velcade®) Carfilzomib (Krypolis®) Delanzomib (CEP-18770) Ixazomib (MLN-9708) Oprozomib (ONX-0912) Salinosporamide A (marizomib)
Inhibition of NEDD8-activating enzyme (NAE)	Small molecule	MLN4924
Inhibition of matrix metalloproteases (MMPs)	Small molecule	Batimastat (BB-94) Marimastat Prinomastat (AG3340) MMI270 (CGS 27023A) ABT-518 Tanomastat (BAY-12-9566) S-3304 Rebimastat (BMS-275291) COL-3 (Metastat®) AE-941 (Neovastat®)
Inhibition of aminopeptidases	Small molecule	Ubenimex (Bestatin®) Tosedostat (CHR-2797)
Inhibition of heparanase	Small molecule	Muparfostat (PI-88)
Inhibition of integrins as cellular adhesion molecules	Small molecule	AS101 Cilengitide (EMD 121974) E7820
	Monoclonal antibody	Etaracizumab (Vitaxin®, Abegrin®)
Inhibitors of methionine aminopeptidase 2 (MetAP2)	Nanoparticle	Fumagillin nanoparticles
	Conjugated drug	XMT-1107
Other inhibitors of angiogenesis	Peptides and peptidomimetics acting as endogenous angiogenesis inhibitors	Endostatin Endostar ABT-510
	Small molecule	Squalamine Thalidomide (Thalomid®) Lenalidomide (CC-5013, Revlimid®) Pomalidomide (CC-4047, Imnovid®) TNP-470

Cys residue in the ubiquitin-activating enzyme (UAE, E1) generating an E1–ubiquitin thioester. Ubiquitin is subsequently transferred to an ubiquitin-conjugating enzyme (UBC, E2), and finally, the ubiquitin is conjugated onto the target substrates by E2 alone or, more often, by interacting with substrate-specific ubiquitin E3 ligases that transfer ubiquitin to the substrate protein (Figure 11.1). An E3 ligase may be considered as a tumor suppressor if it degrades a tumor-inducer protein, or as a tumor promoter if it degrades a tumor-suppressor protein.[3]

Polyubiquitinated proteins carrying long chains or "trees" of ubiquitin are recognized by the 26S proteasome, deubiquitinated, unfolded, and degraded by the peptidase activities in the 20S core particle

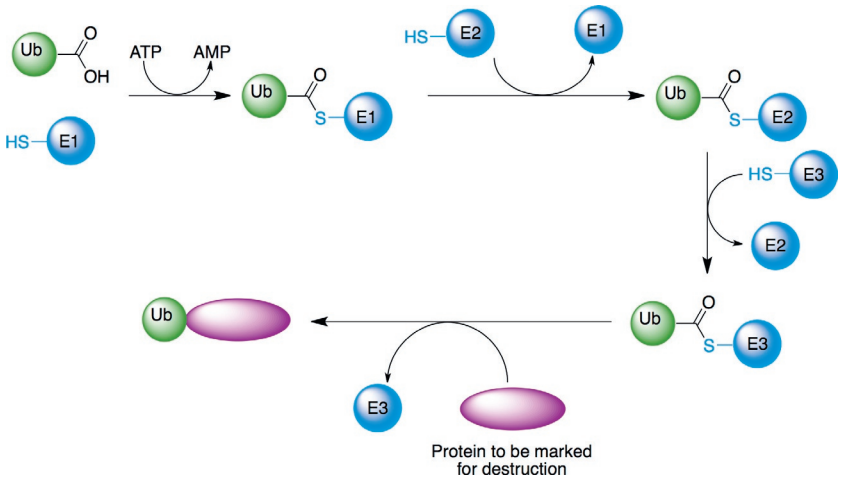


FIGURE 11.1

The ubiquitylation process.

of the proteasome. A key enzyme of this process is the E3 ligase known as anaphase-promoting complex/cyclosome (APC/C; see Section 2.1.3).

The 20S proteasome is a complex of 28 subunits that are organized into four stacked heptameric rings, creating a cylindrical structure (Figure 11.2).[4] The proteins at the top and bottom rings have sequence similarities and are called α subunits, whereas the ones in the two inner rings, three of which perform the enzymatic reaction, are called β subunits.

To be functional *in vivo*, this 20S proteasome must be capped at both ends by the 19S regulatory complex, leading to the 26S proteasome, a 2.4-MDa structure that is the major cellular proteolytic machinery. The 19S regulatory complex, which contains six ATPases and several other polypeptides, recognizes ubiquitinylated proteins and unfolds them, controlling their access to the 26S proteasome core and recycling ubiquitin.

In summary, cells label the proteins to be hydrolyzed by transferring the polypeptide ubiquitin to the ε-NH_2 group of a Lys residue and then attaching more ubiquitin molecules to this covalent adduct, forming ubiquitin chains with different linkages. The Lys-48-linked polyubiquitin chain serves as a recognition marker for the 26S proteasome, which degrades these marked proteins to short peptides (approximately eight amino acids, on average) (Figure 11.3).[5] This degradation is followed by hydrolysis of these peptides by cytoplasmic aminopeptidases, which are also drug targets.

Ubiquitin modification of proteins has also emerged as an important process that regulates cell signaling through proteasome-independent mechanisms. At this respect, bioinformatic and biochemical analyses have identified several ubiquitin-binding domains embedded in a large variety of proteins with diverse cellular functions.[6]

2.1.1 Proteasome Inhibitors in Cancer Therapy

The joint action of ubiquitinylation and the 26S proteasome machinery regulates many cellular functions, including cell cycle progression, development, apoptosis, signal transduction, and antigen presentation. The proteasome is an anticancer target that controls the levels of important regulatory proteins, including

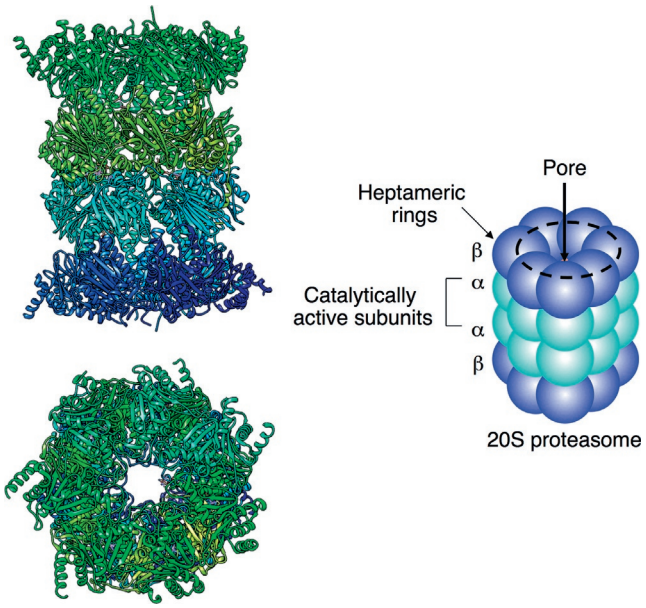


FIGURE 11.2

Structure of the 20S proteasome. The three-dimensional structure, corresponding to the yeast 20S proteasome, was generated from Protein Data Bank reference 1FNT and displayed with Chimera 1.8.1.

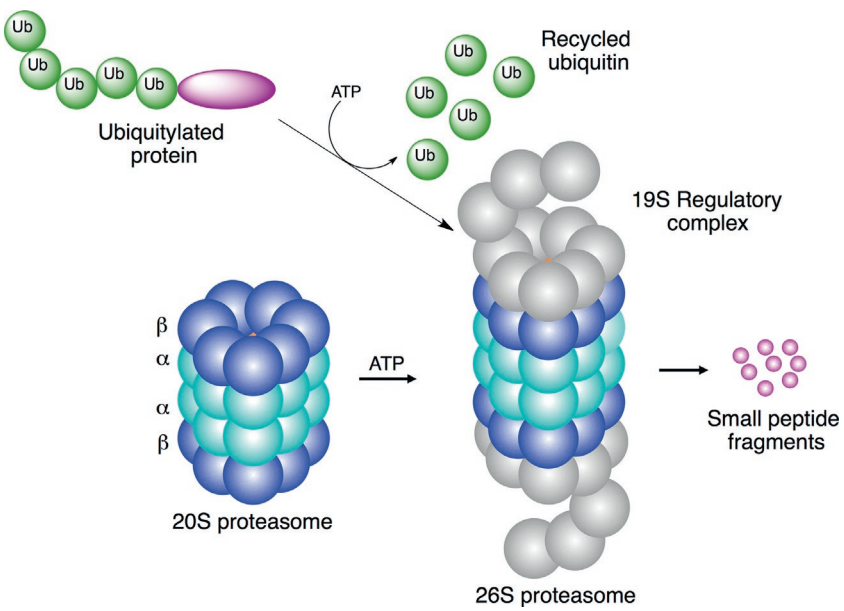


FIGURE 11.3

Structure and function of the proteasome.

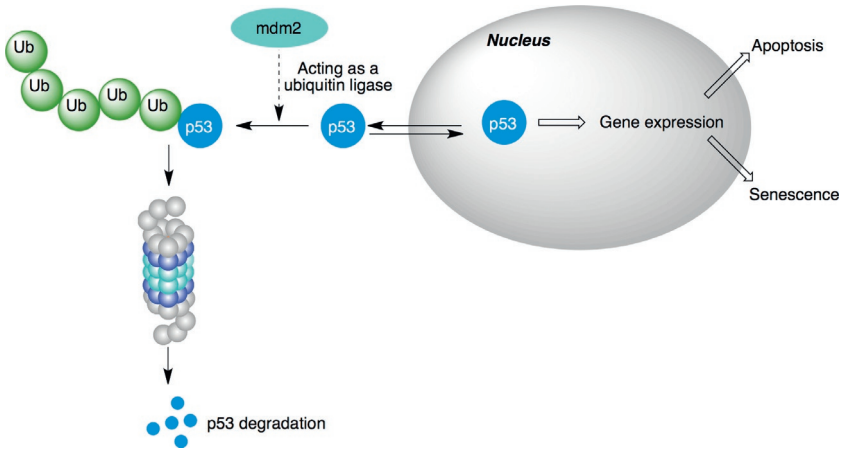


FIGURE 11.4

Negative regulation of p53 by mdm2 and its subsequent degradation by the proteasome.

the tumor-suppressing factor p53 and the nuclear factor κB (NF-κB).[7] Its blocking results in their accumulation, which leads to cell death through a variety of mechanisms. The protein murine double minute 2 homolog (mdm2), also known as hdm2, which is encoded by the *MDM2* oncogene, is the E3 ubiquitin ligase involved in the degradation of p53. For this reason, proteasome inhibitors may provide a good approach to the treatment of tumors that overexpress mdm2 (Figure 11.4). The protein–protein interactions between p53 and hdm2 and their inhibition are discussed in Section 8.1.4.

Proteasome inhibitors may also act as anticancer agents by preventing the expression of pro-survival genes. For instance, NF-κB is a survival factor that is inactivated in the cytoplasm through binding to the IκBα inhibitor protein. Through the intermediacy of membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK), which in turn phosphorylates the I-κBα protein, resulting in the dissociation of IκBα from NF-κB, ubiquitination, and the eventual degradation of IκBα by the proteosome. The NF-κB thus liberated translocates to the nucleus and activates the transcription of a number of factors that protect the cell from apoptosis (Figure 11.5). Therefore, inhibition of proteasome activity avoids the degradation of IκB and prevents the activation of NF-κB, thus promoting apoptosis.

Proteasomes can be described as N-terminal nucleophile (Ntn) hydrolases because the catalytic centers at their β subunits have been identified as N-terminal threonine residues acting as nucleophiles through their hydroxyl groups. The mechanism summarized in Figure 11.6, involving two tetrahedral transition states, has been proposed for the proteasome-catalyzed proteolysis.

Most known proteasome inhibitors (PIs)[8,9] are peptidomimetics containing an electrophilic functional group, normally placed at one end of the molecule, that reacts with the threonine hydroxyl after its activation (see relevant inhibition mechanisms in Figure 11.8). Many of these compounds can be considered as site-directed enzyme inhibitors and bear a close relationship with inhibitors of serine proteases (e.g., HIV protease), as shown in the representative examples given here.

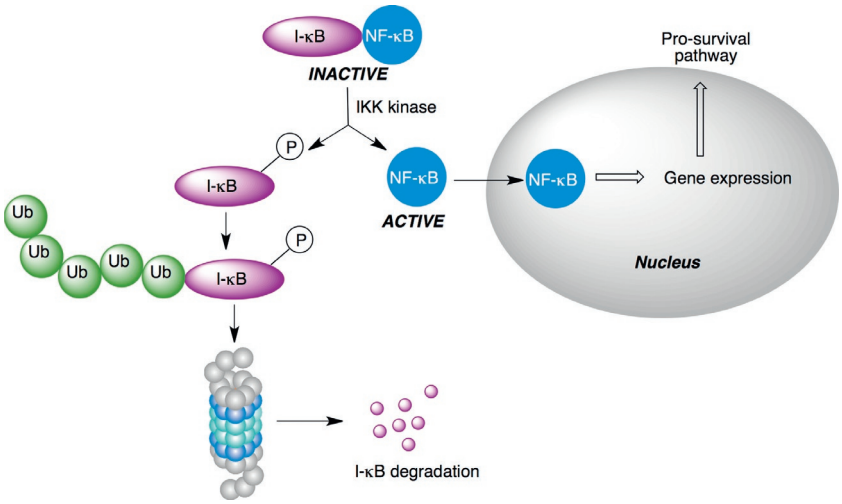


FIGURE 11.5

Proteasome degradation of I-κB stimulates NF-κB-mediated prosurvival pathways in tumor cells.

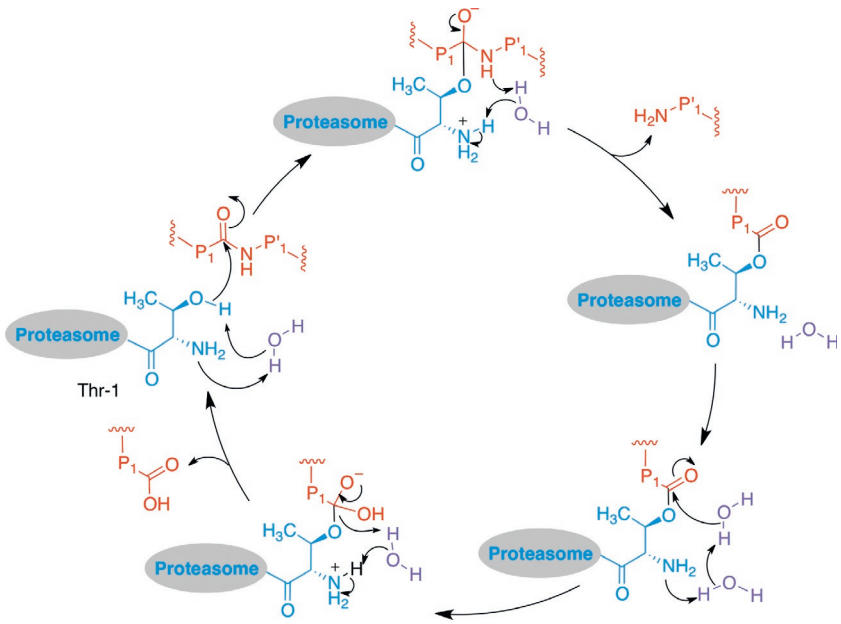
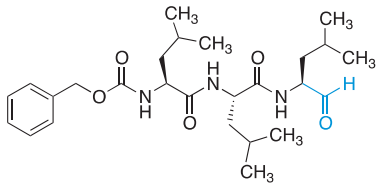
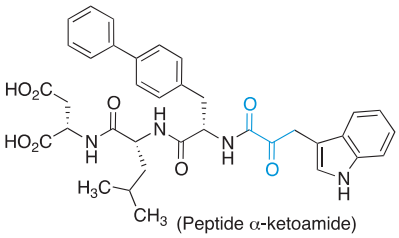
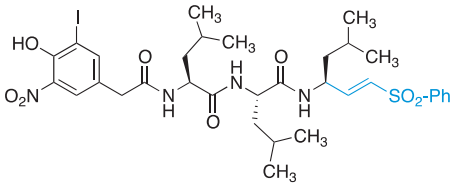


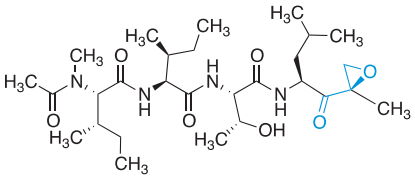
FIGURE 11.6

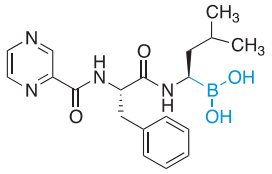
The proteasome catalytic cycle.


MG-132 (peptide aldehyde)


(Peptide α-ketoamide)

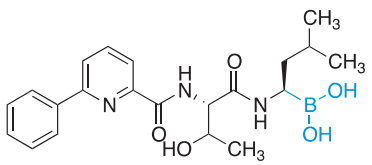

NLVS (peptide vinyl sulfone)


Epoxomycin (peptide epoxy ketone)


Bortezomib (peptide boronic acid)

Several proteasome inhibitors are on the market or under clinical trials for the treatment of multiple myeloma (MM) and other cancers.[10,11] Boronic acids have a high specificity for threonine proteases and a lack of activity on cysteine proteases, and for this reason a large number of peptide boronic acids and boronate esters have been studied as proteasome inhibitors. Among these compounds, bortezomib (PS-341, LDP-341, MLN-341, Velcade®) was approved in 2003 for the treatment of MM, the second most common hematological cancer. This drug is also used for the treatment of mantle cell lymphoma and acute allograft rejection, and it is being evaluated for various other malignancies. It affects multiple signaling cascades in cancer cells (e.g., NF-κB) and induces G_2/M phase arrest followed by apoptosis.[12] Although the use of bortezomib has helped to change the history of MM therapy, it is not universally effective in all patients and its use is associated with reversible peripheral neuropathy, which can limit short- and long-term treatments. One promising adjuvant for bortezomib in MM therapy is the epigenetic drug (+)-JQ1 (see Chapter 8, Section 3.8), which may provide an additional 3–5 years of survival.

Delanzomib (CEP-18770) is an orally active peptidic boronic acid with a tumor-selective pharmacologic profile competitive with bortezomib. It downmodulates the NF-κB activity and the expression of several NF-κB downstream effectors, inducing apoptotic cell death in MM cell lines.


Delanzomib (CEP-18770)

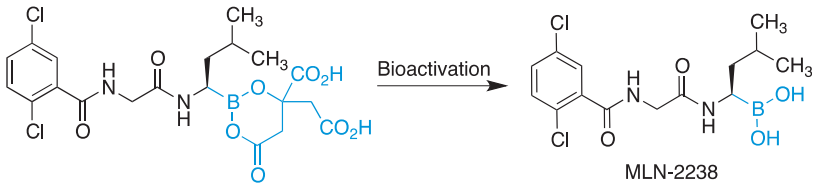
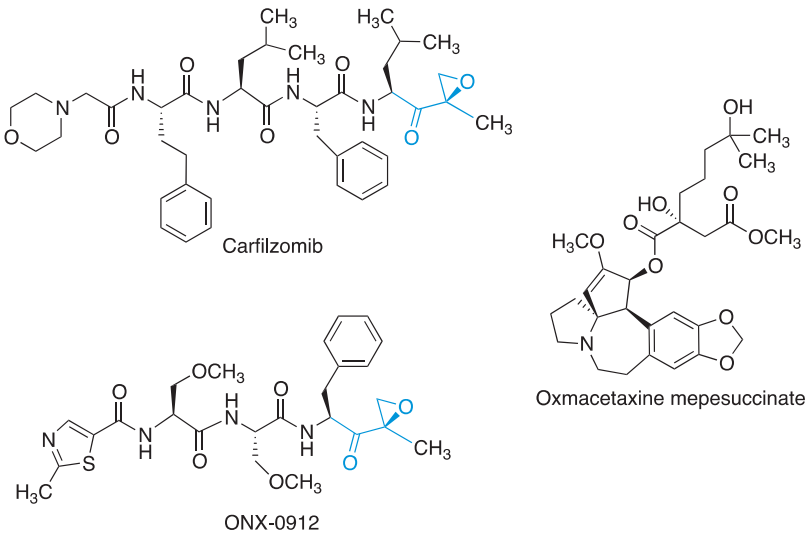


FIGURE 11.7

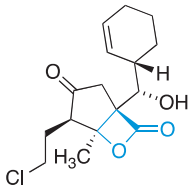
The bioactivation of ixazomib.

The cyclic boronate ester ixazomib (MLN-9708) is an orally bioavailable prodrug that is immediately hydrolyzed when exposed to aqueous solutions to yield its active form, the boronic acid MLN-2238 (Figure 11.7). Phase I/II clinical trials with this drug are ongoing in patients with newly diagnosed and refractory MM, lymphomas, and multiple nonhematologic malignancies.

Epoxy ketones are irreversible inhibitors that elicit a more sustained inhibition than boronic acids. They also have a greater target specificity, which may explain their ability to overcome resistance to bortezomib. Carfilzomib (Krypolis®) was approved by the U.S. Food and Drug Administration (FDA) in 2012 and is in clinical trials to be commercialized in Europe. The structurally related epoxy ketone oprozomib (ONX-0912) is in phase I clinical trials in patients with advanced refractory or recurrent solid tumors. Suppression of induced myeloid leukemia cell differentiation protein (Mcl-1) enhances the killing activities of carfilzomib and ONX-0912, providing a strategy for further improving the efficacies of these proteasome inhibitors against head and neck squamous cell carcinoma (HNSCC).[13] This can be achieved by omacetaxine mepesuccinate (Synribo®), which is used in the treatment of chronic myelocytic leukemia (CML), because this compound is a protein synthesis inhibitor that prevents the synthesis of Mcl-1 (see Chapter 10, Section 4.9.4).



The non-peptide constrained β-lactone salinosporamide A (marizomib), which induces a rapid, broad, and prolonged proteasome inhibition, is being clinically evaluated in MM, lymphomas, leukemias, and solid tumors.[14] It seems to be well tolerated at effective doses, despite several adverse side effects.



Salinosporamide A
(Marizomib)

Regarding the mechanism of the proteasome inhibition, compounds containing aldehyde or ketone functions react reversibly with the catalytic threonine hydroxyl to give the corresponding acetals **11.1** and **11.2**. Vinyl sulfones, which were originally introduced as inhibitors of cysteine proteases,[15] react as Michael acceptors to form covalent adducts **11.3** (Figure 11.8).

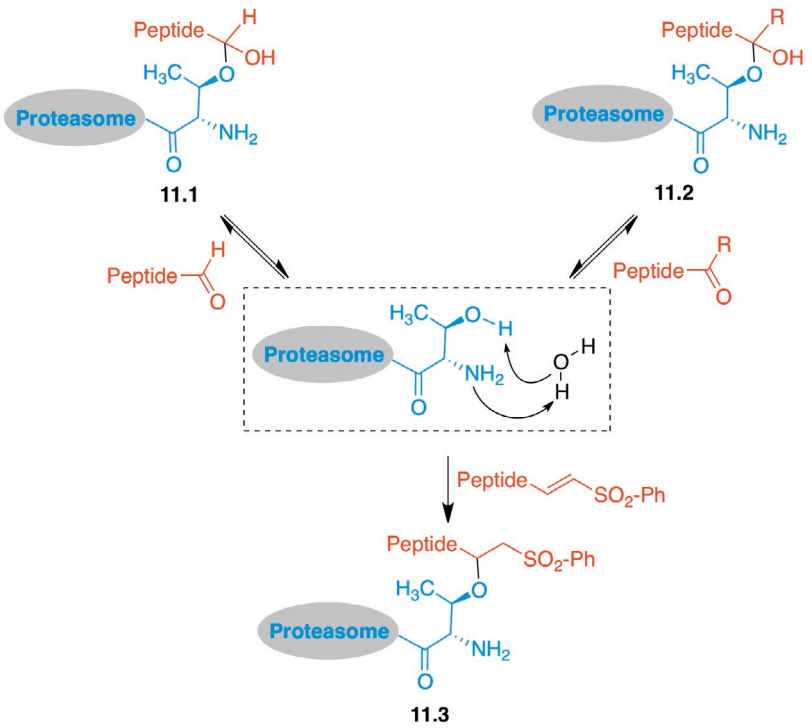


FIGURE 11.8

Mechanisms of proteasome inhibition by aldehydes, ketones, and vinyl sulfones.

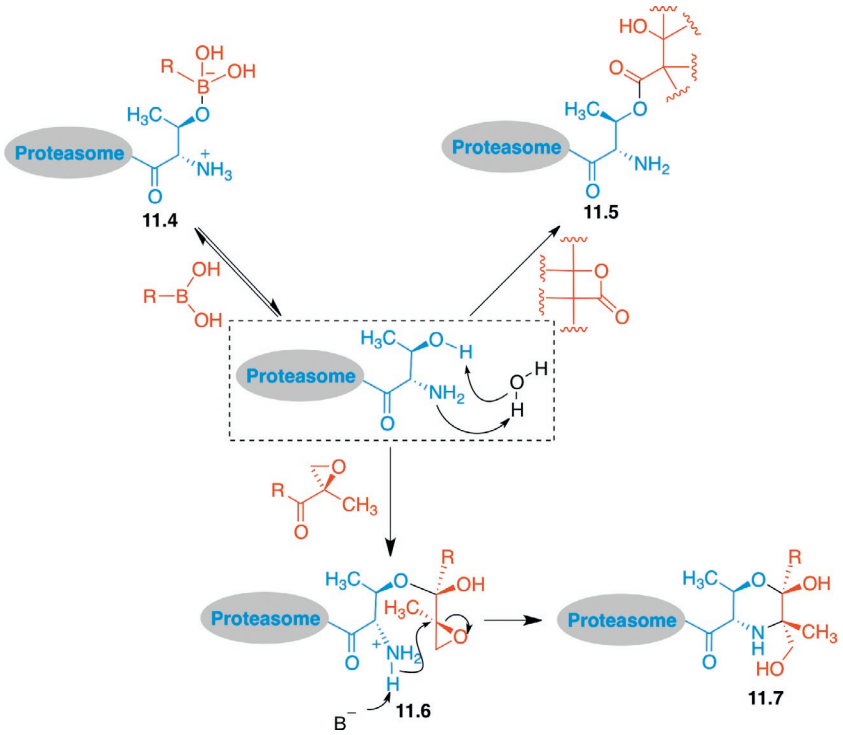


FIGURE 11.9

Mechanisms of proteasome inhibition by boronic acids, β-lactones, and α,β-epoxy ketones.

The interaction of peptide boronic acids is due to the availability of an empty *p*-orbital on boron atoms that can accept the oxygen lone pair of N-terminal threonine residues to form stable tetrahedral intermediates **11.4**,[16] whereas fused β-lactone derivatives are probably opened by the Thr oxygen to give esters **11.5**. Finally, α,β-epoxy ketones generate hemiacetals **11.6** by addition of the Thr oxygen to the carbonyl group, and this is followed by nucleophilic attack of the amino group onto the more hindered epoxide carbon atom with inversion of its configuration to form morfoline derivatives **11.7**. This mechanism has been confirmed by X-ray crystal diffraction and spectrometric analysis of the complex formed by natural proteasome inhibitor epoxomycin and the yeast *Saccharomyces cerevisiae* 20S proteasome (Figure 11.9).[17]

2.1.2 NEDD8-Activating Enzyme Inhibitors

There are several proteins related to ubiquitin that have their own ligases and are known as ubiquitin-like (UbL) proteins. One UbL pathway of particular interest is NEDD8, whose E1-activating enzyme is known as NEDD8-activating enzyme (NAE). In the first step of the activation process, MgATP and NEDD8 yield NEDD8–AMP, which reacts with the thiol of a cysteine residue in the NAE active site to form the NAE–NEDD8 thioester and release AMP. A second NEDD8–AMP is

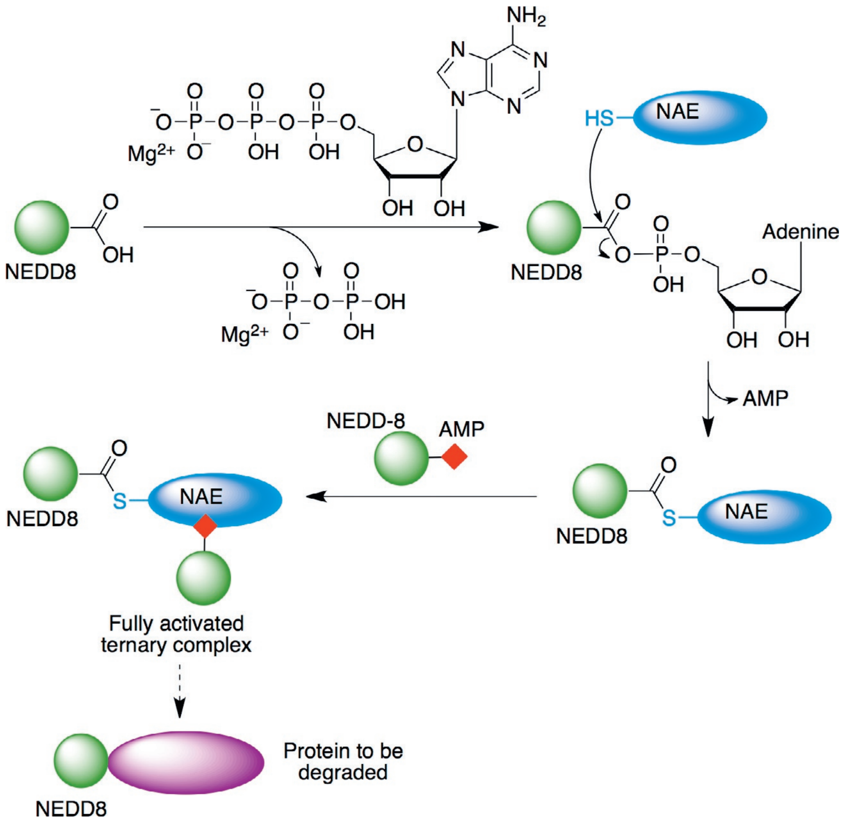


FIGURE 11.10

Attachment of NEDD unit to a protein to be degraded by proteasome by the NEDD8-activating enzyme (NAE).

then bound to give the fully loaded, ternary complex containing two NEDD8 molecules, one of which is covalently bound as a thioester and another occupies the NAE adenylation domain. This form of NAE is responsible for the transthiolation of NEDD8 to an E2-type enzyme known as Ubc12 and then to the cullin subunit of an E3 ubiquitin ligase called cullin-dependent ligase (CDL), which thus becomes activated and finally attaches the NEDD unit to the protein to be degraded by proteasome (Figure 11.10). CDLs control many proteins relevant in oncogenesis because they are involved in cell cycle control, DNA replication, NF-κB signaling, hypoxia signaling, oxidative stress response signaling, and DNA replication and repair.

The adenosine monophosphate (AMP) analog MLN-4924 is a potent and selective NAE inhibitor that has entered phase I trials for both blood and solid tumors. This drug has a significant activity against both cisplatin-sensitive (CS) and cisplatin-resistant (CR) ovarian cancer cells and provokes the stabilization of key NEDD8 substrates. Notably, MLN-4924 significantly augments the activity of cisplatin against CR cells, suggesting that aberrant NEDDylation may contribute to drug resistance. Both drugs cooperate to induce DNA damage, oxidative stress, and increased expression of the apoptosis-inducing BH3-only

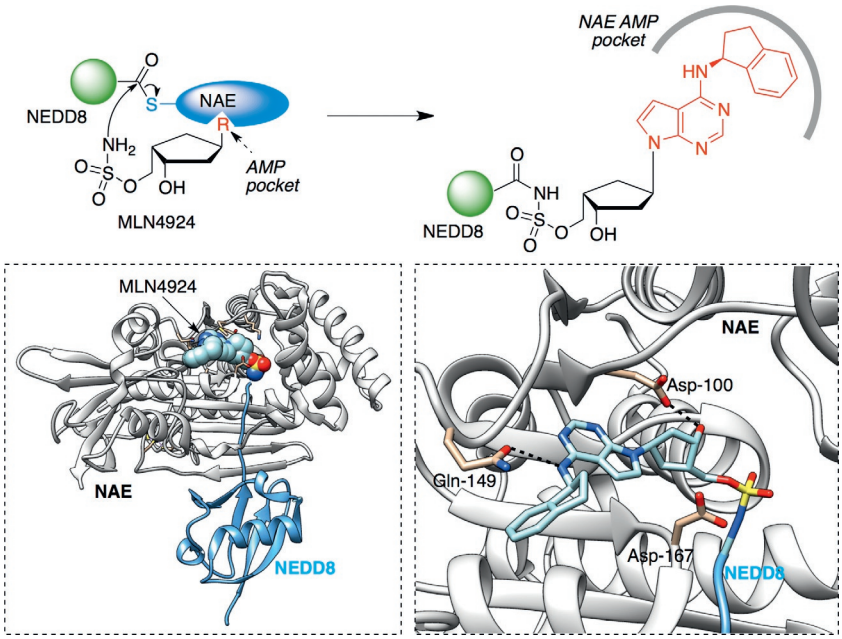


FIGURE 11.11

Mechanism of NAE inhibition by MLN-4924.

protein NBK/BIK.[18] MLN-4924 creates a covalent NEDD8–MLN-4924 adduct under catalysis by the NAE, the same enzyme that becomes inhibited, and can thus be described as a suicide (mechanism-based) inhibitor. By mimicking AMP, MLN-4924 occupies the non-covalent binding site of NAE normally occupied by the second molecule of NEDD8 (Figure 11.11).[19]

2.1.3 APC Inhibitors

The anaphase-promoting complex/cyclosome (APC/C) is a key regulator of the cell cycle and its failure causes improper sister chromatid separation. APC is an E3 ligase that binds the activator proteins Cdh1 (cadherin1) and Cdc20 (cell-division cycle protein 20) at different cell cycle stages. This binding stimulates the APC-dependent ubiquitination of substrates and their subsequent destruction by the 26S proteasome. Initiation of anaphase and exit from mitosis require Cdc20-dependent ubiquitination of APC substrates such as securin and S- and M-phase cyclins, whereas Cdh1 activates APC in early G_1, inducing ubiquitination of several protein substrates like mitotic cyclins and their subsequent destruction by the 26S proteasome, thereby inhibiting the cell cycle at this stage (Figure 11.12).

The Cdc20-dependent ubiquitination of APC substrates begins when the microtubuli are properly attached to the outer kinetochores. Then, Cdc20 activates APC, which in turn ubiquitinylates the separase inhibitor securin, leading to cohesin degradation by the latter and initiating sister chromatid separation. Prior to anaphase, the activity of APC–Cdc20 is inhibited as a consequence of the regulatory mechanism known as the spindle assembly checkpoint (SAC),[20] in which unattached kinetochores

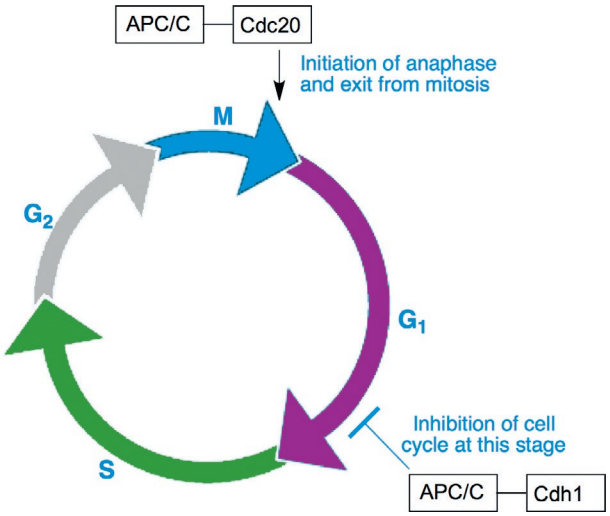


FIGURE 11.12

Cell cycle regulation by anaphase-promoting complex/cyclosome (APC/C).

catalyze the formation of an inhibitory protein complex that sequesters Cdc20 or interferes with its ability to activate APC (Figure 11.13).

In principle, a compound that directly inhibits the proteolysis dependent on ubiquitin ligase APC should induce mitosis arrest without causing the side effects that result from microtubule inhibition, such as peripheral neuropathy (see Chapter 9, Section 2).[21] APC inhibitors induce a more persistent mitotic arrest than microtubule inhibitors[22] because the latter rely on activation of the SAC mechanism by unattached microtubuli, and it is known that SAC cannot fully inhibit APC during mitosis. Therefore, due to the residual APC activity, some cells escape mitotic arrest.[23] Because SAC does not completely inhibit the APC, mitotic arrest induced by microtubule inhibition depends on protein synthesis, which explains the highly variable response of cells to microtubule inhibitors. In contrast, APC inhibitors should be more effective in promoting mitotic arrest, inducing a greater pro-apoptotic effect.

Among APC inhibitors, TAME (tosyl-L-arginine methyl ester) was able to inhibit the cyclin proteolysis in a mitotic *Xenopus* egg extract and also to inhibit cyclin degradation in an interphase extract activated by exogenous Cdh1, but it was not a general inhibitor of the ubiquitin–proteasome system. It was shown later that TAME blocks APC activation by perturbing the interaction between APC and its activator proteins Cdc20 or Cdh1, thus stabilizing all APC substrates during mitosis and early G_1 phase.[24] TAME can be viewed as a structural analog of the C-terminal isoleucine–arginine (IR) tail of Cdc20 and Cdh1; therefore, it is reasonable to believe that TAME binds APC through the IR tail-dependent interactions and inhibits its activation. Because TAME is not uptaken by human cells due to its high hydrophilicity, a more lipophilic prodrug (proTAME) was designed. This compound is an *N*,*N*′-bis(acyloxymethyl carbamate) derivative that can be processed by intracellular esterases to yield the parent compound TAME, as shown in Figure 11.14. As expected, proTAME induces mitotic arrest of human cells stabilizing all APC substrates without spindle damage.

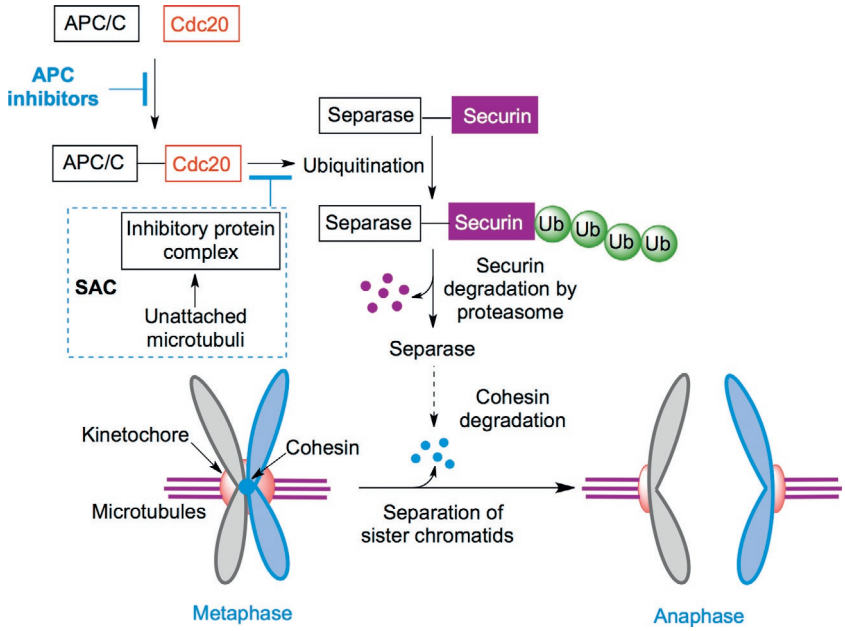


FIGURE 11.13

Cdc20-dependent ubiquitination of APC substrates and its connection to the cell cycle.

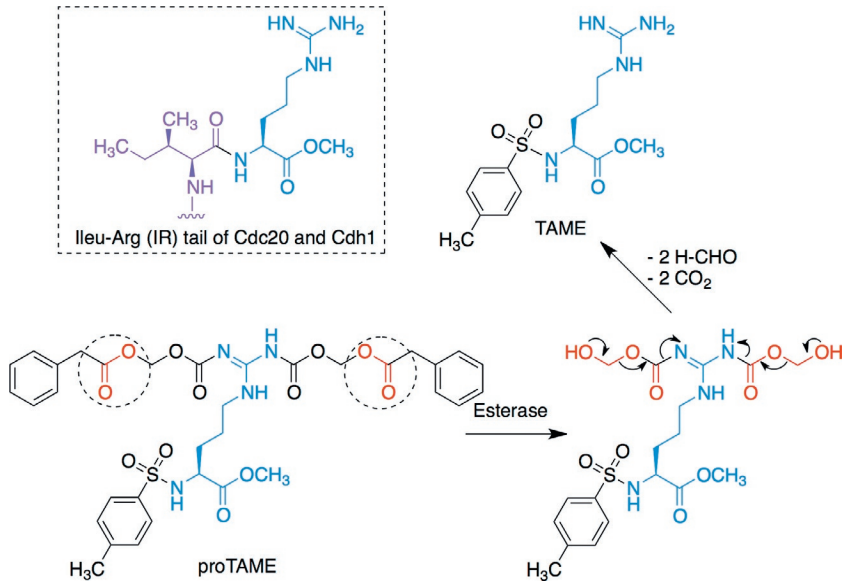


FIGURE 11.14

TAME and its prodrug ProTAME.

Low doses of an APC inhibitor may be useful in combination with microtubule inhibitors to sustain mitotic arrest and enhance cell death. In addition, because topoisomerase 2α is ubiquitinated and degraded in a Cdh1-dependent manner, the lack of Cdh1 (or its inhibition) results in a dramatic increase in sensitivity to topoisomerase 2α poisons, as a consequence of increased levels of trapped topoisomerase α–DNA complexes. For this reason, the combination of proTAME with topoisomerase poisons such as etoposide is being investigated for cancer therapy.[25]

2.2 INHIBITORS OF MATRIX METALLOPROTEINASES

For a tumor to grow beyond a size of approximately 2 mm^3, it needs to develop a network of blood vessels (angiogenesis), a process that is regulated by proangiogenic and antiangiogenic factors. Antiangiogenic drugs[26,27] may have different targets and mechanisms. Those targeting preexisting vasculature through tubulin depolymerization were discussed in Section 5 of Chapter 9. Inhibitors of proangiogenic kinase signaling that compete with proangiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) were discussed in Section 4.7 of Chapter 10. A third mechanism of antiangiogenic drugs is the inhibition of proteolytic enzymes that, in response to angiogenic stimuli, initiate the breakdown of the surrounding extracellular matrix (ECM), a process that allows the migration of proliferating endothelial cells and their growth to form lumens and is therefore associated with both angiogenesis and metastasis. In addition to their importance in cancer treatment,[28] these proteases are mainly being studied as targets for arthritis and emphysema due to their role in collagen degradation.[29] Matrix metalloproteinases (MMPs) are the largest class of human proteinases and the main enzymes involved in these processes.

MMPs are modulated at many different levels by regulatory signals such as soluble factors, ECM–cell interactions, or cell–cell contacts. The interaction of these signals with specific receptors at the cell surface initiates a cascade of events that leads to the generation of functional MMPs, which are localized to the cell surface (MT-MMPs) or secreted to the extracellular medium (pro-MMP).[30] MMPs are zinc-dependent proteolytic endopeptidases in which the zinc cation is coordinated by three imidazole rings from histidine residues and a water molecule. The general mechanism for peptide hydrolysis by zinc MMPs is shown in Figure 11.15 and is based on the enhanced acidity of the water molecule as a consequence of coordination of its oxygen atom with zinc. Their inhibitors replace this water molecule and coordinate to zinc in a monodentate or bidentate manner.

The design of the first generation of MMP inhibitors relied on peptide and peptide-like compounds that combine backbone features that interact with enzyme subsites and functional groups capable of coordination with zinc. Among them, the hydroxamic acid group is a very potent 1,4-bidentate Zn^{2+} ligand that (as an anion) binds with two contacts to the cation and creates a distorted trigonal bipyramidal geometry around the metal.[31] Hydroxamic acid also binds with the MMP protein backbone by its NH group (Figure 11.16). For this reason, peptide-like compounds that contain a hydroxamic acid portion are among the most potent known inhibitors of the MMPs, with potencies in the nanomolar range.

Batimastat (BB-94)[32] and marimastat are hydroxamic acid-based MMP inhibitors with little specificity. Batimastat reached phase III clinical trials, but it cannot be given orally and it is no longer considered for clinical testing. Marimastat is orally active and underwent several phase III assays, but its

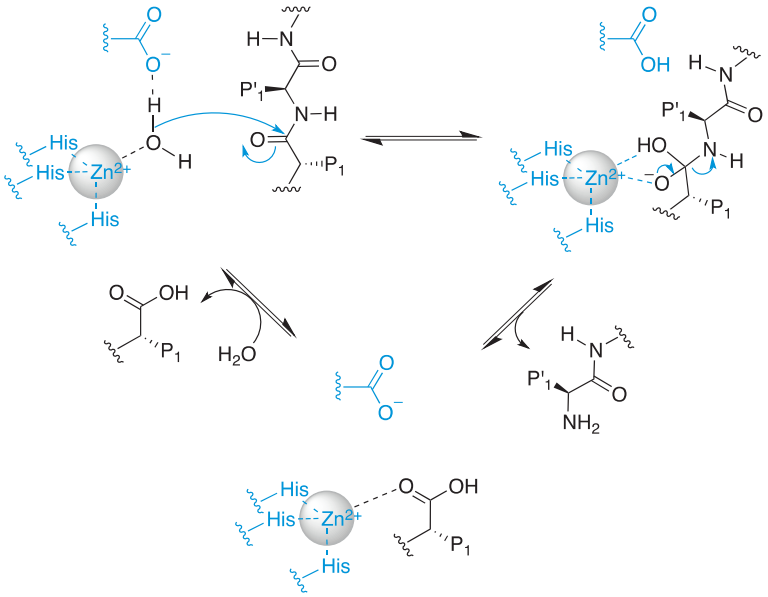


FIGURE 11.15

Catalytic mechanism of MMPs.

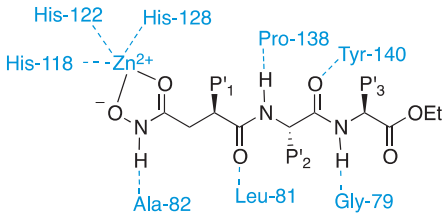
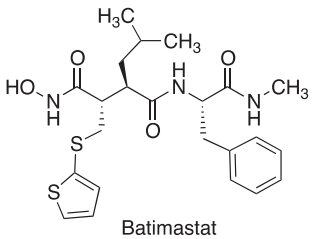
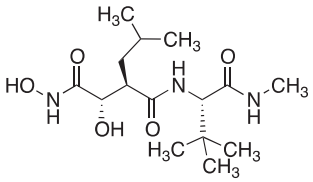


FIGURE 11.16

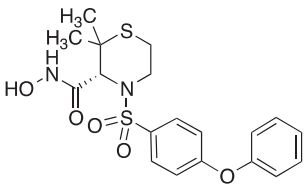
Interaction of hydroxamic acid peptidomimetics with MMPs.

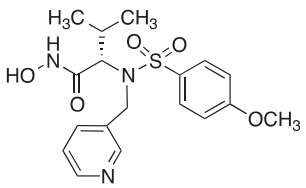
development was halted because of its poor performance.[33] These disappointing results led many investigators to conclude that MMPs were not suitable targets for the treatment of human cancer, whereas others argued that because MMP inhibitors would decrease the rate of tumor progression, the therapeutic benefit obtained from their administration would be minimized for patients undergoing clinical trials, who are normally in the late stages of their disease.[34] Indeed, later results from marimastat trials in patients with gastric carcinoma and pancreatic cancer have been encouraging.

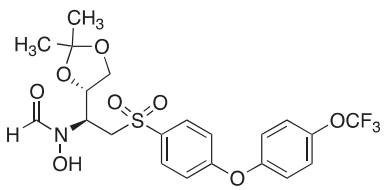

Batimastat


Marimastat

The second generation of MMP inhibitors includes non-peptidic compounds that are more specific, probably because they have been designed on the basis of structural studies of the MMP active site by nuclear magnetic resonance (NMR) and X-ray crystallography. Several of these compounds entered phase III clinical trials to treat many types of cancer, but those that showed only partial selectivity failed. In the first subgroup, bearing a hydroxamic function, are prinomastat (AG3340) and MMI-270 (CGS-27023A). Phase II clinical studies of prinomastat for early stage cancers are still ongoing, but phase III trials for advanced prostate and non-small cell lung cancer (NSCLC) were stopped because they did not show beneficial effects.[35] Clinical studies with MMI-270 were advanced to phase II, but they were interrupted because of poor patient tolerance. The reverse hydroxamate ABT-518 was designed to overcome the metabolic instability of hydroxamates, which is due to reduction of this group to an amide. This compound, which is a potent, orally bioavailable, selective inhibitor of MMP-2 and -9, entered phase I/II clinical trials for some solid tumors.[36]

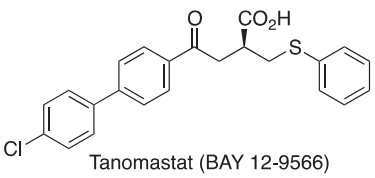

Prinomastat (AG-3340)


MMI-270 (CGS-27023)

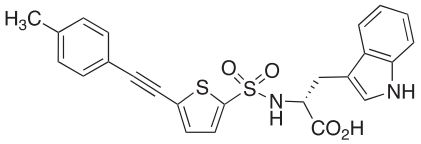

ABT-518

Other functional groups that can interact with the Zn^{2+} cation are the carboxy and mercapto moieties. Among the carboxylic acid-based specific inhibitors, the development of tanomastat (BAY 12-9566) was discontinued after phase III studies for treatment of several cancers, whereas S-3304 (a potent, orally active, non-cytotoxic inhibitor of MMP-2 and -9)[37] entered phase II trials for the treatment of some solid tumors. Rebimastat (BMS-275291) is a thiol-based inhibitor that selectively inhibits MMP-1, -2, -8, -9, and -14 and has entered phase II/III clinical trials in advanced NSCLC.

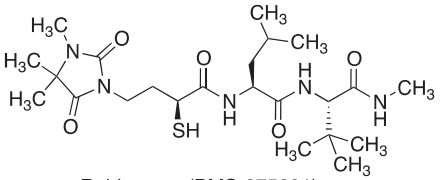
Because of their chelating activity, some tetracyclines, such as COL-3 (Metastat®), inhibit MMPs. COL-3 inhibits MMP-1, -2, -8, -9, and -13 and downregulates various inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin IL-8. It has entered phase II trials for Kaposi's sarcoma and advanced brain tumors.[38]



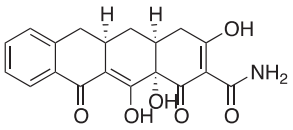
Tanomastat (BAY 12-9566)



S-3304



Rebimastat (BMS-275291)



COL-3 (Metastat)

AE-941 (Neovastat®) is an orally bioavailable standardized extract prepared from shark cartilage that shows significant antiangiogenic and antimetastatic properties *in vivo* by a complex mechanism that includes inhibition of various members of the MMP family. It is orally bioavailable and has been studied in patients with lung and renal carcinoma and MM.[39]

In summary, MMP inhibitors have been pursued as clinical candidates for the treatment of cancer, arthritis, and cardiovascular diseases since the late 1970s.[40] In the first years, the therapeutic strategy of controlling cancer by broadly targeting MMPs was founded on reducing degradation of basement membrane in metastasis and angiogenesis. The hope that antiangiogenic drugs would convert malignant cancer to a survivable benign disease has been realized in some animal tumor models, but in clinical practice, antiangiogenic drugs only extend the life span of advanced cancer patients by less than 1 year, on the average. This failure may be due in part to the plasticity of tumors to induce alternate proangiogenic factors that bypass the targets of the existing drugs, making it necessary to either develop compounds that target all possible angiogenic factors produced by the tumor or to identify conserved aspects of the signal transduction pathways used by these factors that can be the targets for universal angiogenesis inhibitors.[41]

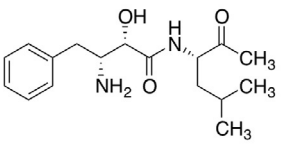
The failure of human trials with MMP inhibitors has also been attributed to insufficient knowledge of the multifunctional role of these proteases. For example, one early study involving the MMP inhibitor tanomastat to treat small cell lung cancer inadvertently targeted MMP-2, an enzyme subclass that now is thought to have no role in this particular tumor type. Moreover, because MMPIs are designed to halt the development of a tumor rather than to kill it, better tools are required to establish whether they are actually working and what doses are needed. It is clear that the role of MMPs is not simply to degrade the extracellular matrix because they participate in many deregulated signaling pathways. Some MMPs, especially MMP-3, -8, and -9, are antitarget proteins that, when genetically eliminated in knockout animal models, induce enhanced tumorigenesis and metastasis. Consequently, for successful cancer therapy based on MMP inhibition, compounds must be selective against validated MMP targets.[42]

2.3 INHIBITORS OF AMINOPEPTIDASES

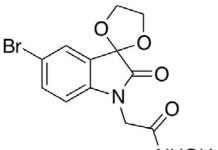
Aminopeptidases are proteolytic enzymes that hydrolyze the peptide bond involving the N-termini of peptide substrates, thereby releasing a single amino acid residue. Although they are anticancer targets, their development lags far behind that of MMP inhibitors.

Aminopeptidase N (APN, CD13) is a Zn^{2+}-dependent membrane-bound ectopeptidase that degrades preferentially proteins and peptides with an N-terminal neutral amino acid. The active sites of APN and MMP-2 are similar, and both enzymes contain two hydrophobic cavities (the S1 and S1′ pockets) around the catalytic zinc ion, although APN possesses another binding site rich in electropositive amino acid residues that provide H-bonding interactions. APN has been associated with the growth of different human cancers as a cell-surface marker for malignant myeloid cells, and it is also regarded as a good target for cancer therapy.[43] The key angiogenesis regulator VEGF induces APN expression at an early stage of tumor growth,[44] and high levels of APN are associated with the progression of several tumors, including breast, ovarian, and prostate cancer.

Although to date no inhibitors of these proteolytic enzymes (APNIs) are available as clinical agents, some compounds have shown considerable interest. For instance, ubenimex (Bestatin®), a peptidomimetic obtained from *Streptomyces olivoreticuli*, is an inhibitor of APN that entered clinical trials for acute myeloid leukemia, CML, lymphomas, and stage I squamous cell lung carcinoma.[45] Compound **11.8**, which was discovered in a virtual screening of a specifically filtered commercial database, exhibited good antiproliferative activities against a broad spectrum of human cancer cell lines.[46]



Ubenimex



11.8

Tosedostat (CHR-2797) is an ester prodrug that liberates by hydrolysis the poorly membrane-permeable active metabolite CHR-79888, a hydroxamic acid derivative that acts as an inhibitor of the M1 family of aminopeptidases (Figure 11.17). It has given encouraging clinical responses in patients with acute leukemia and several other blood-related cancers, and it is also under clinical study for solid tumors.[47]

The tosedostat active metabolite acts by a unique mechanism of action involving amino acid depletion in cancer cells by disruption of the cycle summarized in Figure 11.18, in which the action of aminopeptidases is essential to recycle the peptide fragments derived from the action of proteasome on proteins. This amino acid deprivation disrupts the turnover of cell cycle intermediates and prevents cancer cell survival or proliferation.

2.4 INHIBITORS OF CATHEPSINS

Other proteases that increase the metastatic potential of cancer cells are the cathepsins, which are cysteinyl and aspartyl proteases normally present inside the lysosomes as inactive pro-enzymes. When released at the extracellular space and activated, they facilitate cell migration and invasiveness. They are also transported into the cell nucleus, where they enhance the expression of genes involved in the

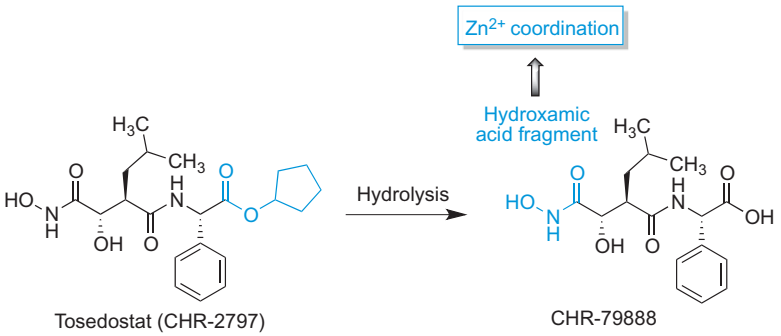


FIGURE 11.17

Bioactivation of tosedostat.

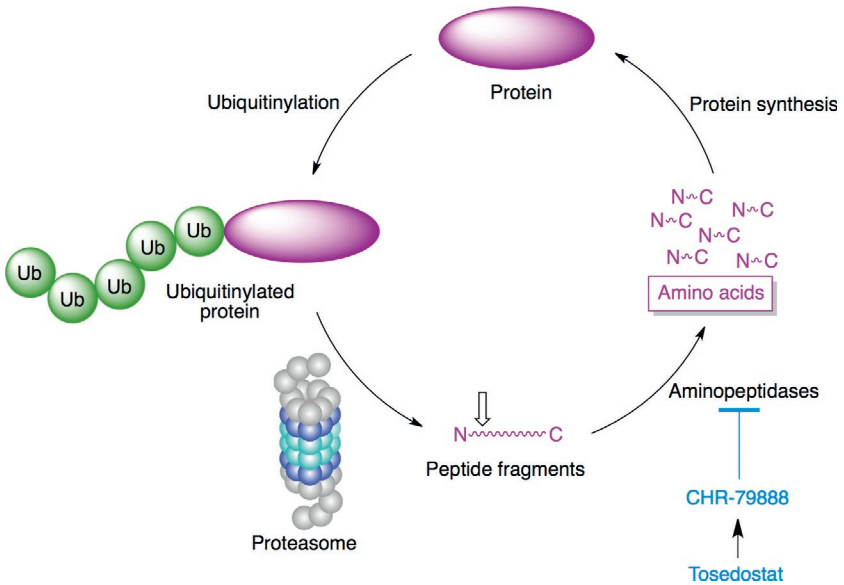


FIGURE 11.18

The tosedostat active metabolite induces amino acid depletion in cancer cells.

epithelial–mesenchymal transitions (EMTs). Cathepsin-L (Cts-L) is upregulated in a wide range of human cancers and pathophysiological tumor microenvironments, characterized by hypoxia and acidic pH, augment metastatic aggressiveness because they trigger elevation of invasive potential associated with Cts-L activation. It has been shown that its inhibition diminishes the spreading ability of different primary and metastatic melanoma cell lines.[48] Consequently, it holds great promise to delay tumor growth and metastasis, although its study is still in a preliminary stage.[49]

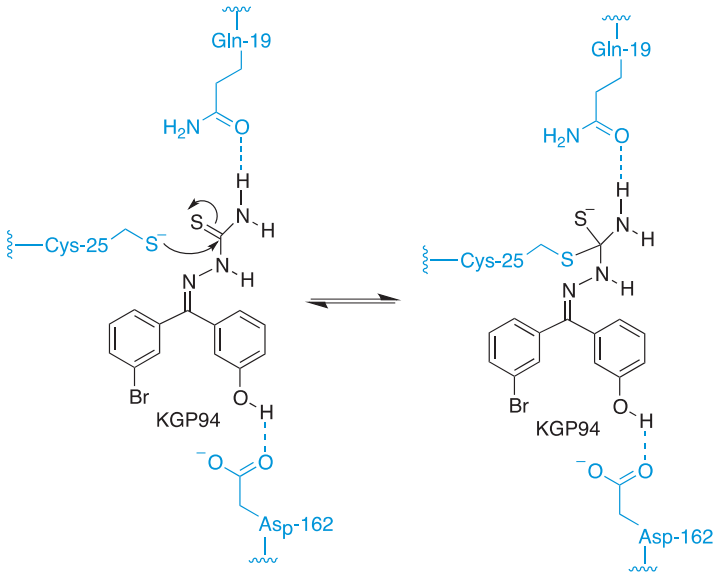
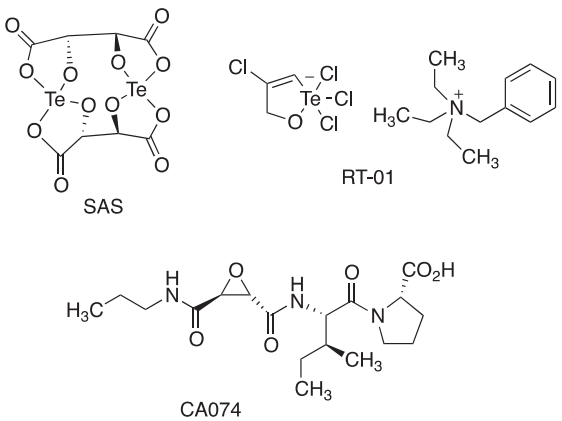


FIGURE 11.19

Cathepsin inhibition by KGP94.

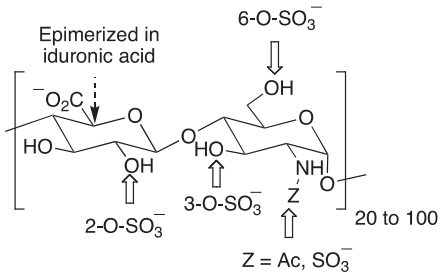
KGP94, a lead compound discovered among a library of benzophenone thiosemicarbazone derivatives,[50] is a reversible and competitive inhibitor of Cts-L and a potent inhibitor of invasion and migration in breast cancer MDA-MB-231 and prostate DU-145 cell lines.[51] Molecular modeling of the best interaction energy between Cts-L and KGP94 showed that the thiocarbonyl group is in close proximity with the Cys-25 residue of the target, and in this position, it is ready for attack by the enzyme thiolate to form a transient tetrahedral intermediate (Figure 11.19).[52]

Several other cathepsin A inhibitors are in preclinical studies. Octa-*O*-bis-(*R*,*R*)-tartrate ditellurane (SAS) and RT-01 are organotellurium (IV) compounds[53] that have displayed selectivity toward the cysteine protease cathepsin B. The epoxide CA-074A has also been characterized as a cathepsin B inhibitor.[54]

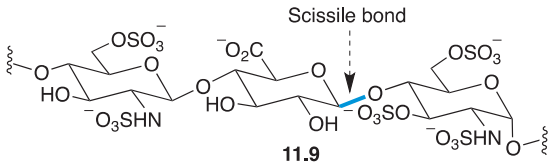


3 HEPARANASE INHIBITORS

Heparanase is an endo-β-D-glucuronidase that degrades polymeric heparan sulfate (HS), a polysaccharide formed by alternating, repetitive units of D-glucosamine and D-glucuronic acid/L-iduronic acid. HS proteoglycans are important components of the endothelial cell layer and are formed by a protein core covalently bound to HS side chains. Its cleavage affects the integrity and functional state of tissues and is involved in the response to changes in the extracellular microenvironment.



Heparanase is preferentially expressed in human tumors, in which it confers a highly invasive phenotype by releasing angiogenic factors. It recognizes sequences as small as a trisaccharide provided they are highly sulfated, as is the case with structure **11.9** (Figure 11.20a).[55] Its catalytic mechanism involves two acidic residues, a proton donor at Glu-225 and a nucleophile at Glu-343 (Figure 11.20b).[56]



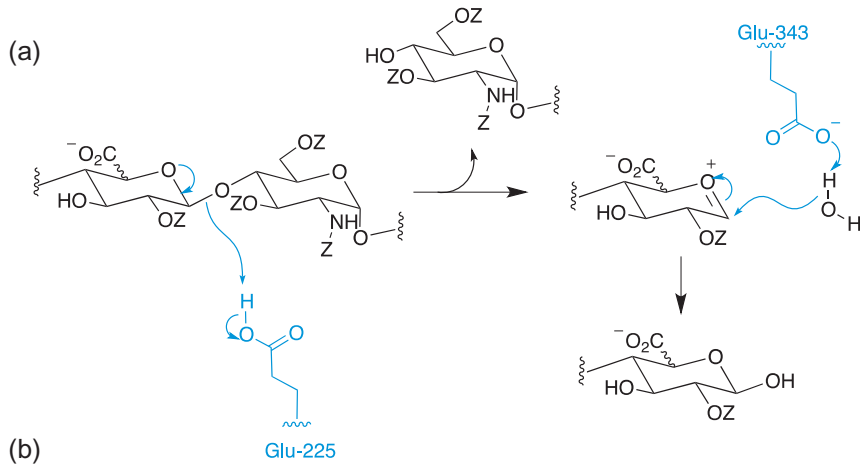


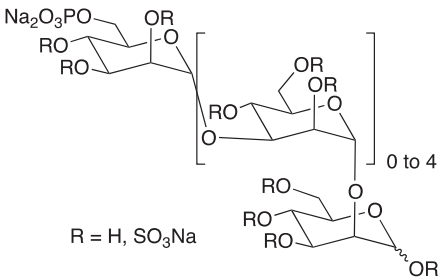
FIGURE 11.20

(a) Recognition sequence of heparan sulfate. (b) Mechanism of the reaction catalyzed by heparanase.

A variety of heparanase inhibitors have been developed, including peptides, modified non-anticoagulant species of heparin, and several polyanionic molecules such as suramin and PI-88.[57,58] They are structurally very heterogeneous and can be classified into four categories: polysaccharides with *O*- or *N*-sulfate groups, compounds with *C*-sulfate groups, neutral inhibitors, and monoclonal antibodies.

3.1 POLYSACCHARIDES WITH *O*- OR *N*-SULFATE GROUPS

Heparins and other sulfated polysaccharides, or synthetic polymers that mimic heparin have a broad range of biological activities, although their main uses are outside the anticancer field. The most important goal in this area is the development of compounds with reduced molecular sizes, as is the case with the phosphosulfomannan muparfostat (PI-88), which is a mixture of highly sulfated monophosphorylated mannose oligosaccharides derived from the extracellular phosphomannan of the yeast *Pichia* (*Hansenula*) *holstii*. In addition to being a heparanase inhibitor, muoarfostat may also bind with high affinity to the HS-binding domains of VEGFs and FGFs 1 and 2, thus inhibiting the stimulation of tumor angiogenesis.

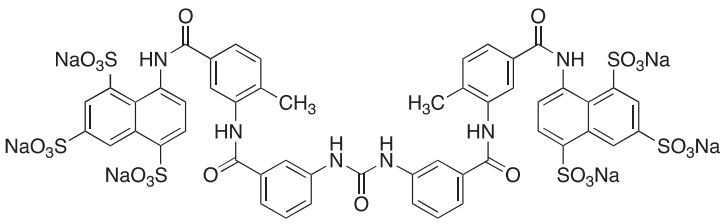


Muparfostat (PI-88)

Muparfostat entered clinical trials for the treatment of several cancers,[59] including studies in combination with docetaxel in patients with metastatic castrate-resistant prostate cancer.[60] The FDA granted its fast track designation in the prevention of tumor recurrence following curative liver resection in patients with hepatocellular carcinoma, an indication studied in phase III trials.

3.2 COMPOUNDS WITH *C*-SULFONATE GROUPS

In addition to inhibition of PDGF receptors (see Chapter 10, Section 4.7.3), suramin (Metaret®) shows angiogenesis inhibition activity through its noncompetitive heparanase inhibition.[61]



Suramin

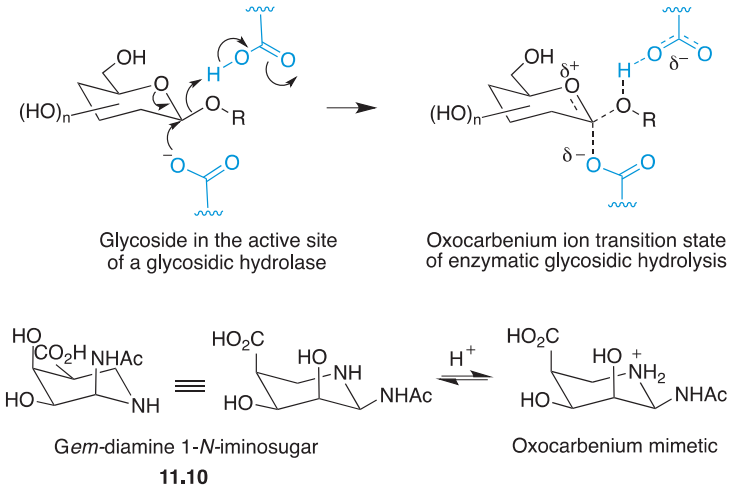


FIGURE 11.21

gem-Diamine 1-*N*-iminosugars as heparanase inhibitors.

3.3 NEUTRAL INHIBITORS

These compounds do not contain sulfate moieties and are structurally very diverse. Among them, some natural and unnatural *gem*-diamine 1-*N*-iminosugars, such as **11.10**, are reversible heparanase inhibitors acting as putative transition state analogs.[62]

These glycomimetics carry a basic nitrogen in place of the anomeric carbon in the carbohydrate ring and are easily accessible from siastatin B, a secondary *Streptomyces* metabolite. The protonated form of 1-*N*-iminosugars mimics the charge at the anomeric position in the transition state of enzymatic glycosidic hydrolysis, resulting in a strong and specific inhibition of glycosidases and glycosyltransferases. These compounds have been recognized as a new source of therapeutic drug candidates in a wide range of diseases associated with the carbohydrate metabolism of glycoconjugates, including tumor metastasis (Figure 11.21).[63]

3.4 MONOCLONAL ANTIBODIES

Several anti-heparanase antibodies have been shown to neutralize heparanase enzymatic activity, but their clinical application has not been reported. Recently, the clinically relevant novel splice variant of heparanase T5 was preferentially recognized by the monoclonal antibody 9c9 in patients with renal cancer.[64]

4 INTEGRIN ANTAGONISTS AND INHIBITORS OF CHEMOKINE RECEPTORS

Another approach to antiangiogenic therapy is based on the inhibition of the adhesive interactions required by vascular endothelial cells. The migration of these cells is dependent on their adhesion to ECM proteins.[65] Cells in animals are linked directly to each other by cell adhesion molecules

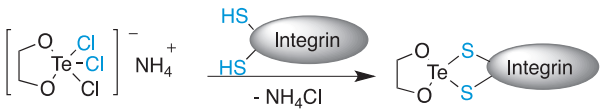
(CAMs) at the cell surface. The main classes of CAMs are cadherins, the immunoglobulin (Ig) superfamily, selectins, mucins, and integrins. The integrins mediate cell–matrix interactions, whereas the other types of CAMs participate in cell–cell adhesion. Integrin αvβ3 is one of the most prevalent integrins, and its overexpression onto proliferating endothelial cells of the tumor vasculature is associated with a high rate of neovascularization and invasive potential.[66] On the other hand, integrin VLA-4 (α4β1) is a key cell receptor expressed on most leukocytes that plays an important role in the process of adhesion, migration, and activation of inflammatory leukocytes at sites of inflammation. VLA-4 also plays a critical role in the resistance of certain leukemias to chemotherapy. Anti-VLA-4 antibodies or small-molecule antagonists inhibit leukocyte infiltration into extravascular tissue and prevent tissue damage in models of inflammatory diseases, such as asthma, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. Integrin antagonists have shown encouraging activity in anticancer clinical trials.[67]

Among them, the nontoxic organotellurium-IV compound ammonium trichloro(dioxoethylene-*O, O′*)tellurate (AS101), the most studied tellurium compound, has potential therapeutic applications in clinical conditions involving immunosuppression. It has also anticancer interest which is related to its reaction with cysteine thiols within specific protein subunits of integrins (Figure 11.22).

In addition, AS101 induces a significant reduction in the thrombocytopenia that accompanies cancer therapy through direct inhibition of the anti-inflammatory cytokine IL-10, and it shows no major toxicity. It has entered phase II clinical studies to evaluate its efficacy for the treatment of this secondary effect in patients with various solid tumors. It also has significant bone marrow (BM)-sparing effects, prevents hair loss in chemotherapy-treated patients,[68] and shows potential as an ovarian-protective agent toward cancer.[69]

Integrin αvβ3 binds to an arginine–glycine–aspartic acid (RGD) sequence that can be found in several ECM proteins and in some angiogenic growth factors. For this reason, a number of RGD-derived peptides and peptidomimetics have been studied as integrin antagonists. The cyclic peptide *cyclo*-(Arg-Gly-Asp-D-Phe-D-NMeVal) (cilengitide, EMD 121974) was the first antiangiogenic small molecule with a potent αvβ3-integrin antagonist activity. This compound was developed in the early 1990s through a screening that resulted in the active cyclic peptide *c*-(RGDfV), which was later modified by *N*-methylation of one of the amide nitrogens to yield cilengitide.[70] After its entry into clinical trials, the European Medicines Agency (EMA) granted cilengitide orphan drug status in 2008. However, a phase III trial with glioblastoma patients, in which cilengitide was added to the standard temozolomide and radiotherapy regimen, did not significantly increase overall survival.

αvβ5 is another RGD-dependent adhesion integrin that plays a critical role in angiogenesis, and for this reason dual αvβ3/αvβ5 antagonists such as SCH-221153 were proposed as angiogenesis and tumor-growth inhibitors.[71] Another interesting compound is the sulfonamide derivative E7820, which suppresses

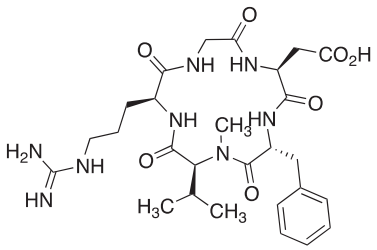


AS101

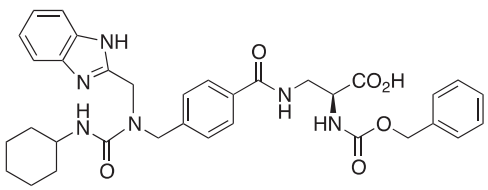
FIGURE 11.22

Integrin inhibition by AS101.

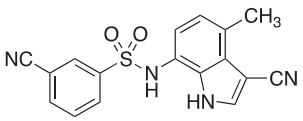
the expression of integrin α_2 subunit on endothelium.[72] After a phase I assay,[73] several phase II studies in patients with advanced malignancies are ongoing.



Cilengitide

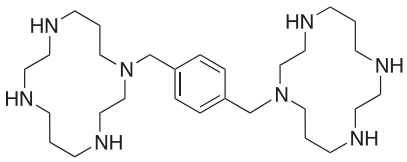


SCH-221153



E7820

Peripheral blood stem cell mobilization, which is important as a source of hematopoietic stem cells for autologous transplantation in non-Hodgkin's lymphoma and MM cancer patients, is generally performed using granulocyte colony-stimulating factor (G-CSF), but this technique is ineffective in some patients. The interaction between the stromal-derived factor-1 (SDF-1) and the chemokine receptor CXCR4 (also known as fusin) plays an important role in holding hematopoietic stem cells in the bone marrow; consequently, inhibitors of CXCR4 are able to mobilize hematopoietic stem cells into the bloodstream. The percentage of patients who produce enough stem cells for transplantation increases with the combination of G-CSF with the CXCR4 inhibitor plerixafor (Mozobil®), which was approved by the FDA in 2008. Plerixafor has a curious history. It was synthesized in 1987 as part of basic studies on the redox chemistry of dimetallic coordination compounds, but later it was discovered that this compound could have a potential use in the treatment of HIV because it inhibits CXCR4, one of several chemokine receptors that certain strains of HIV uses to infect T-cells. The lack of oral availability and cardiac disturbances prevented the development of plerixafor for this indication, but further studies led to its approval as a subcutaneous injection for the previously mentioned cancer patients.[74]



Plerixafor

5 ENDOGENOUS INHIBITORS OF ANGIOGENESIS

Some endogenous compounds, including endostatin, angiostatin, thrombospondin-1 (TSP-1), and the platelet-derived factor 4 (PF-4), are angiogenesis inhibitors. Endostatin is an inhibitor of integrin $\alpha v\beta 3$ whose activity can be mimicked by short arginine-rich peptides.[75] It is a polypeptide of 184 amino acids

that corresponds to the globular domain found at the C-terminal fragment of type XVIII collagen and may be obtained by recombinant DNA technology. In early trials, endostatin was shown to be safe and exhibited low toxicity without development of drug resistance over time, reaching phase II clinical trials in patients with advanced neuroendocrine tumors.[76] Endostar is a new recombinant endostatin with an additional 9-amino acid sequence that is more stable, easier to manufacture, and more potent;[77] it is currently in clinical trials. Endostar also played an efficient anticancer role in malignant pleural effusion, a common complication of lung cancer, through its suppressive effect on angiogenesis and lymphangiogenesis.[78] Since 2005, when it was approved by the Chinese State Food and Drug Administration, it has been used in China for the treatment of cancer.

Angiostatin is a 57-kDa fragment of plasmin, which is in turn a fragment of plasminogen. It was studied in combination with paclitaxel and carboplatin in patients with NSCLC,[79] but it is no longer in clinical trials.

The first and most studied naturally occurring tumor growth suppressor and angiogenesis inhibitor is the extracellular glycoprotein trombospondin-1 (TSP1). Its expression in adult organisms is limited to the sites of tissue remodeling, where it determines cell phenotype and ECM structure and composition. Genetic mutations of tumor cells are associated with decreased expression of TSP1. The core of TSP1 contains three thrombospondin type 1 repeats (TSRs) that interact with proteins such as the CD36 receptor (cluster of differentiation 36) on the surface of endothelial cells. This interaction leads to the expression of the Fas ligand (FasL), a transmembrane protein that belongs to the TNF family and induces apoptosis through caspase activation.

Peptidomimetics of TSRs showed their ability to block angiogenesis in early stage clinical trials.[80] For instance, the heptapeptide NAc-Gly-Val-D-Ile-Thr-Arg-Ile-ArgNHEt, a structurally modified fragment derived from the second type-1 repeat of TSP-1, showed antiangiogenic activity but its therapeutic utility could not be demonstrated because of its very short half-life. Structural modifications (Figure 11.23) led to the nonapeptide ABT-526 as a promising lead, and substitution of D-alloIle in place of D-Ile provided ABT-510 (Ac-Sar-GV-DalloIle-T-Nva-IRP-ethylamide) with increased water solubility and slower clearance.[81] Both peptidomimetics exhibited antiangiogenic properties and tumor growth inhibition in preclinical models, and ABT-510 entered phase II clinical trials. Unfortunately, its evaluation for the treatment of metastatic melanoma did not demonstrate definite clinical efficacy.[82,83] The substituted octapeptide ABT-898 (*N*-acetyl-glycine-valine-D-alloisoleucine-serine-glutamine-isoleucine-arginine-proline-ethylamide) is a second-generation mimetic of TSP-1 with a greatly increased potency over that of ABT-510 as well as slower clearance;[84] it is promising for the treatment late-stage ovarian cancer.

Another possible approach to antiangiogenic therapy related to endogenous inhibitors is based on the observation that certain orally active small molecules raise the plasma levels of endogenous angiogenesis inhibitors by increasing their expression or by alternative means, such as mobilization from matrix or platelets.[85] These molecules include celecoxib, doxycycline, and rosiglitazone, which can increase serum endostatin (celecoxib) or thrombospondin-1 expression. Celecoxib (Celebrex®), a well-known anti-inflammatory drug acting by cyclooxygenase-2 inhibition, has shown potential activity against most carcinomas, being the only nonsteroidal anti-inflammatory drug that has been approved for adjuvant treatment of patients with familial adenomatous polyposis. It has been demonstrated that its antiproliferative effect is not extended to other coxibs, indicating the lack of a role for COX-2.[86] Because its side effects restrict its generalized use for cancer therapy, celecoxib-loaded nanoparticles are being studied as a possible effective and safe mode of using celecoxib for colon cancer therapy.[87]

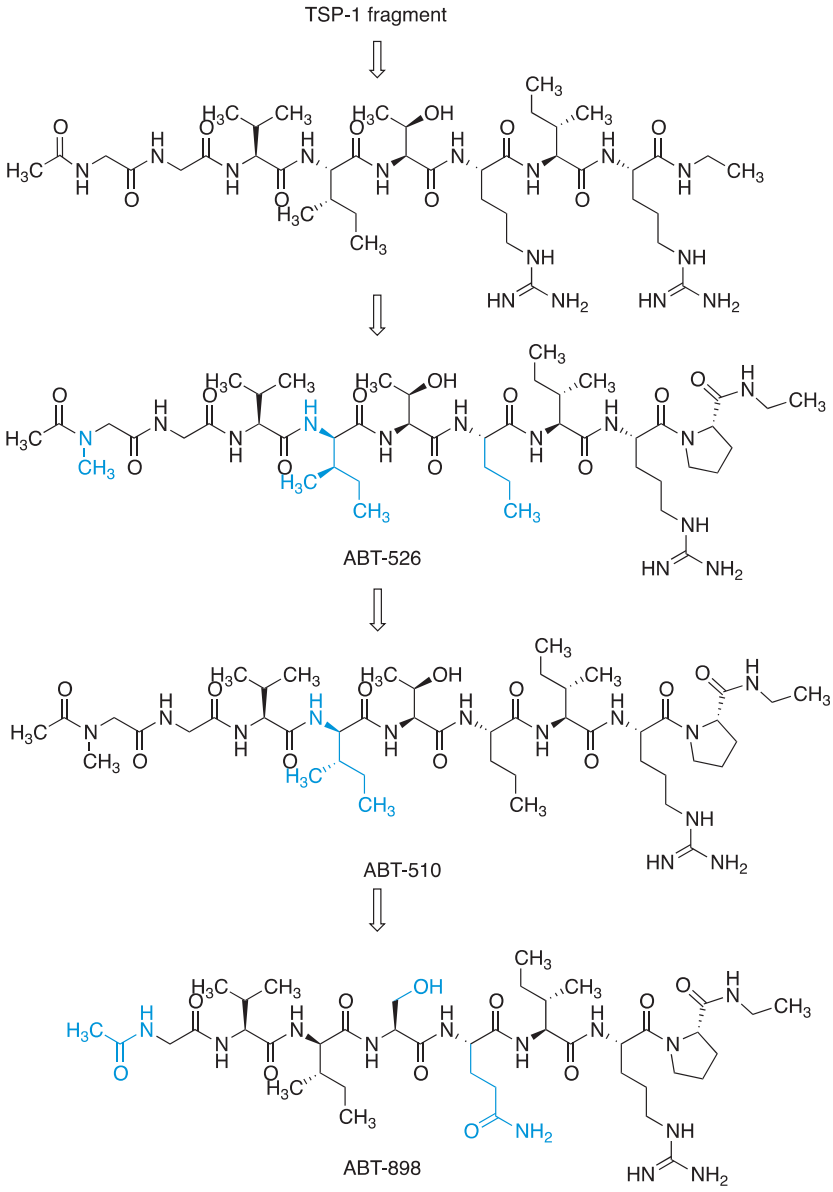
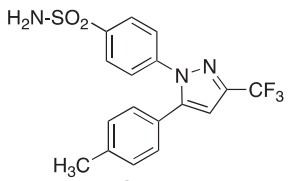


FIGURE 11.23

Antiangiogenic peptides designed as thrombospondin mimetics.

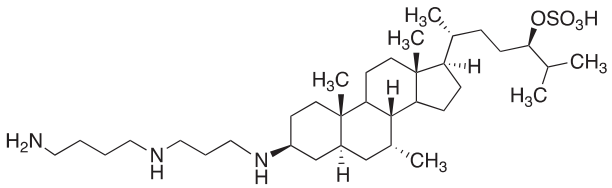


Celecoxib

6 MISCELLANEOUS ANTIANGIOGENIC COMPOUNDS

6.1 SQUALAMINE

Squalamine is an antiangiogenic aminosteroid isolated from tissues of several species of dogfish shark (*Squalus acanthias*). When discovered in 1993, it was reported to exhibit broad-spectrum antibiotic and fungicidal activity, being also an inductor of osmotic lysis in protozoa. In 2005, the FDA gave it fast track status for the treatment of "wet" age-related macular degeneration, which is mediated by inhibition of the choroidal neovascularization associated with this eye condition. In addition, squalamine has been the subject of clinical studies for several types of cancer.[88]



Squalamine

Similarly to the previously mentioned neovastat, squalamine interrupts and reverses multiple facets of the angiogenic process, such as VEGF activity, but it also induces endothelial cell inactivation and apoptosis through inhibition of integrin expression and disruption of cytoskeletal formation. In addition, it interacts with calmodulin and possibly other signaling pathways, and it specifically inhibits the isoform NHE3 of the Na^+/H^+ exchanger protein present on cell surfaces,[89] leading to changes in intracellular pH and subsequent inhibition of MAPK activity (Figure 11.24).[90]

6.2 THALIDOMIDE AND ITS ANALOGS

Thalidomide (Thalomid®) was introduced in the 1950s as a sedative prescribed for nausea and insomnia in pregnant women. Later, it was found to be the cause of severe birth defects in children whose mothers had taken the drug during the first trimester of pregnancy. In 1965, it was serendipitously discovered that thalidomide was effective at improving the symptoms of patients with erythema nodosum leprosum (ENL), and it was approved for this use in 1998. In 1994, thalidomide was found to inhibit angiogenesis through a complex mechanism that includes inhibition of the synthesis of TNF-α, which has a role in angiogenesis by upregulation of the expression of the endothelial integrin and VEGF growth factors, among others. In addition to the inhibition of angiogenesis, thalidomide is also involved

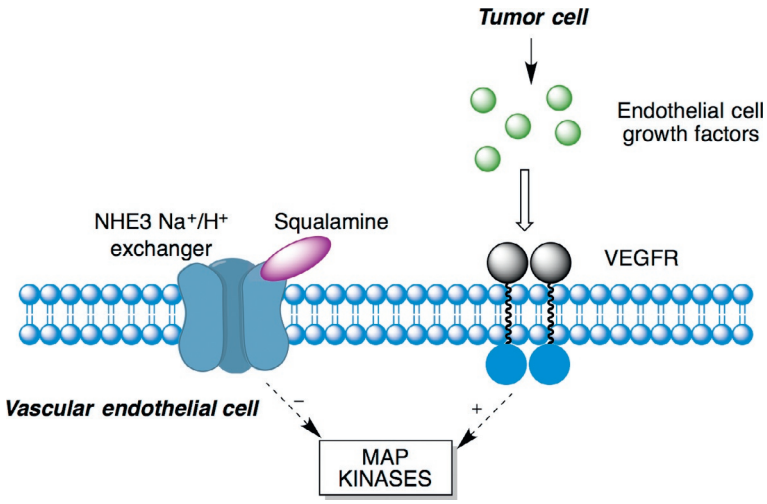
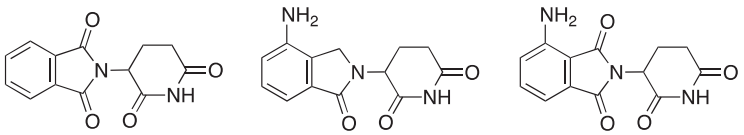


FIGURE 11.24

Inhibition of the NHE3 Na^+/H^+ exchanger by squalamine.

in other mechanisms, such as apoptosis induction.[91] On these bases, it was approved by the FDA in 2006 for the treatment of MM[92] and ENL in association with dexamethasone[93] and cytotoxic agents such as cyclophosphamide.[94] The EMA approved thalidomide in 2008 to treat MM in combination with prednisone[95] and/or melphalan. Orphan indications for this drug include primary brain malignancies, Kaposi's sarcoma, and myelodysplastic syndrome. Thalidomide is also a lead compound in the development of a class of drugs known as immunomodulatory drugs (IMIDs®).[96]

Since it was demonstrated that inhibition of angiogenesis by thalidomide requires prior metabolic activation,[97] a large number of potential metabolites have been evaluated. Lenalidomide (CC-5013, Revlimid®) is a thalidomide analog that was approved by the FDA in 2006 in combination with dexamethasone for the treatment of MM patients who have received at least one prior therapy.[98] It has also shown efficacy in the hematological disorders known as the myelodysplastic syndromes and has undergone numerous clinical trials alone or in combination with other drugs. Pomalidomide (CC-4047, Pomalyst®, Imnovid®) is another antiangiogenic and immunomodulator thalidomide analog that entered phase II assays for prostate cancer and was approved in 2013 by the the FDA and the EMA as a treatment for some cases of MM.[99,100] The *S*-isomer of pomalidomide is the more potent enantiomer, but it has been shown to undergo rapid racemization in human plasma—a finding that supports the development of these drugs in their racemate form.[101]

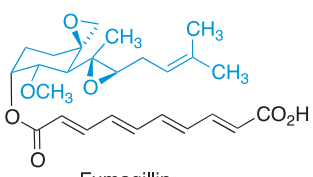


6.3 FUMAGILLIN ANALOGS

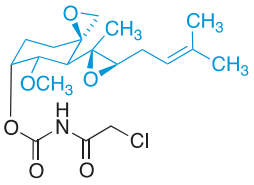
Among several natural products with activity as angiogenesis inhibitors,[102] one of the best studied is fumagillin, a mycotoxin isolated from *Aspergillus fumigatus* that is one of the most potent angiostatic agents widely employed as an antifungal in honeybees infected with *Nosema apis*. Fumagillin is a covalent inhibitor of methionine aminopeptidase-2 (MetAp2),[103] a cytosolic metalloenzyme and one of two methionine aminopeptidases responsible for protein stability and post-translational modifications that catalyze the hydrolytic removal of N-terminal methionine residues from nascent proteins. Current data support the hypothesis that MetAp2 may play a central role in endothelial cell proliferation and tumorigenesis, although the mechanism by which inhibition of MetAp2 function inhibits angiogenesis has not been clearly defined.[104] Fumagillin also inhibits the expression of the transcription factor ETS1, one member of the E26 transformation-specific transciption factors, which regulates the expression of VEGFs.[105]

Among the studied fumagillin analogs, the covalent binding inhibitor TNP-470 progressed to clinical trials, showing significant antitumor activity in cervical, breast, and lung cancers. However, its dose-limiting neurotoxicity and short half-life prevented its incorporation into further clinical studies.[106] In order to prevent their access to the central nervous system through the blood–brain barrier conjugate prodrugs have been developed.[107] These conjugates are accumulated selectively in tumor vessels because of the enhanced permeability and retention effect (see Chapter 13, Section 4).[108] Caplostatin is a water-soluble conjugate of TNP-470 in which this active compound is bonded to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer through a Gly-Phe-Leu-Gly linker. In the oral formulation of TNP-470 known as lodamin, this drug was conjugated to monomethoxy polyethylene glycol–polylactic acid to form an amphiphilic molecule that forms micelles in aqueous solution. These nanopolymeric micelles can be absorbed by the intestine and selectively accumulate in tumors (see Chapter 13, Section 7.2).[109]

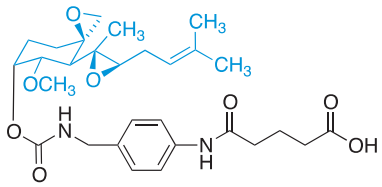
XMT-1107 is a conjugate of the fumagillol derivative XMT-1191 with Fleximer®, a biodegradable, hydrophilic polymer that does not cross the blood-brain barrier. This conjugate has entered clinical trials for advanced solid tumors.



Fumagillin



TNP-470



XMT-1191

These compounds bind covalently to their target by alkylating the His-231 residue through its ring epoxide (Figure 11.25).[110]

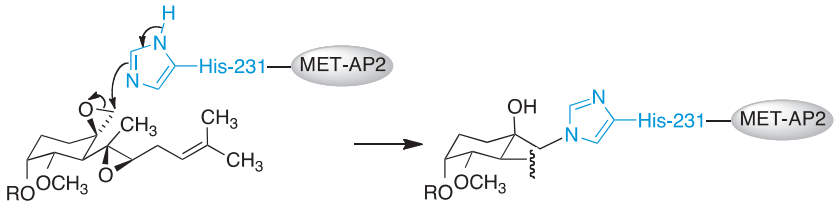
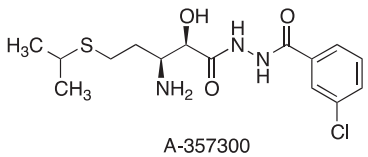


FIGURE 11.25

Covalent inhibition of methionine aminopeptidase-2 (MetAp2) by fumagillin and its analogs.

The reversible MetAp2 inhibitor A-357300 was designed from the aminopeptidase inhibitor ubenimex through iterative optimization of 2-hydroxy-3-amino amide series with the aid of crystal structures of the enzyme–inhibitor complexes and parallel synthesis. This compound has shown single-agent efficacy against a variety of human cancer xenografts, including neuroblastoma.



A-357300

7 DRUGS TARGETING CANCER STEM CELLS

The current therapies for cancer have shown a lack of long-term efficacy and limited survival benefits when used for most advanced stage cancers because of their induced toxicity on normal cells and their failure to target cancer stem cells (CSCs). These are a class of cancer cells with stem cell-like characteristics, such as self-renewal, and are capable of differentiating to give rise to all cell types found in a particular cancer. They are associated with chemo- and radioresistance, and they have the potential to generate a tumor when present in small numbers compared with thousands of normal cancer cells usually required. CSCs have been identified in hematopoietic cancers and in solid tumors. They are recognized by the expression of cell surface markers (e.g., CD24, CD44, or CD133); aberrant Wnt, Notch, or Hedgehog signal pathways; and other signatures such as a high aldehyde dehydrogenase ($ALDH^+$) activity or the presence of the ABCG2 transporter. It has been recently discovered that these cells show autofluorescence due to riboflavin accumulation in membrane-bound cytoplasmic structures bearing ATP-dependent ABCG2 transporters. This intrinsic autofluorescent phenotype may permit an easy procedure to identify and characterize CSCs.[111] Targeting CSCs offers a promising approach to improve cancer survival or even to cure cancer;[112] however, although some progress has been made in this direction, an effective therapy based on this concept is still not available.[113]

7.1 WINGLESS/β-CATENIN SIGNALING

Signaling by the Wnt family of secreted glycolipoproteins via the transcription co-activator β-catenin controls embryonic development and adult homeostasis. It was recognized for its function in embryonic development when genetic mutations produced abnormal fruit fly embryos. Later research found that the genes responsible for these abnormalities also influenced breast cancer development in mice. The clinical importance of this pathway has been demonstrated by mutations that lead to a variety of diseases, including breast and prostate cancer and glioblastoma. Because Wingless/β-catenin (Wnt/β-Cat) signaling is a key feature of epithelial cancers and seems to be critical for metastasis and EMTs, its specific modulation may help to eliminate drug-resistant cancer stem cells.[114]

All Wnt signaling pathways are activated by the binding of a Wnt protein ligand to a receptor belonging to the Frizzled family (FRZ), which passes the biological signal to the disheveled protein (DSH) inside the cell. In the *on* state, Wnt is associated with membrane-bound FRZ receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), leading to sequestration of the β-catenin phosphorylation complex, which is composed of DSH proteins, adenomatous polyposis coli (APC), axin, and GSK-3β. Consequently, phosphorylation of β-Cat is suppressed, and the free β-Cat escapes from the degradation and translocates from the cytoplasm to the nucleus, where it binds to the TCF/LEF (tumor cell factor/lymphoid enhancer factor) with the participation of a coactivator called CBP, activating the transcription of several target genes such as cyclin D1, c-myc, c-Jun, and fibronectin (Figure 11.26a). In the *off* state, the Dickkopf-1 (DKK1) protein, which is especially expressed in

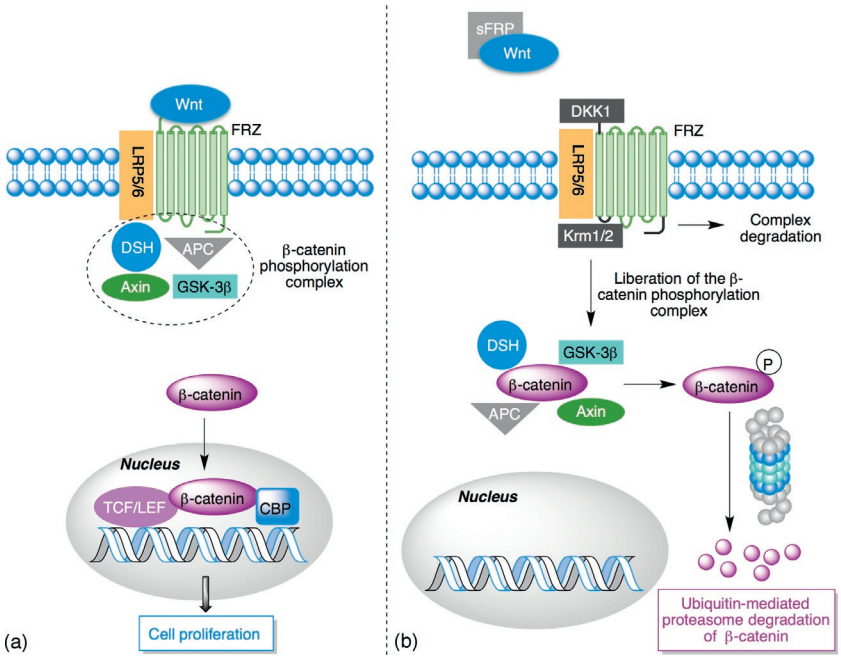


FIGURE 11.26

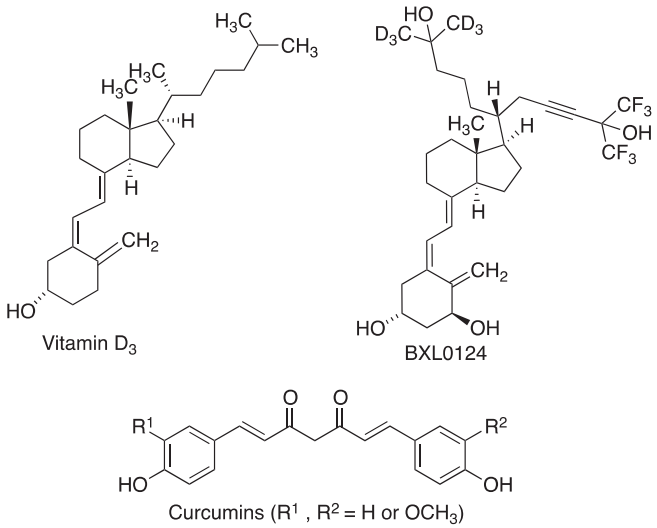
Processes taking place in the Wnt signaling pathway: (a) *on* state; (b) *off* state.

myeloma tumor cells, interacts with LRP5/6, leading to association of the Kremen 1/2 (Krm1/2) protein with the receptor. The subsequent FRZ–LRP5/6 degradation allows for the axin, DSH, APC, and GSK-3β complex to phosphorylate β-Cat, which is degraded by the proteasome. In addition, secreted Frizzled-related glycoproteins (sFRPs) function as soluble antagonists of Wnt signaling by binding directly to Wnt proteins and preventing their interactions with Frizzled receptors (Figure 11.26b).

This signaling is always disrupted in colorectal tumors and other cancers, in which mutations in the *APC* gene are common, resulting in β-Cat stabilization. An APC deficiency or β-Cat mutations that prevent its degradation can lead to excessive stem cell renewal and proliferation, predisposing the cells to the formation of tumors.

Much effort has been made to identify small molecules capable of disrupting aberrant Wnt/β-Cat pathway responses. Among them, natural compounds such as vitamins A and D_3 and their derivatives compete with β-Cat/TCF interactions and allow E-cadherin to relocate β-Cat to the membrane.[115] Ligand-activated vitamin D receptor competes with TCF-4 for β-Cat binding, thereby reducing levels of c-Myc, peroxisome proliferator-activated receptor, TCF-1, and the CSC marker CD44. Vitamin D reduces the incidence of human breast, prostate, and colon cancers and induces the apoptosis and cell cycle arrest of various cancer cells. Its analog, BXL0124, also decreases the expression of CD44 through vitamin D receptor and p53-dependent mechanisms.[116]

Curcumin, a mixture of dietary polyphenols that possesses anti-inflammatory and antioxidant activities, has been studied as a chemopreventive agent in several cancer models. It is a modulator of the ABCG2 transporter and can induce caspase-3-mediated cleavage of β-Cat, leading to inactivation of Wnt/β-Cat signaling. It also decreased β-Cat/TCF transcription activity in all tested cancer cell lines, which has been attributed to the reduction of the nuclear concentration of both proteins.[117]



Some nonsteroidal anti-inflammatory drugs, such as aspirin and sulindac, interfere with Wnt signaling by directly inhibiting the Wnt target COX2, whereas others, such as celecoxib, promote the degradation of TCF.

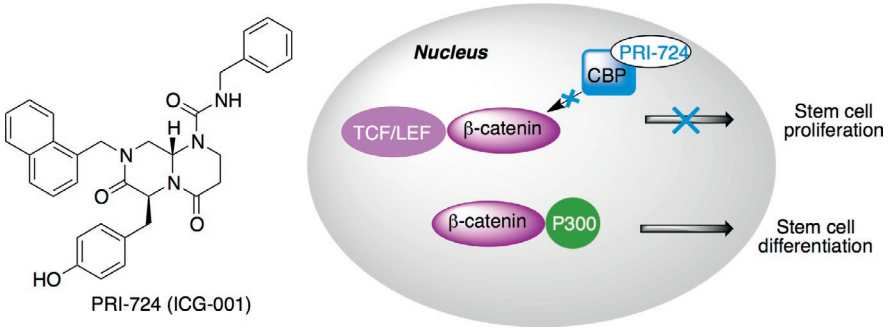
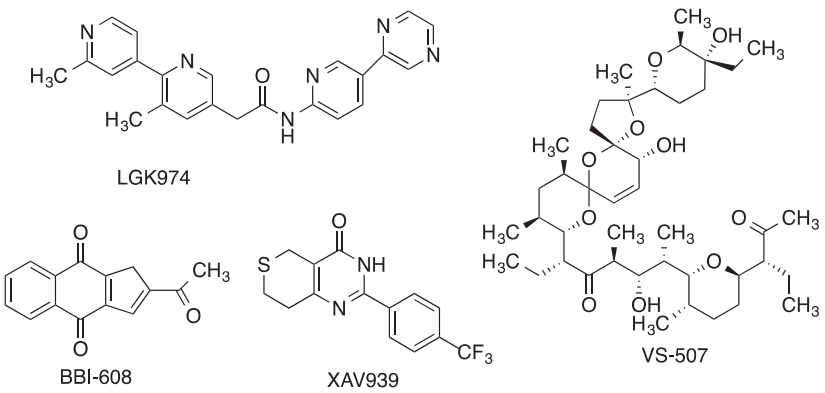


FIGURE 11.27

Mechanism of action of PRI-724.

PRI-724 (ICG-001) has entered clinical trials (phases I and II). It inhibits the interaction of β-Cat with its coactivator CBP (cyclic AMP response element-binding protein), which would promote stem cell proliferation. It increases instead the binding of β-Cat to another protein called P300, which promotes stem cell differentiation (Figure 11.27).[118]

Some additional compounds, identified through high throughput screening that act on this pathway are CWP232291 (structure not publicly available), which was identified in a high throughput screen for inhibitors of Wnt/β-catenin mediated transcriptional activity and promotes β-Cat degradation; LGK974, an inhibitor of a membrane-bound *O*-acyltransferase that prevents palmitoylation of Wnt;[119] BBI-608, whose development was halted at phase III clinical trials for colorectal cancer; the polyether natural product VS-507, which reduces the expression of receptors LRP5/6; and XAV939, that stimulates β-Cat degradation by stabolizing axin through inhibition of the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2. Some additional inhibitors of Wnt/β-Cat signaling have entered preclinical trials, including monoclonal antibodies against Wnt glycolipoproteins;[120] small interfering RNAs against sFRPs; and recombinant adenoviruses that constitutively express the β-Cat binding domain of APC, enabling its tumor-suppressor activity and preventing the β-Cat translocation to the nucleus.



7.2 INHIBITORS OF THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway plays a critical role in cell fate, tissue patterning, and morphogenesis, as well as in the maintenance of the stem cells in glioblastoma, breast cancer, and some other tumors. The Notch1 receptor is a transcriptional activator of multiple genes, and its upregulation facilitates the activation of a number of signals leading to apoptosis inhibition and proliferation activation (Figure 11.28). This pathway has been correlated with increased tumor cell growth and survival.[121,122]

Notch inhibition can be achieved at different levels. First, inhibitors of the SERCA (sarco/endoplasmatic reticulum calcium ATPase) channels have been identified as indirect Notch1 inhibitors because the early maturation of Notch1 in the endoplasmic reticulum, in which the receptor is processed by proteases, requires SERCA-mediated Ca^{2+} influx for their proper folding.[123] The best-known SERCA inhibitors are thapsigargin, a toxin isolated from *Thapsia garganica*, and its analog 12-ADT that is contained in the prodrug G-202 specifically activated in prostate cancer cells (see Chapter 13, Section 2.1).

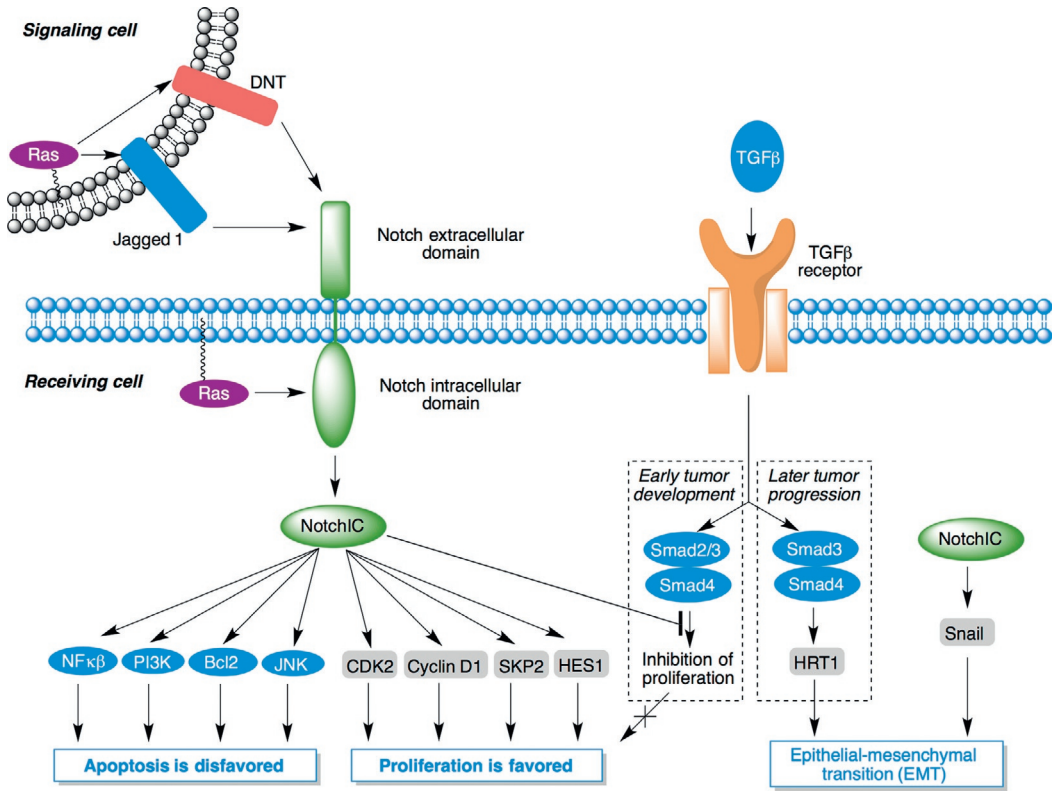
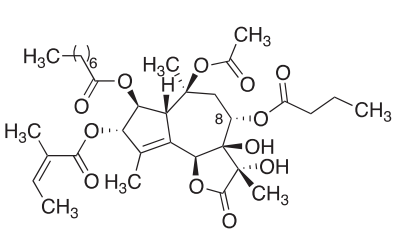
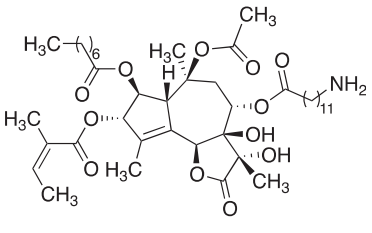


FIGURE 11.28

The Notch signaling pathway.





In resting conditions, Notch1 is located in the membrane as a heterodimeric complex comprising an N-terminal extracellular subunit that interacts with different ligands, a transmembrane portion, and an intracellular subunit. The binding of ligands induces a conformational change that in turn triggers the cleavage of the heterodimer by α-secretase, a metalloprotease at the cell surface, followed by a second proteolytic cleavage catalyzed by the γ-secretase complex in the transmembrane region. This releases the intracellular domain into the cytosol and allows its translocation into the nucleus, where it activates gene expression through interactions with different coactivators (Figure 11.29). The proteolytic steps are similar to those involved in the generation of the β-amyloid protein (which plays a major role in Alzheimer's disease), from the amyloid precursor protein. This mechanism allows targeting the Notch pathway by blocking the proteolytic activation of Notch receptors with γ-secretase inhibitors (GSIs) previously studied as potential therapies for Alzheimer's disease.[124,125]

One of these compounds is MK-0752, which was studied for the treatment of acute lymphoblastic leukemia of T cells, following the observation that activating mutations in the *NOTCH1* gene have been

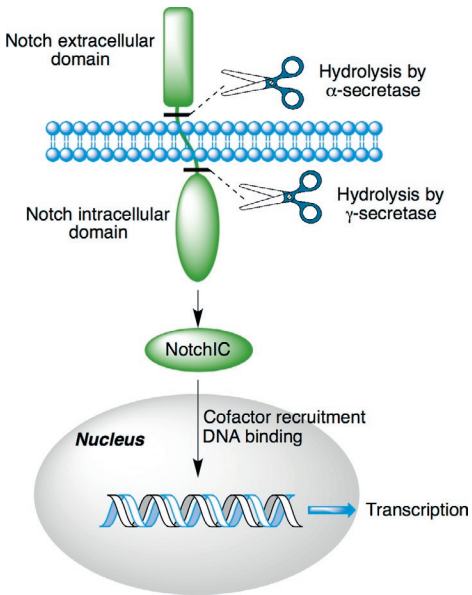
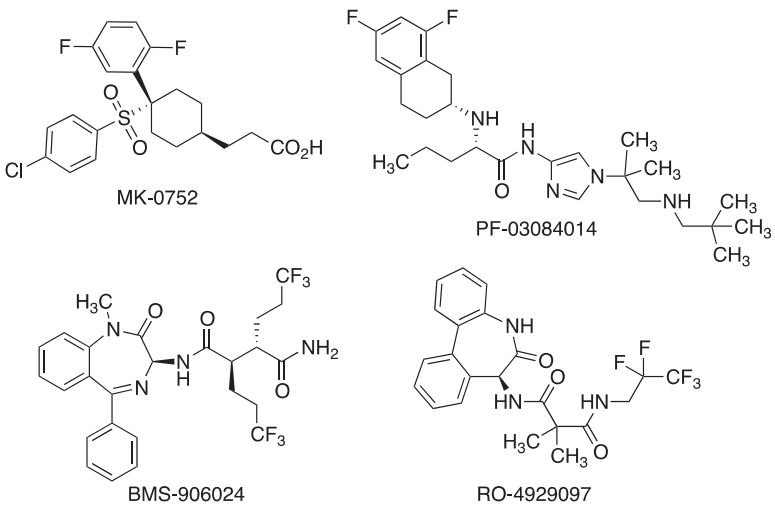


FIGURE 11.29

Hydrolytic activation of the Notch intracellular domain.

found in more than 50% of patients with this disease. Although a phase I clinical trial in these patients revealed an unfavorable toxicity profile related to inhibition of Notch signaling in the gut there are several ongoing clinical studies involving MK-0752—alone or in combination with tamoxifen, docetaxel, or letrozole—in breast cancer.[126]

Other studies have shown that intermittent doses reduce the toxicity associated with PF-03084014, another selective GSI,[127] and that combination therapies and glucocorticoid treatment increase the antileukemic effects of different pan-Notch inhibitors, ameliorating its intestinal toxicity. The combination of PF-03084014 with docetaxel demonstrated early stage synergistic apoptosis, which provides a strong preclinical rationale for its clinical utility to improve taxane therapy.[128] The benzodiazepine pan-Notch inhibitor BMS-906024[129] is other GSI that has entered clinical trials alone or in combination to treat leukemia and breast, lung, and colon cancers. RO4929097 is another GSI that has entered clinical trials of patients with refractory metastatic or locally advanced solid tumors.[130]



The limitations of GSIs in the clinic have suggested the use of synthetic peptides to block the Notch transcriptional complex directly in the cell nucleus[131] or the use of highly specialized antibodies that block the receptor in an "off" conformation.[132] Several of these antibodies have undergone phase I/II clinical trials, including OMP-59R5, OMP-52 M51, and MEDI0639.

Furthermore, because activation of Notch receptors by the vascular-specific ligand DLL4 (delta-like 4) stimulates the proteolytic cleavage of the Notch intracellular domain, targeting DLL4 provides an alternative way to inhibit the Notch pathway.[133] Demcizumab (OMP-21 M18) is a humanized monoclonal antibody directed against the N-terminal end of DLL4 that received orphan drug status from the FDA in 2014 for the treatment of pancreatic cancer.

7.3 HEDGEHOG SIGNALING/SMO RECEPTOR INHIBITORS

Hedgehog (Hh) signaling has been found to play multiple roles in the proper development of embryonic cells and adult organ homeostasis and repair, and its activation is linked to tumorigenesis of several cancers.[134] Data from many tumors, including glioblastoma, pancreatic adenocarcinoma, breast

cancer, MM, and CML, suggest that Hh signaling regulates cancer stem cells. This pathway activates the 7-pass transmembrane protein Smoothended (Smo), a G protein-coupled receptor whose activation results in the nuclear translocation of the Hh transcription factors Gli1 and Gli2, which initiate transcription of Hh-responsive genes. It is inactive in the absence of ligands because Smo is inhibited by the 12-pass transmembrane spanning receptor Patched 1 (Ptch 1). Its activation occurs when Ptch is bound by one of the Hh family of ligands, such as Sonic Hh (Shh).

Among Hh antagonists, the most developed are those targeting Smo. The discovery of cyclopamine, a highly teratogenic natural product, and the subsequent assignment of its activity to the Hh pathway, paved the way for the rapid development of synthetic inhibitors with druglike properties and improved bioactivity.

The semisynthetic derivative saridegib (IPI-926), which showed greater chemical stability, solubility, potency, selectivity, and bioavailability compared to cyclopamine,[135] entered clinical trials, but its development was interrupted. In order to identify small-molecule Hh antagonists of a different chemical class, a high-throughput screen based on murine embryonic fibroblast cells containing a plasmid with a luciferase reporter gene was performed. When these cells are stimulated with the ligand Shh, the luciferase activity can be optically measured, as can the reduction of this signal induced by antagonists of the Hh pathway. As shown in Figure 11.30, hit-to-lead optimization of the screening hits produced compound **11.1** that, after studying the replacement of the benzimidazole ring by a broad variety of heterocycles, led to the 2-piridylamide **11.2**, which eventually led to GDC-0449 (vismodegib, Erivedge®) following examination of a wide variety of amide substituents.[136]

Vismodegib is a competitive antagonist of the Smo receptor that was approved by the FDA for the treatment of basal cell carcinoma in 2012; it is also undergoing clinical trials for metastatic colorectal cancer, small cell lung cancer, advanced stomach cancer, pancreatic cancer, medulloblastoma, and chondrosarcoma.[137] Erismodegib (LDE-225, NVP-LDE225) is a selective, orally bioavailable Smo antagonist that inhibits the Hh- and Smo-dependent proliferation and is currently in a phase II trial in advanced basal cell carcinoma and in a phase I trial in medulloblastoma. Erismodegib monotherapy in chemonaive tumors seems to have little effect, but it is highly effective in preventing the recurrence

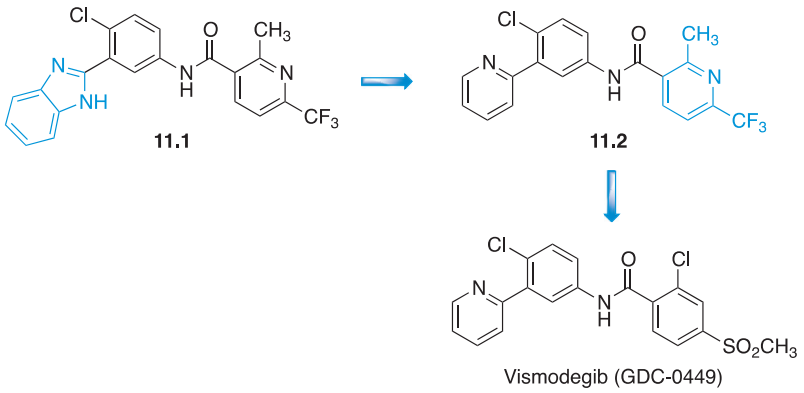
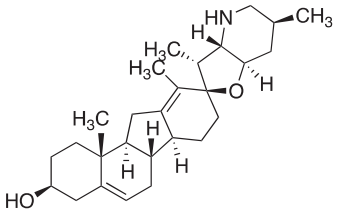


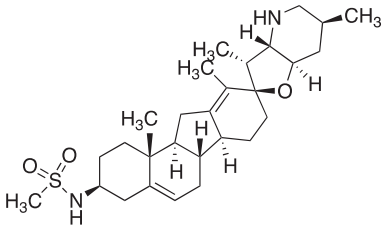
FIGURE 11.30

Development of vismodegib.

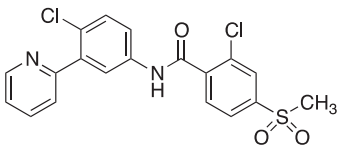
of residual tumors following chemotherapy. NVP-LEQ-506 is being investigated in a phase I trial of patients with advanced solid tumors.[138]



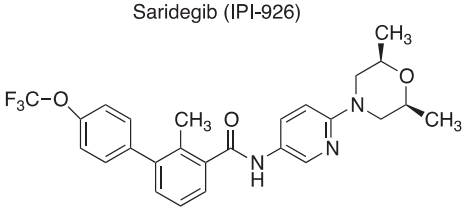
Cyclopamine



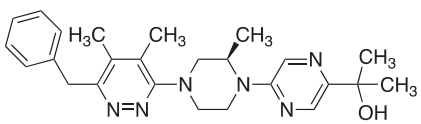
Saridegib (IPI-926)



Vismodegib (GDC-0449, Erivedge®)



Erismodegib (LDE225, NVP-LDE225)



NVP-LEQ506

7.4 MESENCHYMAL STEM CELL-MEDIATED GENE THERAPY FOR CANCER

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes. After their systemic delivery, MSCs are integrated into the tumor sites mostly by the chemokine receptor CXCR4. This property allows for engineered MSCs targeting a given tumor to potentially be used for *in situ* delivery of therapeutic proteins, genes, or replicating oncolytic viruses.[139]

8 INHIBITORS OF ONCOGENIC PROTEIN–PROTEIN INTERACTIONS

Although drug development has been mainly focused on enzymes and receptors, these targets represent only approximately 1% of the proteins that comprise the human proteome.[140] Furthermore, only a few of the oncogenes encode proteins that are receptors or have enzymatic activities, whereas many others participate in protein–protein interactions (PPIs) that are different from those of noncancer proteins[141] and play essential roles in linking networks that relay oncogenic signals in cancer cells. For instance, mdm2–p53 and CDK4–Rb interactions are involved in neutralizing tumor-acquired mechanisms to evade growth suppression,[142] human papillomaviruses induce tumors through the binding of their

oncoproteins E7 and E6 to p53 and Rb, and the catalytic activity of telomerase reverse transcriptase (TERT) dimers enables replicative immortality.

Proteins are key components of the cellular machinery, and most cellular functions are executed by groups of them acting in concert. In contrast to the well-defined and normally hydrophilic ligand-binding cavities observed in the crystal structures of enzymes and G protein-coupled receptors, the affinity between two proteins is due to numerous weak interactions between large interfaces, which are commonly hydrophobic and lack deep grooves where a small molecule can dock. Therefore, it is inherently difficult for a small molecule to compete for binding on such an extensive surface and such a large number of complementary interactions. Because the interfaces have been regarded as essentially flat and featureless and therefore undruggable, interference with protein–protein interactions via small molecules has long been considered impossible. Thus, PPIs were viewed as being able to be targeted only with large molecules (e.g., peptides and antibodies). However, the analysis of three-dimensional (3D) structures obtained by X-ray crystallography or NMR[143] has shown that PPIs are normally mediated by a centralized region of "hot spot" residues, with comparable dimensions to those of a small organic molecule. PPIs involve small secondary structural domains such as α-helices, β-sheets, and β-strands, which may be used as templates in the design of small molecules acting as structural and functional mimics.[144] In particular, α-helix-mediated PPIs are present in a wide array of cellular signaling pathways, and for this reason, short helical peptides are common PPI modulators. These recent advances in the knowledge of PPI surfaces have led to the identification of protein interaction hubs and nodes that are critical for cancer and have thus become promising therapeutic targets, opening a new paradigm in anticancer drug development.[145] Several small-molecule modulators of PPIs are already in clinical trials.[146]

The scarcity of natural products active as PPI modulators is a problem for structure-based drug design, although there are some promising examples (e.g., rapamycin for mTOR and taxol for tubulin). However, diversity oriented synthesis, a methodology that goes beyond total synthesis and combinatorial chemistry and in which the design of libraries takes into consideration relevant parameters that define compound properties such as Lipinski rules or molecular weight,[147] has been successfully used in the discovery of several lead compounds targeting α-helix-mediated PPIs. In the fragment-based screening approach, X-ray crystallography or NMR are used to identify molecular fragments with binding activity for a target protein, and these fragments are later built into a druglike compound.[148] The discovery of X-linked IAPs (XIAPs)[149] and ligands of K-Ras are examples of the successful application of NMR fragment screening.[150] In pharmacophore- or structure-based approaches, the 3D computational screening of compound libraries attempts to identify compounds whose conformation and chemical structure match the requirements of a pharmacophore model and have a high binding affinity.[151]

The high-throughput screening (HTS) techniques most widely employed for characterizing interference with PPIs are fluorescence polarization, which measures the change in emitted polarization signals on association of a small fluorescent solved molecule with a relatively large binding partner, and Föster/fluorescence resonance energy transfer (FRET), in which the energy absorbed by a donor fluorophore is transferred to a coupled acceptor fluorophore inducing a FRET signal.

8.1 ANTICANCER DRUGS ACTING ON APOPTOTIC SIGNALING PATHWAYS

8.1.1 Programmed Cell Death and Its Relevance in Cancer

Apoptosis is normally defined as type I programmed active cell death (PCD). At first sight, cell death might be viewed as a pathological phenomenon; however, each day, approximately 50–70 million defective cells in a human body undergo apoptosis. Two other forms of PCD are autophagy (PCD type II)

and necrosis (PCD type III). The main morphological feature of apoptosis is shrinkage of the cell and nuclear fragmentation, whereas in autophagy, cells generate energy by digesting their own organelles and macromolecules and necrosis is characterized by the swelling of the cell and its organelles. These alternative mechanisms leading to cell death play a fundamental role in development, environmental adaptations, and survival, and are carefully regulated.[152]

The apoptosis mechanism involves many pathways along which different defects can occur and plays a pivotal role in the pathogenesis of many diseases. It has been estimated that either too little or too much cell death contributes to approximately half of all medical illnesses, for many of which an adequate therapy is lacking. In some diseases, such as in degenerative processes, the problem is due to too much apoptosis, whereas in others such as cancer too little apoptosis occurs. In this case, inhibitors of apoptotic signals (IAPs) are overexpressed, leading to malignant transformation of the affected cells, tumor metastasis, and resistance to anticancer drugs. Evasion from apoptosis mainly occurs by a disrupted balance of pro-apoptotic and anti-apoptotic proteins, a reduced caspase function, or impaired death receptor signaling. Genes encoding IAPs can act as oncogenes, although their effect is not achieved by increasing the rate of cell proliferation but, rather, by reducing the rate of cell death. Several genes involved in the apoptosis process, especially the *BCL2* (B-cell lymphoma protein 2) and the caspase family genes, have been found to be defective in cancer cells.[153]

The majority of chemo/radiotherapy strategies inhibit cancer cell growth by activating cell death pathways, such as apoptosis, necrosis, and autophagy. However, as the disease progresses, cancer cells can acquire a variety of genetic and epigenetic alterations, which leads to dysregulation of cell death-associated signaling pathways and chemo/radioresistance. Tumor cells generate increased levels of reactive oxygen species (ROS), which elevate stress signaling and oncogenic stimulation, promoting tumor cell proliferation that increases the demand for ATP from the inefficient glycolytic pathway in mitochondria and affects greater leakage of injury-causing electrons. Tumor cells survive this sustained stress by increasing ROS tolerance through the upregulation of redox-buffering systems, including glutathione, thioredoxin, superoxide dismutase, peroxidase, and catalase. However, because these adaptive mechanisms have limited capacities, some specific therapeutic agents are aimed at further increasing oxidative stress. Bleomycin, doxorubicin, bortezomib, or cisplatin, among other anticancer drugs, disrupt the mitochondrial respiratory chain and promote the leakage of electrons to further increase ROS and induce apoptosis. Other ROS-inducing agents are in clinical trials. In this context, it is worth noting that the response to those anticancer agents that act by damaging DNA or cellular signaling pathways related to stress induction requires the existence of an intact apoptosis machinery. Therefore, defects in any part of the apoptotic pathways will lead to tumor resistance, including cross-resistance phenomena to structurally unrelated drugs that share the same pathway. Several mechanisms may be responsible for defects in apoptosis, including the following:

1. Failure to detect DNA damage
2. Decrease in levels of pro-apoptotic factors such as p21
3. Defects in the activation of pro-apoptotic proteins or receptors such as caspases
4. Upregulation of proteins involved in survival pathways such as EGFR, mdm2, MDM4, Bcl-2, MCL-1, and Bcl-xl

In this context, this section is devoted to those anticancer drugs that are aimed at specific targets in the apoptotic pathways.[154] Two commonly described initiation pathways to apoptosis eventually lead to the execution phase: the intrinsic or mitochondrial pathway, triggered by internal signals, and the extrinsic or death receptor pathway, triggered by external signals (Figure 11.31).

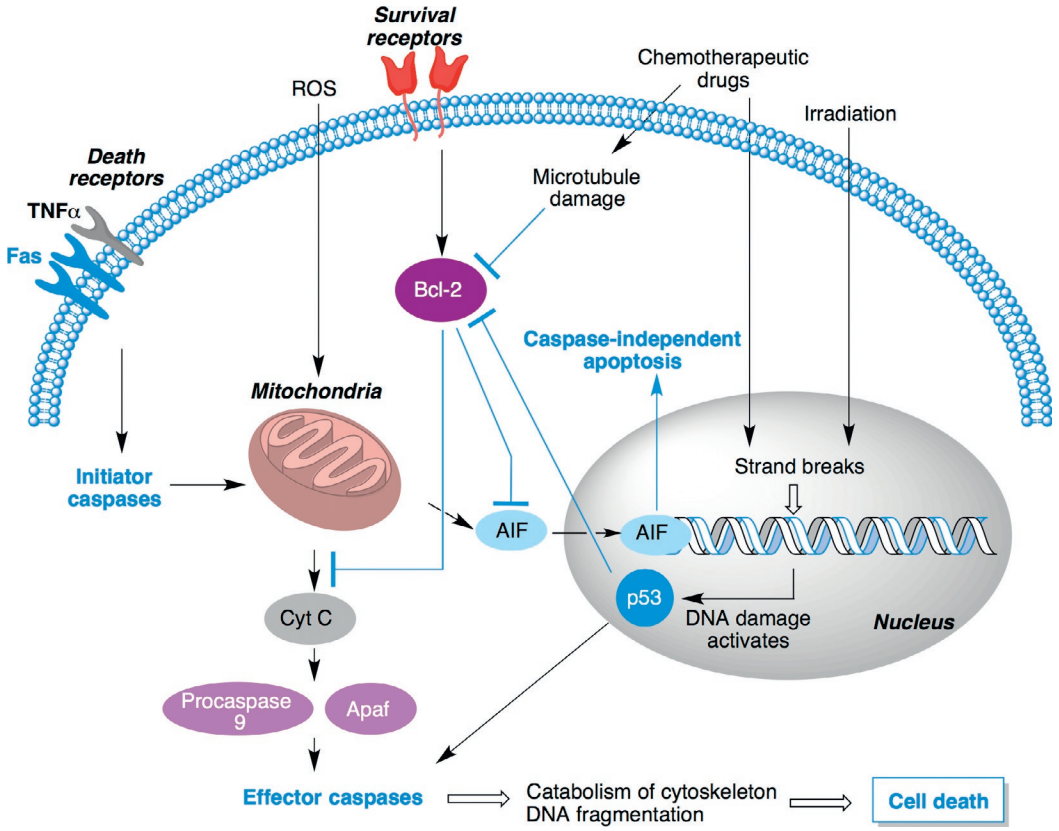


FIGURE 11.31

Some drug targets in apoptotic pathways.

A third, less well-known initiation pathway is the intrinsic endoplasmic reticulum (ER) pathway,[155] which is believed to be caspase-12-dependent and mitochondria-independent. In this case, when the ER is injured by cellular stress, protein synthesis is reduced, many proteins unfold, and the adaptor protein known as TNF receptor-associated factor 2 (TRAF2) dissociates from procaspase-12, resulting in activation of the latter.

Caspases are a group of specific proteases produced as inactive zymogens (pro-enzymes) that are activated and cleave their substrates by a hydrolysis reaction at Asp sites, inducing proteolytic cascade processes. They are central to the mechanism of apoptosis, being both its initiators and its executioners. Low levels of caspases or impairment in caspase function may lead to a decrease in apoptosis and carcinogenesis. However, most caspases and related proteins are not typical drug targets, and small-molecule drugs are of limited value.

Mitochondria are indispensable for energy production and hence for the survival of eukaryotic cells, but they are also crucial regulators of the intrinsic pathway of apoptosis. Internal stimuli, such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+}, and severe

oxidative stress, are triggers of the initiation of the intrinsic mitochondrial pathway through an increase of mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome C (cyt C) into the cytoplasm, together with second mitochondria-derived activator of apoptosis (Smac), also known as DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low pI), and the serine protease HTRA2 (Omi). The functions of these proteins are coordinated to activate or enhance potency of the corresponding effector caspase.

The mitochondrial pathway is closely regulated by a group of proteins belonging to the Bcl-2 family. One branch of this group, which includes mammalian Bcl-2, is anti-apoptotic by blocking the mitochondrial release of cyt C, and its overexpression promotes tumorigenesis. In contrast, two other subgroups of the Bcl-2 family, termed Bax and Baf, can function as tumor suppressors by promoting the release of cyt C, and they are required for apoptotic cell death.

The cytosolic release of cyt C promotes its binding to the adapter protein Apaf-1 and recruitment of procaspase-9 to form the "apoptosome" structure. The resultant active caspase-9 converts procaspase-3, -6, and -7 to the corresponding active form. On the other hand, the already mentioned Smac/DIABLO and HTRA2 proteins potentiate the apoptotic process by inhibiting or inactivating IAPs such as XIAP. Mitochondria may also release the apoptosis-inducing factor (AIF) when the cell receives a death signal that may be triggered by ROS. AIF migrates into the nucleus and binds to DNA, which induces the destruction of the DNA and cell death. This mitochondrial pathway is regulated by a group of proteins belonging to the Bcl-2 family. One branch of this group, including mammalian Bcl-2, is anti-apoptotic by blocking the mitochondrial release of cyt C, and its overexpression promotes tumorigenesis. In contrast, two other subgroups of the Bcl-2 family, Bax and Baf, function as tumor suppressors by promoting the release of cyt C. Activation of the cell death machinery by inhibiting tumor-specific alterations of the mitochondrial metabolism or by stimulating mitochondrial membrane permeabilization could be promising therapeutic approaches in cancer.[156]

Other apoptotic factor that is released from the mitochondrial intermembrane space into the cytoplasm is the second mitochondria-derived activator of caspase (Smac; see later). AIF is released when the cell receives a death signal that may be triggered by ROS. Then, it migrates into the nucleus and binds to DNA, which induces the destruction of the DNA and cell death.

The extrinsic apoptotic pathway is activated by extracellular signals that are transduced into cells when specific death-inducing ligands bind to transmembrane death receptors on the cell surface. The most common interactions are between TNF, TNF-related apoptosis-inducing ligands (TRAILs), or Fas ligands and their cognitive receptors TNF-R1 (TNF receptor type 1), DR4 (TRAIL-R1), and DR5 (TRAIL-R2), or Fas (also known as Apo-1 or CD95) (see Figure 11.31). This binding induces oligomerization of the receptor and allows its intracellular extension to recruit the corresponding TNF or TRAIL receptor-associated death domain (TRADD) or the Fas-associated death domain (FADD) adapter protein. These interactions are followed by the binding of procaspase-8 to form the death-inducing signaling complex, which allows autoactivation of procaspase-8 to the initiator caspase-8. Then, this caspase activates the downstream effector caspase-3, -6, and -7, which ultimately target cellular structures to effect cell death. Caspase-3 is most often implicated in the cell death process following exposure to therapeutic agents. Downregulation of the receptor or impairment of its function, as well as a reduced level in the death signals, contribute to impaired signaling and reduce apoptosis.[157]

Some viruses associated with cancers produce proteins to prevent apoptosis of the cells that they have transformed, enabling them to continue to proliferate. Thus, a human papillomavirus implicated

in causing cervical cancer produces the protein E6 that binds and inactivates the apoptosis promoter p53. This protein, called the "guardian of the genome," is involved in the induction of apoptosis, cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and cellular senescence.[158] It has a dual and conflicting role in the regulation of autophagy because nuclear p53 promotes the transcriptional activation of autophagy-related genes, whereas cytoplasmic p53 acts as a repressor of autophagy. Epstein–Barr virus, which causes mononucleosis and is associated with some lymphomas, inhibits cell apoptosis by producing a protein similar to Bcl-2 and another that causes the cell to increase its own production of Bcl-2. Cancer cells may avoid apoptosis without the participation of viruses. For example, some B-cell leukemias and lymphomas express high levels of Bcl-2, which block the apoptotic signals that they may receive; melanoma cells avoid apoptosis by inhibiting the expression of the gene encoding APAF-1; and other cancer cells secrete elevated levels of a soluble "decoy" molecule that binds to FasL, avoiding the binding of this ligand to Fas receptors. Alternatively, cancer cells may express high levels of FasL that activate the Fas receptors of cytotoxic T lymphocytes that try to kill them.

The IAPs are endogenous inhibitors of caspase activity. They are a group of structurally and functionally similar proteins characterized by the presence of a baculovirus IAP repeat (BIR) protein domain. The binding of these BIR domains to caspases promotes their degradation, or keeps them away from their substrates. Dysregulated IAP expression has been reported in many cancers, and the overexpression of XIAP (also known as BIRC4), which occurs in many NSCLCs, and survivin has been associated with resistance against a variety of apoptosis-inducing conditions.[159] XIAP is the most potent inhibitor of the intrinsic and extrinsic pathways of apoptosis, mainly by binding and inhibiting upstream caspase-9 and the downstream caspases-3 and -7, being highly expressed in many human tumor cell lines.[160] Survivin, also called baculoviral inhibitor of apoptosis protein repeat-containing 5 (BIRC5), is another crucial target in cancer therapy.[161] Its name reflects its ability to promote cell survival in cancer by blocking programmed cell death.[162] With a single BIR domain, it is the smallest IAP, and its levels and localization can be regulated by changes in transcription, physical association with chaperones, altering proteosomal degradation, and other post-translational mechanisms. Survivin inhibits apoptosis by interacting with multiple regulators of both intrinsic and extrinsic apoptosis pathways, including caspase-dependent and caspase-independent mechanisms. Because IAPs suppress apoptosis, enhance survival signaling, and are upregulated in many cancer types, they may be excellent therapeutic targets, opening the possibility that IAP antagonists might specifically target cancer cells over normal cells (Figure 11.32).

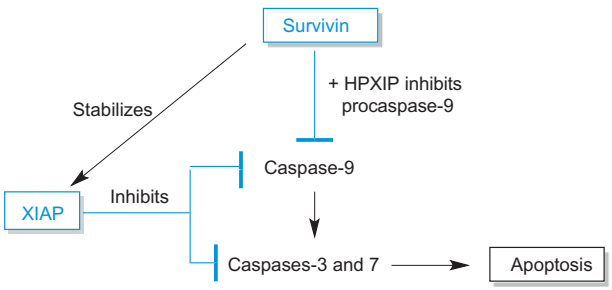


FIGURE 11.32

Apoptosis inhibition by survivin.

8.1.2 Apoptosis-Targeted Therapy: Death Receptor Ligands

Apoptosis-targeted drug development is still in its infancy, but identification of the major regulators of apoptosis has laid the foundation for intense research using different therapeutic approaches. The extracellular death signals sensed by death receptors can be mimicked by recombinant ligands or agonistic antibodies, caspases can be either inhibited or activated, undesirable proteins can be specifically downregulated by antisense oligonucleotides, and important protein–protein interactions can be interfered by peptides or by organic compounds. Several strategies have progressed to clinical testing,[163] and many of them have been incorporated into combination therapies involving conventional anticancer drugs. Currently, targeting apoptosis in cancer is feasible, but the use of new drugs designed to enhance apoptosis has led to many troubling questions about their safety.

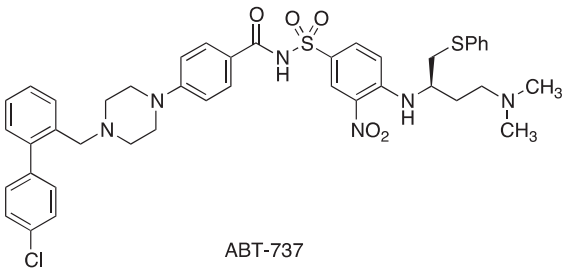
The extrinsic apoptotic pathway has attracted much attention for the development of apoptosis-targeted therapy because death receptors are easily accessible targets for apoptotic agonists and because this pathway can bypass upstream signaling defects, such as those derived from mutations in p53. Dulanermin (AMG-951) is a recombinant human TNF-related apoptosis-inducing ligand that has entered phase II clinical trials.[164] The natural FasL is a cytokine that needs to trimerize to mediate a cell death signal. Two unnatural ligands, APO-010 (a synthetic hexameric FasL) and Fasaret (a recombinant adenoviral construct encoding FasL), are being tested in clinical trials against solid tumors.[165] Several agonistic antibodies targeting death receptors DR4 and DR5, such as apomab, mapatumumab, lexatumumab, TRA-8/CS-1008, AMG-655, and LBY135, are in various stages of clinical development.[166] Other clinical trials have demonstrated that the therapeutic potential of agents targeting death receptors is enhanced in combination with other antitumor drugs.[167]

8.1.3 Drugs Targeting the Bcl-2 Family of Proteins: BH3 Mimetics

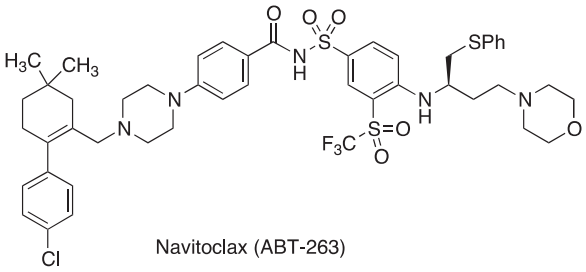
Strategies used in targeting the anti-apoptotic Bcl-2 proteins include the use of therapeutic agents to inhibit them or the silencing of the upregulated involved genes. Since apoptosis is impaired in malignant cells overexpressing pro-survival Bcl-2 proteins, drugs mimicking their natural antagonists BH3-only proteins might overcome chemoresistance. Analysis of the 3D structure of the most important proteins that regulate this death-signaling cascade revealed that they have a binding groove that interacts with an amphipathic α-helix of anti- or pro-apoptotic Bcl-2 family members. This interaction antagonizes the survival activity of anti-apoptotic proteins or activates the pro-apoptotic proteins Bax and Bak.[168] Natural or synthetic pro-apoptotic BH3 mimetics may improve the effectiveness of cancer treatments,[169] although they are not sufficient to cure patients when used as a single agent.[170] Examples of small-molecule inhibitors of Bcl-2 anti-apoptotic proteins include gossypol (AT-101), ABT-737, navitoclax (ABT-263), ABT-199, obatoclax (GX15-070), and HA14-1.[171] Some inhibitors of the anti-apoptotic protein Bcl-xl (B-cell lymphoma–extra large) have been identified by 3D computational screenings of compound libraries.[172]

ABT-737 is one of the best-characterized BH3 mimetics, being able to reverse resistances by inhibiting anti-apoptotic proteins Bcl-2 and Bcl-xl.[173] For hematological malignancies and solid tumors, it works with radiotherapy and chemotherapy, and its combination with the PI3K inhibitor BEZ235 (see Chapter 10, Section 5.4.1) reduces the expression of the anti-apoptotic myeloid cell leukemia 1 (Mcl1) protein.[174] Its orally available derivative, navitoclax (ABT-263), was developed to enhance the clinical potential of ABT-737, and it is in phase I/II trials as monotherapy[175] or in combination with chemotherapeutics or monoclonal antibodies for CLL, lymphoma, and SCLC.[176] Its clinical utility has been compromised by dose-limiting thrombocytopenia due to its high affinity for Bcl-xl, which is critical for platelet survival. ABT-199 is another orally bioavailable, less toxic, selective inhibitor of the Bcl-2

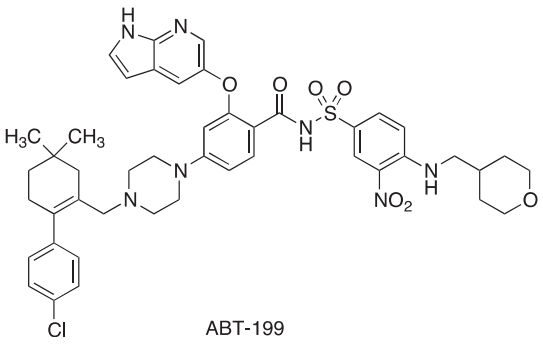
protein. It induced remission in patients with relapsed/refractory CLL and small lymphocytic lymphoma, but clinical assays were suspended after the death of a patient due to tumor lysis syndrome—an effect caused by breakdown products liberated from the sudden death of a large number of leukemia cells.



ABT-737

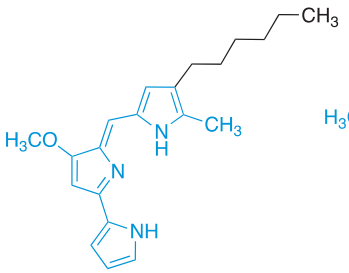


Navitoclax (ABT-263)

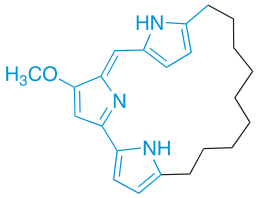


ABT-199

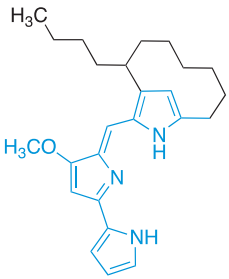
Obatoclax (GX15-070) is another BH3 mimetic in clinical evaluation as a single-agent or combination regimen for the treatment of hematological malignancies and solid tumors[177] that was designed by structural manipulation of bacterial secondary metabolites of the prodigiosin class. Prodigiosin and its analogs, nonylprodigiosin and streotorubin B, are blood red-colored natural products from bacteria such as *Serratia marcescens* or *Streptomyces coelicolor* that have demonstrated potent antimicrobial, antifungal, immunosuppressive, and cytotoxic activities.[178] Streptorubin B was found to be responsible for the activity of a natural extract in a biactivity-guided assay aimed at identifying inhibitors of the interaction of Bcl-2 with the pro-apoptotic protein Bax. Structure–activity studies and further optimization led to obatoclax, one of the first Bcl-2 antagonists to enter clinical trials. Further studies showed that obatoclax is a pan-Bcl-2 inhibitor that blocks BH3-mediated binding of Bax and Bak to their binding partners (Bcl-2, Bcl-xl, Mcl-1, and A1), thereby resulting in pro-apoptotic activity.[179] Additional biological targets have been proposed for this compound.[180]



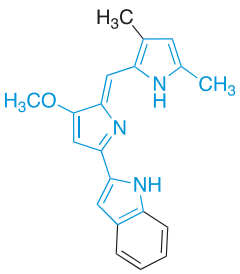
Prodigiosin



Nonylprodigiosin

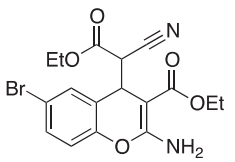


Streptorubin B

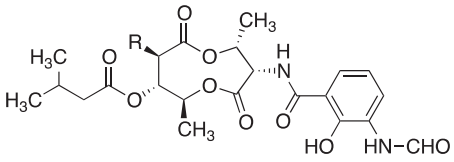


Obatoclax

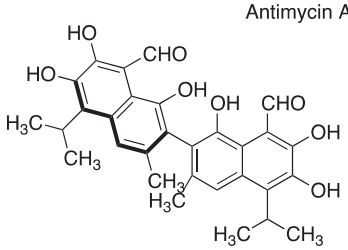
HA14-1 decreases Bcl-2 and Bcl-xl expression and increases the expression of p53, suggesting that this compound may provide therapeutic potential for the treatment of human cervical cancer.[181] Members of the antimycin A family, a group of closely related bis-lactones previously known as inhibitors of mitochondrial electron transfer, are also anti-apoptotic Bcl-2 inhibitors, although they have not reached the clinical stage yet.[182] Another anti-apoptotic Bcl-2 inhibitor is AT-101 [(–)-gossypol], which has entered phase I/II trials. It may have clinical utility in patients with CLL and high-risk features and to enhance radiation-induced apoptosis.[183]



HA14-1



Antimycin A_1: R = nC_4H_9
Antimycin A_3: R = nC_6H_{13}



AT-101 [(-)-Gossypol]

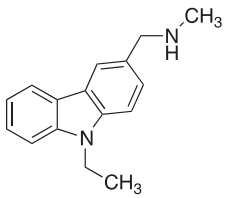
Some antisense oligonucleotides that reduce the expression of *BCL-2* genes are undergoing clinical trials.[184] One of them is oblimersen sodium (Genasense®), an 18-mer oligonucleotide that was the first agent targeting the Bcl-2 pathway to enter clinical trials. It has shown chemosensitizing effects and an

improvement in survival in combined treatment with conventional anticancer drugs in CML,[185] MM,[186] and relapsed/refractory myeloma patients.[187]

8.1.4 Drugs Targeting p53 Proteins: Inhibitors of the mdm2–p53 Interaction

The protein p53, also known as the guardian of the human genome, is a transcription factor that controls the cellular response to DNA damage or other stress stimuli through the induction of cell cycle arrest, DNA repair, apoptosis, or senescence. Several p53-induced genes, expressed only during apoptosis, are involved in the cellular synthesis of ROS,[188] playing major roles in downstream processes through induction of the synthesis of different mitochondrial proteins such as Bax and death receptors DR4 and DR5. The p53 transcription-mediated apoptotic response is slow because it must first be stabilized, activated, and translocated to the nucleus to transcriptionally increase the levels of mRNA of target genes, which subsequently translate into higher levels of the corresponding proteins to activate the respective cell death pathway. In contrast, the transcription-independent mechanism of apoptosis is relatively fast because it is only dependent on translocation of p53 to the mitochondria, where its negative interaction with anti-apoptotic Bcl-xl and Bcl-2 proteins indirectly enhances pro-apoptotic oligomerization of Bak and Bax. The *P53* gene is mutated or deleted in approximately 55% of human cancers. Approximately one-third of the mutations lower the melting temperature of the protein, leading to its rapid denaturation. For this reason, small molecules that bind to those mutants and stabilize them could be effective anticancer drugs.

The p53-based strategies investigated for cancer treatment include gene therapy, drug therapy, and immunotherapy.[189] Many of them attempt the reactivation of the remaining wild-type p53 or the reversion of the mutant conformation.[190] The frequent mutation Y220C creates a surface cavity in p53 that destabilizes the protein. Based on an *in silico* analysis of the crystal structure using virtual screening and rational drug design, the carbazole derivative PhiKan083 was discovered.[191] This compound binds to this cavity, raising the melting temperature of the mutant and slowing its rate of denaturation and also restoring the p53 wild-type functions.



PhiKan083

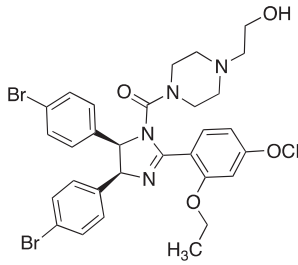
Among the mechanisms that regulate the function of p53, much effort has been directed at blocking the interaction of p53 with mdm2 (also known as hdm2), the previously mentioned E3 ubiquitin ligase that targets p53 for degradation by the proteasome pathway (see Figure 11.4). Because the *MDM2* gene is amplified in human cancer cells as a mechanism to block their death, inhibition of the p53–mdm2 protein–protein interaction provides the possibility to activate the p53 responsive reporter genes in malignant cells containing wild-type p53. The identification and study of inhibitors of the p53/mdm2 PPI have improved the understanding of the p53 pathway, demonstrating that p53 wild-type status is necessary but not sufficient for antitumor activity and that amplification of *MDM2* may predict the tumor cell sensitivity to apoptosis induction. However, although the first clinical results showed efficacy, they also denoted on-target toxicities; therefore, additional studies are needed to establish the therapeutic application of these compounds.[192]

The X-ray structure of the N-terminal binding domain of mdm2 bound to a 15-residue transactivation domain of p53 revealed the complementarity between the mdm2 cleft and the hydrophobic face of the α-helix of p53.[193] In particular, the side chains of three amino acids of this helix (Phe-19, Trp-23, and Leu-26) make extensive van der Waals contacts with the pockets of hdm2, and additional interactions involve two intermolecular hydrogen bonds—one between the Phe-19 backbone amide of p53 and the Gln-72 side chain of hdm2 and the other between the nitrogen of p53 Trp-23 indole and the carbonyl group of the hdm2 Leu-54 backbone. The critical role of these residues was further demonstrated in experiments with phage display peptide libraries.[194]

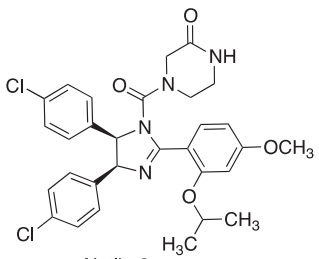
Despite the great interest in compounds able to antagonize the p53/mdm2 interaction,[195] reports of small molecules with this activity are limited. In the screening of a compound library, several 3,4-diphenylbenzodiazepine-2,5-diones showed affinity for the p53 binding pocket in mdm2.[196] However, these compounds have poor bioavailability and rapid clearance, and further optimization attempts were not very successful.

Imidazoline derivatives—known as nutlins because they were originally discovered in Nutley, New Jersey—were also found by HTS of libraries of synthetic molecules and subsequent structure optimization. They inhibit the mdm2–p53 interaction, stabilize p53, and induce senescence in cancer cells.[197] Nutlin-3, another inhibitor of the mdm2–p53 interaction discovered by high-throughput screening, leads to p53 stabilization and subsequent activation of cell cycle arrest and apoptosis. It shows a synergistic cytotoxic effect when used in combination with drugs such as bortozemib, and it is currently in phase I trials for the treatment of retinoblastoma; however, its effects on normal cells remain largely unknown.[198]

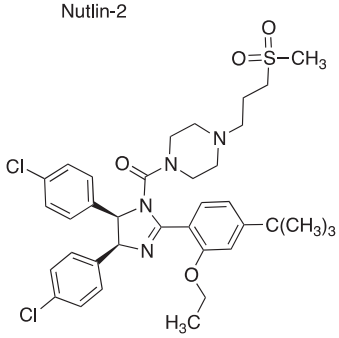
The limited cellular activity and pharmacological properties of the first-generation nutlins prompted the preparation and biological evaluation of further analogs. This work led to the discovery of RG7112 (also known as RO5045337), which was the first p53–mdm2 inhibitor to enter clinical trials that used the measurement of macrophage inhibitory cytokine-1 in blood samples as a surrogate pharmacodynamic readout to demonstrate target modulation (this protein is secreted in serum when p53 is activated).[199] RG7112 has been tested in patients with advanced solid tumors or hematological malignancies, with the most common adverse events being neutropenia and thrombocytopenia.



Nutlin-2



Nutlin-3



RG-7112

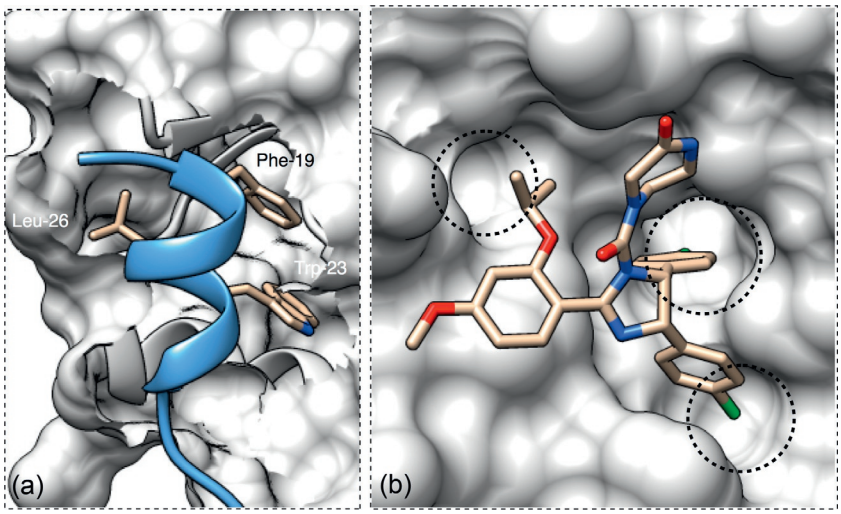


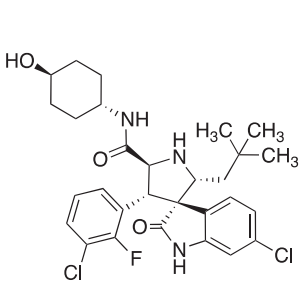
FIGURE 11.33

(a) Structure of the mdm2 protein bound to the p53 transactivation domain. (b) Crystal structure of an mdm2/nutlin-3a complex. Both structures were generated from Protein Data Bank (1YCR and 4HG7, respectively) and displayed with Chimera 1.8.1.

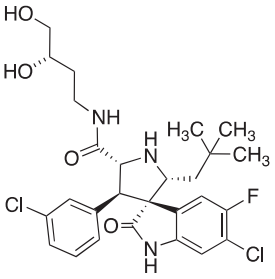
The crystal structures of the complexes formed in the interaction of p53 with nutlin-2 and of p53 with the N-terminal domain of mdm2 showed that the ethoxy and bromophenyl groups of nutlin-2 match the positions of Phe19, Trp23, and Leu26 residues of p53 that normally occupy a hydrophobic pocket of mdm2.[200] A similar binding mode was found for nutlin-3 (Figure 11.33).[201]

Molecular modeling approaches have identified oxindole and isoindolinone derivatives as novel p53–mdm2 leads that display the critical pharmacophores in a suitable orientation to bind the hdm2 cleft.[202] The family of spiro(oxindole-3,3′-pyrrolidine) derivatives was especially important, leading to MI-773 (SAR299155), which entered clinical trials.[203] The starting point of this strategy was to mimic the previously mentioned van der Waals and hydrogen bond contacts while building additional interactions. The related MI-219 selectively inhibits growth of wild-type p53-containing lung cancer cells by induction of G_1 or G_2 arrest in a p53-dependent manner and has been proposed as a chemosensitizing agent.[204]

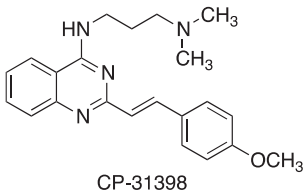
CP-31398 acts as a p53-stabilizing agent that restores p53 functions in mutant p53-expressing cells. It increases the protein levels of wild-type p53 by blockade of ubiquitination and degradation of p53 without interrupting the physical association between p53 and mdm2 *in vivo*. Another p53–mdm2 inhibitor (RG7338, RO5503781) started phase I clinical trials in 2011, but its structure has not yet been disclosed.



MI-773 (SAR-299155)



MI-219



CP-31398

In parallel, efforts have been made to design peptides derived from the transactivation domain of p53 that contain an all-hydrocarbon cross-link to restore α-helical conformation, confer protease resistance, and favor cellular uptake. Many of these compounds have specific mdm2 binding affinity. In the field of oligonucleotides, OL(1)p53 is a 20-mer phosphorothioate oligodeoxynucleotide complementary to a portion in exon 10 of *p53* mRNA, which results in leukemic cell death.[205] Regarding gene therapy, early attempts using exclusively the *p53* gene showed that this treatment is not enough to eliminate all tumor cells. For this reason, later studies investigated the use of *p53* gene therapy concurrently with other anticancer strategies, although no final approval for *p53*-based gene therapy has been granted.[206] The adenoviral *p53* gene vector INGN201 (Ad-p53, Advexin®) showed broad-spectrum antitumor activity in combination with chemotherapy,[207] and its clinical development was expanded into multiple phase II studies in several cancers, but the FDA refused its approval in 2008. SCH-58500 (ACN53), another replication-deficient recombinant adenovirus that expresses human p53 protein has shown promising results in phase I and phase III clinical trials for ovarian, bladder, lung, and liver cancer. Another interesting *p53* gene-based strategy uses engineered viruses to eliminate *p53*-deficient cells. Thus, the genetically engineered oncolytic adenovirus ONYX-015, which is able to survive and replicate in *p53*-deficient cancer cells producing their selective destruction, has been extensively tested in clinical trials, although its therapeutic effect is low. In addition, several clinical trials have been carried out using *p53* vaccines.[208] It is hoped that advances in targeting of *p53* will eventually lead to success of these approaches to tumor treatment.[209]

8.1.5 Drugs Targeting Caspases

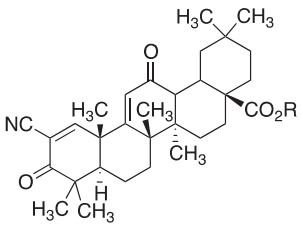
Among the several drugs designed to activate caspases, some are peptides that contain the arginine–glycine–aspartate motif and are able to induce autoactivation of pro-caspase-3. However, to interfere with the activity of caspases, the use of monoclonal antibodies, antisense oligonucleotides, or indirect caspase activation are often needed (see Section 8.1.1). Caspase-based gene therapy has also been attempted.[210]

8.1.6 Inhibitors of NK-κB and TNF-α

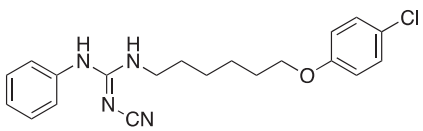
NF-κB is a family of heterodimeric transcription factors that control hundreds of genes and are present in many, if not all, cell types of the body. These factors can be activated by various physiological agents and stress situations, such as those produced in cancer cells due to the higher levels of ROS originated by their high rate of metabolism and inefficient respiration. The chronic activation of NF-κB, which is characteristic of many cancers, is a critical adaptation to these higher levels of oxidative stress that allows cancer cell survival by preventing activation of the pro-apoptotic c-Jun N-terminal kinases (JNKs; see Chapter 10, Section 6.5.4) through an increased expression of JNK–MAPK phosphatase 1 (MKP1).[211] NF-κB also induces inhibition of Smad expression, leading to subsequent inhibition of the transforming growth factor-β (TGF-β) signaling (see Chapter 10, Section 7). However, in certain tumors in which other oncogenes provide pro-survival signals, NF-κB enhances instead the sensitivity to cytotoxic chemotherapy, thereby exerting a tumor-suppressor function.[212]

Some synthetic triterpenoids, especially CDDO and CDDO-Me, inhibit NF-κB activity, leading to sustained activation of JNKs and triggering caspase-mediated apoptosis. These compounds have shown potent activity in multiple cancer animal models and in treatment-resistant cancer cell samples. CDDO-Me was able to prevent the progression of pre-neoplastic lesions to the adenocarcinoma of the prostate in a mouse model.[213]

NF-κB activity can also be inhibited by interference with its activation processes, which depends on a group of proteins known as IκB (inhibitory proteins of κB family, also known as IKK). For instance, the anticancer activity of CHS-828 (a compound evaluated in clinical trials without notorious results in solid tumors)[214] is due to inhibition of the IKK activity leading to apoptosis promotion.[215] In unstimulated cells, IKK proteins sequester NF-κB in the cytoplasm, but when cytokines such as TNF-α and IL-1β are triggered, they induce IκB degradation, thus permitting the translocation of NF-κB to the nucleus (see Figure 11.5).[216]



R = H CDDO (RTA-401)
R = CH_3 CDDO-Me (RTA-402)



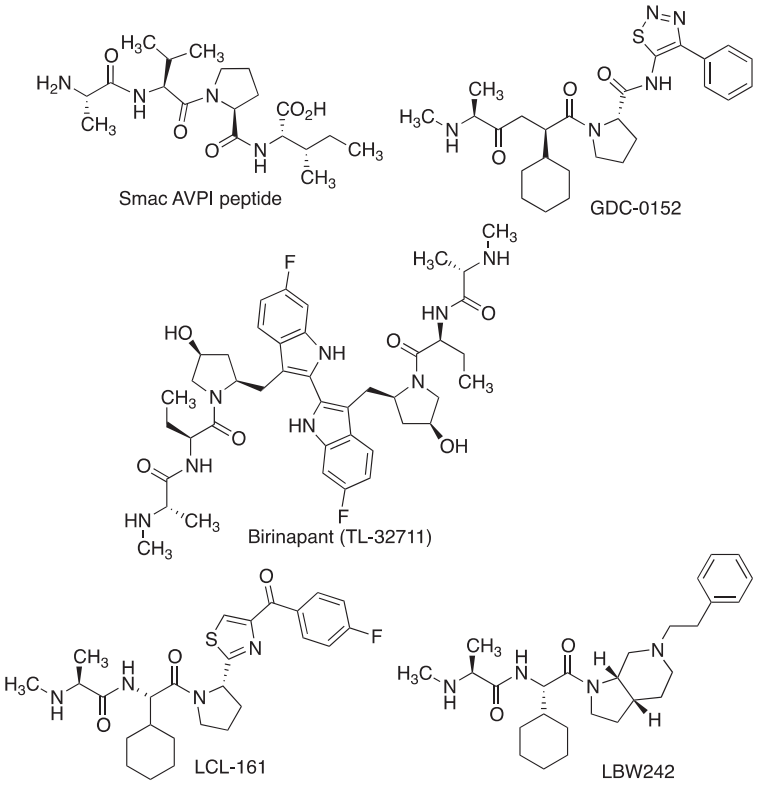
CHS-828

On the other hand, TNF-α is a multifunctional cytokine highly expressed in tumors, in which it is thought to be proangiogenic. Paradoxically, it is a potent antivascular cytokine at higher doses, which may be clinically used to destroy tumor vasculature. TNF-α is also able to initiate cellular apoptosis, but this apoptotic pathway is deactivated in tumor cells. Due to its very toxic systemic side effects, administration of TNF-α has limited uses, and to target TNF-α specifically to tumors, the human recombinant hTNF-α gene (rhTNF-α) has been tested. Its success was limited to the regional treatment of locally advanced solid tumors. More complex treatments to overcome these limitations include the hybrid adeno-associated virus phage vector AAVP-TNF-α that induces the tumor endothelium to express TNF-α[217] and also the use of a conjugate protein obtained by fusion of a recombinant mutant human TNF-α with the peptide GX-1, which binds selectively to the human gastric cancer vasculature.[218] Both strategies have been studied in human melanoma and gastric tumor models.

8.1.7 Drugs Targeting IAPs: Smac Mimetics

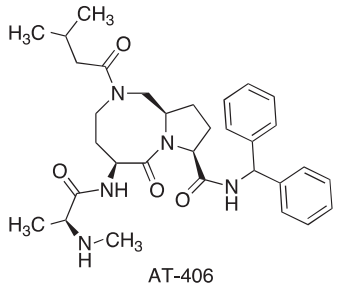
Numerous preclinical studies have shown that targeting IAPs with either small interfering RNA (siRNA) or mimetics of the naturally occurring IAP antagonist Smac/DIABLO (see Section 8.1.1) increases sensitivity of cancer cells to therapies that are widely used in the clinic. Consequently, multiple IAPs have been developed, and some of them have entered clinical trials.[219] The most widely used approach is based on mimicking the IAP binding motif of Smac, which regulates the apoptosis mediated by caspase-3, -7, and -9.[220] The elucidation of the interaction between Smac and IAPs[221] has permitted the design of Smac mimetics that facilitate apoptosis by promoting the auto-ubiquitylation of IAPs and their subsequent proteasomal degradation or the activation of the autocrine TNF stimulation. Smac mimetics resemble the binding motif required for Smac function, which consists of four amino acids at the N-terminal domain of the protein (the AVPI binding motif). The tetrapeptide AVPI is able to trigger caspase activation; however, its clinical value is greatly hampered by membrane impermeability. Recently, the cell-penetrating chimeric apoptotic peptide AVPIR8 was synthesized, in which AVPI is strategically blended with the cell-penetrating sequence of octa-arginine (R8).[222]

The first Smac mimetic to enter clinical trials was GDC-0152, and the most advanced one is birinapant (TL-32711).[223] This compound has been studied both alone and in combination with irinotecan and gemcitabine, being developed for the treatment of solid tumors and hematological malignancies. Other Smac mimetics in clinical development include LCL-161, which is being evaluated in advanced solid tumors in combination with paclitaxel,[224] and LBW-242, which sensitizes XIAP-overexpressing neuroblastoma cells for TNF-α-independent apoptosis.[225]

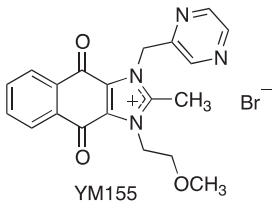


Another Smac mimetic is AT-406, which entered in phase I clinical trials in patients with advanced solid tumors and lymphomas.[226] YM-155 and SM-164 are nonpeptidic compounds that strongly enhance TRAIL activity, concurrently targeting XIAP and cIAP1.[227] The combination of Smac mimetics with TRAIL inducers may be particularly attractive; for this reason, YM-155 is undergoing clinical trials.[228]

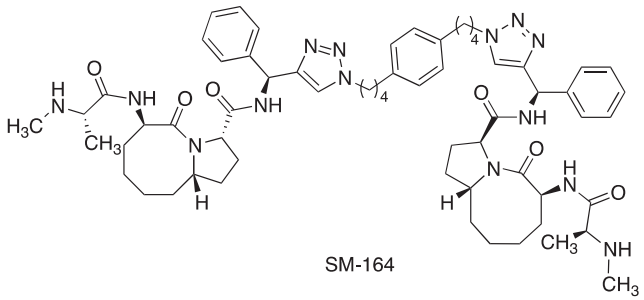
Alternative strategies targeting XIAP or Survivin[229] include transcriptional repression by antisense oligonucleotides[230–232] such as EZN-3042,[233] AEG-35156, and LY-2181308/ISIS-23722, which increases sensitivity to radiation and to chemotherapeutic agents.[234] An alternative is the use of siRNA molecules. The use of Hsp90 inhibitors (see later), gene therapy, and immunotherapy has been also attempted to antagonize Survivin.[235]



AT-406



YM155



SM-164

8.1.8 Other Apoptosis Inducers

In the 1930s, arsenic trioxide (Trisenox®) was found to be effective for treating CML. Interest in arsenic trioxide as an anticancer agent was renewed when promising results were reported in patients with acute promyelocytic leukemia, leading to its FDA approval in 2000 for this indication. Additional clinical trials are in progress for the use of this compound for the treatment of other blood disorders, including myeloma. This compound acts by multiple mechanisms, the main one of which is apoptosis induction because it causes the morphological changes and DNA fragmentation characteristic of apoptosis in NB4 human promyelocytic leukemia cells *in vitro*. Arsenic trioxide also damages the fusion protein PML–RARα, inhibits angiogenesis, and stimulates the immune system killer cells known as LAK (lymphokine-activated killer) and NK (natural killer) cells.

Table 11.2 Main Anticancer Treatments Targeting Apoptosis That Have Entered Clinical Trials

Name	Mechanism	Type of Tumor	Clinical Stage
Navitoclax (ABT-263)	Bcl-2 inhibition	Solid tumors, lymphoid cancers	Phases I/II
Obatoclax (GX15-070MS)	Bcl-2 inhibition	Leukemia, lymphoma, unspecified childhood solid tumor	Phases I/II
Gossypol (AT-101)	Bcl-2 inhibition	Lymphocytic leukemia, chronic B-cell leukemia	Phases I/II
Oblimersen sodium, Genasense®	Bcl-2 antisense oligonucleotide	Melanoma, multiple myeloma, chronic lymphocytic leukemia, Non-small cell lung cancer, hormone-refractory prostate cancer	Phase III
Nutlin-3	Inhibition of mdm2/p53 interaction	Retinoblastoma	Phase I
INGN-201, Advexin®	p53 gene vector	Broad spectrum	Phase II
SCH-58500	p53 gene vector	Ovarian, bladder, lung, and liver cancer	Phase II
RO-5458640	TWEAK antibody	Advanced solid tumors	Phase I
GDC-0152	Smac mimetic	Advanced solid tumors, lymphoma	Phase I
Birinapant (TL-32711)	Smac mimetic	Colorectal cancer	Phase II
LCL-161	Smac mimetic	Solid tumors	Phase I
LBW-242	Smac mimetic	Solid tumors	Phase I
AT-406	Smac mimetic	Solid tumors, lymphoma, acute myelogenous leukemia	Phase I
YM-155	Survivin inhibitor	Non-small cell lung cancer	Phase II
EZN-3042	Survivin antisense oligonucleotide	Acute, childhood, and T cell lymphoblastic leukemia	Phase I
LY-2181308/ ISIS-23722	Survivin antisense oligonucleotide	Non-small cell lung cancer	Phase II
AEG-35156	XIAP antisense oligonucleotide	Acute myeloid leukemia	Phase II
AEG-40826 (HGS-1029)	IAP inhibitor	Advanced solid tumors	Phase I

A summary of the main therapies targeting apoptosis is given in Table 11.2. Among them, RO-5458640 is a humanized monoclonal antibody that blocks the TWEAK/Fn14 signaling through its interaction with the apoptotic ligand TWEAK (TNF-like weak inducer of apoptosis), which prevents its binding to the receptor Fn14 (FGF-inducible molecule 14).

8.2 INHIBITORS OF HEAT SHOCK PROTEINS AND OTHER CHAPERONES

Protein folding is catalyzed *in vivo* by isomerases and chaperones, which are ubiquitous proteins that assist folding, assembly, transport, and degradation of proteins within the cell. The first identified chaperones were the heat shock proteins (Hsp), whose name is derived from the elevated levels produced

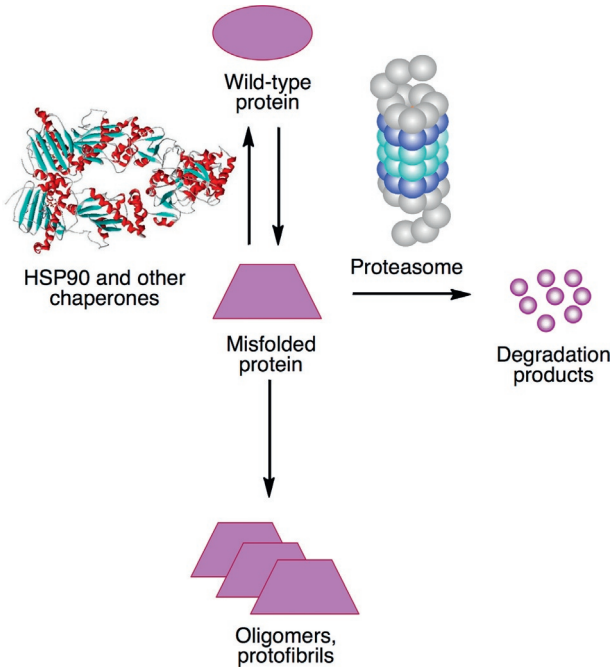


FIGURE 11.34

Function of Hsp90.

when cells are grown at higher than normal temperatures. The best known among heat shock proteins is Hsp90, associated with stress responses, whose activity is coupled to an ATPase cycle that is controlled by several cofactors. It assists the structural folding of a wide variety of oncogenic client proteins to achieve their active conformation and correct cellular location.[236] These proteins include human epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER), N-Ras, AKT, PDGF receptor, and b-Raf. Another Hsp90 client protein is the hypoxia-inducible factor-1 (HIF-1), which plays an important role in reprogramming cancer metabolism toward aerobic glycolysis and is critical to supplying cancer cells with the biomass needed for proliferation.[237] As shown in Figure 11.34, the inhibition of the Hsp90 ATPase activity targets client proteins for their proteasomal degradation, which may be favorable if they are previously mutated and hence are dangerous for the survival of the cell or may become a problem if the proteins are necessary for its normal functioning.

Hsp90 has three major domains, namely a highly conserved N-terminal ATPase domain, a middle domain, and a C-terminal dimerization domain. The crystal structure of Hsp90 bound to ATP indicates that the ATP site greatly differs from that found in other kinases and has also provided insight into how this nucleotide is hydrolized.[238] The conformational changes that occur after binding and hydrolysis of ATP regulate the stabilization and maduration of client proteins, but the detailed mechanism of protein folding remains unknown. The design and study of selective inhibitors of Hsp90 was initially

controversial because this protein is critical for the survival of both normal and sick cells. However, Hsp90 is contained preferentially in mitochondria of cancer cells, where it forms a superchaperone complex with the tumor necrosis factor receptor-associated protein 1 (TRAP1). This complex is much more sensitive to inhibition than the uncomplexed form that predominates in normal cells. The molecular basis for this preferential localization in malignant cells is still elusive, but some oncogenes, such as Ras and AKT, seem to favor this mitochondrial import. Furthermore, due to the effects of oncogenes and microenvironmental factors, the dependence of tumors on Hsp90 and other chaperones is increased with respect to normal cells.

The main strategy employed in the design of Hsp90 inhibitors was based on the synthesis of analogs of the natural antitumor geldanamycin, a benzoquinone derivative belonging to the ansamycin class that is an ATP-competitive inhibitor of Hsp90, although some companies working in this field have designed entirely synthetic molecules not related to this compound. Crystallographic studies revealed that geldanamycin binds inside a deep pocket at the Hsp90 ATP-binding site in a U-shaped conformation, with the ansa ring and the benzoquinone folded nearly parallel to each other and the lactam moiety in a *cis* configuration (Figure 11.35). This knowledge has allowed the use of structure-based design strategies by incorporating structural features that favor the *cis*-amide bond structure.[239]

Geldanamycin was the first Hsp90 inhibitor to enter clinical trials, although it was not advanced because of its unacceptable hepatotoxicity, probably associated with the presence of the

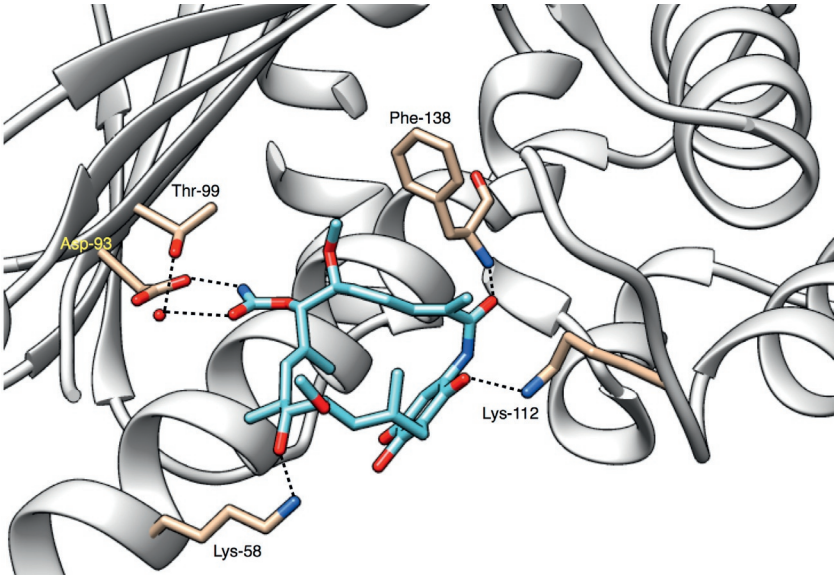
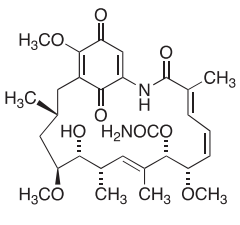


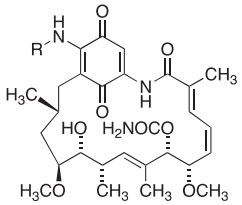
FIGURE 11.35

Binding of geldanamycin to Hsp90. The structure was generated from Protein Data Bank reference 1YET and displayed with Chimera 1.8.1.

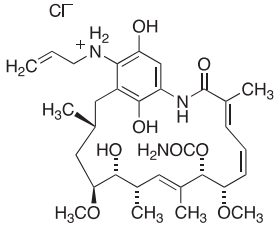
electrophilic methoxybenzoquinone moiety. For this reason, replacement of the 17-methoxy group by other substituents led to less toxic analogs such as tanespimycin (17-allylaminogeldanamycin, 17-AAG).[240] Another problem associated with geldanamycin is its very low solubility; this problem was solved with the development of the water-soluble analog alvespimycin (17-dimethylaminoethylaminogeldanamycin, 17-DMAG).[241] The development of its analog, tanespimycin, as a potential treatment for MM was halted after phase III clinical trials, whereas alvespimycin is in phase II for patients with HER-2-positive metastatic breast cancer. The problematic quinone moiety of tanespimycin was reduced to the hydroquinone stage to give retaspimycin (IPI-504), a compound that can be formulated as a soluble salt suitable for intravenous or oral formulations and that has shown encouraging results in phase I/II trials in patients with gastrointestinal stromal tumors resistant to imatinib and other cancers.[242]



Geldanamycin

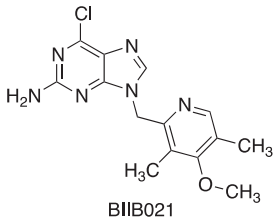


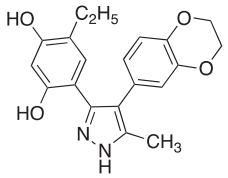
R = $CH_2CH{=}CH_2$ Tanespimycin (17-AAG)
R = $(CH_3)_2NCH_2CH_2$ Alvespimycin (17-DMAG)
(as HCl salt)

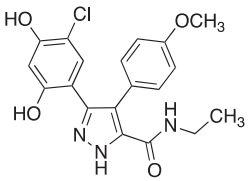


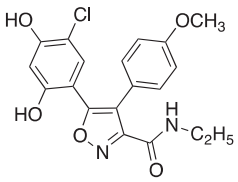
Retaspimycin (IPI-504)

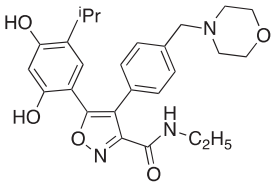
Geldanamycin and its analogs have provided proof-of-concept that Hsp90 inhibition can be an effective approach to anticancer therapy, inspiring significant efforts to identify nonbenzoquinone compounds by structure-based design, HTS, virtual screening, and fragment-based screening. Among several purine scaffold derivatives, BIIB021[243] entered a phase II study against gastrointestinal stromal tumors refractory to imatinib and sunitinib.[244] Pyrazole resorcinols are a second class of synthetic Hsp90 inhibitors that was initially exemplified by CCT018159, a hit discovered by HTS. Subsequent structure-based design generated the amidopyrazole VER-49009, with significantly increased potency due to the additional hydrogen bonding to the protein surface in the ATP pocket. Further optimization to increase its inhibitor affinity while improving pharmacokinetic properties led to the isoxazole VER-50589, which provided the first evidence of antitumor efficacy *in vivo* for this chemotype. The pyrazole-to-isoxazole switch in VER-50589 does not affect the critical hydrogen bonding network exhibited by the pyrazole resorcinol unit of VER-49009, which anchors this class of inhibitors to the HSP90 NH_2-terminal ATP site.[245] NVP-AUY922 (VER-52296) is another significantly improved isoxazole resorcinol that has shown *in vivo* pharmacokinetic, pharmacodynamic, and efficacy profiles in a wide spectrum of human tumor cell lines and xenografts. It is currently in phase II studies for hematologic malignancies and solid tumors, including breast cancer and MM.[246,247] Ganetespib (STA-9090) is a unique triazolone-containing Hsp90 inhibitor that exhibits potent antitumor activity and is being clinically evaluated in non-small cell lung, breast, colorectal, gastric, prostate, pancreatic, melanoma, and hematologic cancers.[248] A combination of X-ray and NMR fragment-based HTS led to identification of the Hsp90 inhibitor AT13387, which entered phase II studies in patients with refractory gastrointestinal stromal tumors, castration-resistant prostate cancer, and ALK^+ lung cancer.[249] Clinical trials were also initiated with the oral Hsp90 inhibitor NVP-HSP990.[250]

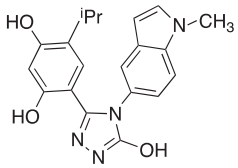

BIIB021

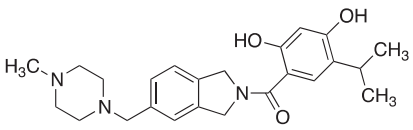

CCT018159

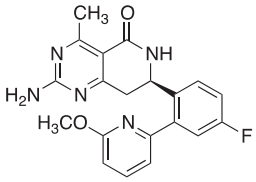

VER-49009


VER-50589


NVP-AUY922 (VER-52296)


Ganetespib (STA-9090)


AT13387


NVP-HSP990

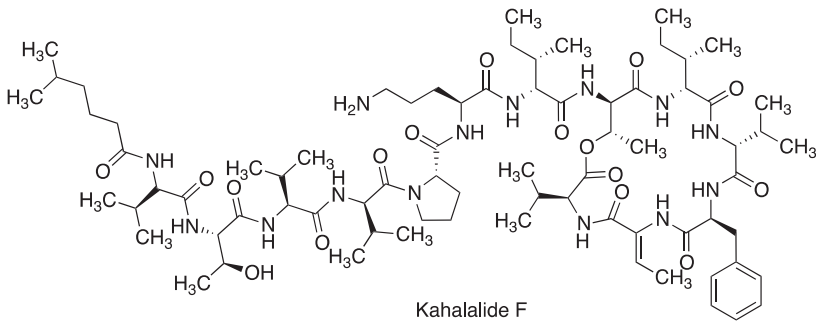
In addition to Hsp90, it has been recently found that inhibition of Hsp27 (also known as heat shock protein beta-1) may be an effective therapeutic approach to inhibit SPARC-induced glioma cell invasion and survival. SPARC is an extracellular Ca(2+)-binding matricellular glycoprotein whose principal functions *in vitro* are counteradhesion and antiproliferation. Its down-regulation is essential for ovarian carcinogenesis, while in gliomas, the loss of tumor supresor gene PTEN enables SPARC-induced migration and invasion via phosphorylation of HSP27 (for the role of the SPARC protein as a modulator of cells and the extracellular matrix interactions, as well as its involvement in anticancer drug resistance, see Section 7.5 of Chapter 13 and Section 7 of Chapter 14, respectively).[251] Clusterin is a stress-induced cytoprotective chaperone overexpressed across a number of cancers that confers broad-spectrum treatment resistance. Custirsen® (OGX-011) is a promising novel second-generation antisense oligonucleotide inhibitor of clusterin in clinical development.[252] HSPPC-96 is a protein–peptide complex consisting of Hsp96 and an array of associated cellular peptides that induces T-cell-specific immunity against these peptides.[253] This autologous tumor-derived heat shock protein peptide–complex has reached advanced clinical trials as an individualized cancer vaccine in patients with glioblastoma multiforme.

9 ANTICANCER AGENTS TARGETED AT THE LYSOSOMES

Kahalalide F is a cyclic depsipeptide derived from the sea slug *Elysia rufescens*. This compound alters the function of the lysosomal membranes, a mechanism that distinguishes it from all other known antitumor agents. Other mechanisms of action are inhibition of TGF-α expression, blockade of intracellular

signaling pathways downstream of the ErbB2 receptor family, and induction of non-p53-mediated apoptosis.

Kahalalide F is in phase II clinical trials in hepatocellular carcinoma, NSCLC, and melanoma. It is also being evaluated for the treatment of severe psoriasis. In these studies, kahalalide F has shown limited activity but an excellent tolerability profile that merits further clinical evaluation in combination with other anticancer compounds.



Kahalalide F

REFERENCES

1 For reviews, see. (a) Abbenante GM, Fairlie DP. *Med Chem* 2005;**1**:71; (b) Gluza K, Kafarski P. Rundfeldt C, editor. *Drug development: a case study based insight into modern strategies*. Rijeka, Croatia: Intech; 2011. p. 39–74.
2 Nalepa G, Rolfe M, Harper JW. *Nature Rev Drug Discov* 2006;**5**:596.
3 Wang Z, Liu P, Inuzuka H, Wei W. *Nature Rev Cancer* 2014;**14**:233.
4 Almond JB, Cohen GM. *Leukemia* 2002;**26**:433.
5 Neefjes J, Dantuma MP. *Nature Rev Drug Discov* 2004;**3**:58.
6 Hurley JH, Lee S, Prag G. *Biochem J* 2006;**399**:361.
7 Adams J. *Nature Rev Cancer* 2004;**4**:349.
8 Myung J, Kim KB, Crews CM. *Med Res Rev* 2001;**21**:245.
9 García-Echeverría C. *Mini Rev Med Chem* 2002;**2**:247.
10 For a review, see Moreau P, Richardson PG, Cavo M, Orlowski RZ, San Miguel JF, Palumbo A, et al. *Blood* 2012;**120**:947.
11 Kisselev AF, van der Linden WA, Overkleeft HS. *Chem Biol* 2012;**19**:99.
12 Paramore A, Frantz S. *Nature Rev Drug Discov* 2003;**2**:611.
13 Zang Y, Thomas SM, Chan ET, Kirk ChJ, Freilino ML, DeLancey HM, et al. *Clin Cancer Res* 2012;**18**:5639.
14 Richardson PG, Spencer A, Cannell P, Harrison SJ, Catley L, Underhill C, et al. *Blood* 2011;**118**:140abstr 302.
15 Palmer JT. *J Med Chem* 1995;**38**:3193.
16 Adams J, Behnke M, Chen S, Cruickshank AA, Dick LR, Grenier L, et al. *Bioorg Med Chem Lett* 1998;**8**:333.
17 Groll M, Kim KB, Kairies N, Huber R, Crews CM. *J Am Chem Soc* 2000;**122**:1237.
18 Nawrocki ST, Kelly KR, Smith PG, Espitia CM, Possemato A, Beausoleil SA, et al. *Clin Cancer Res* 2013;**19**:3577.
19 Brownell JE, Sintchak MD, Gavin JM, Liao H, Bruzzese FJ, Bump NJ, et al. *Mol Cell* 2010;**37**:102.
20 Musacchio A, Salmon ED. *Nature Rev Mol Cell Biol* 2007;**8**:379.
21 Zeng X, Sigoillot F, Gaur S, Choi S, Pfaff KL, Oh DC, et al. *Cancer Cell* 2010;**18**:382.

22 Nilsson J, Yekezare M, Minshull J, Pines J. *Nature Cell Biol* 2008;**10**:1411.
23 Huang HC, Shi J, Orth JD, Mitchison TJ. *Cancer Cell* 2009;**16**:347.
24 Zeng X, Sigoillot F, Gaur S, Choi S, Pfaff KL, Oh DC, et al. *Cancer Cell* 2010;**18**:382.
25 Eguren M, Álvarez-Fernández M, García F, López-Contreras AJ, Fujimitsu K, Yaguchi H, et al. *Cell Rep* 2014;**6**:670.
26 Gourley M, Williamdon JS. *Curr Pharm Des* 2000;**6**:417.
27 Dhanabal M, Jeffers M, LaRochelle WJ. *Curr Med Chem Anticancer Agents* 2005;**5**:115.
28 Rao BG. *Curr Pharm Des* 2005;**11**:295.
29 Borkakoti N. *Biochem Soc Trans* 2004;**32**:17.
30 Overall CM, López-Otín C. *Nature Rev Cancer* 2002;**2**:657.
31 Cross JB, Duca JS, Kaminski JJ, Madison VS. *J Am Chem Soc* 2002;**124**:11004.
32 Botos I, Scapozza L, Zhang D, Liotta LA, Meyer EF. *Proc Natl Acad Sci U S A* 1996;**93**:2749.
33 Sparano JA. *J Clin Oncol* 2004;**22**:4683.
34 Coussens LM, Fingleton B, Matrisian LM. *Science* 2002;**295**:2387.
35 Bissett D, O'Byrne KJ, von Pawel J, Gatzemeier U, Price A, Nicolson M, et al. *J Clin Oncol* 2005;**23**:842.
36 Wada CK. *Curr Top Med Chem* 2004;**4**:1255.
37 Chiappori AA, Eckhardt SG, Bukowski R, Sullivan DM, Ikeda M, Yano Y, et al. *Clin Cancer Res* 2007;**13**:2.
38 Konstantinopoulos PA, Karamouzis MV, Papatsoris AG, Papavassiliou AG. *Int J Biochem Cell Biol* 2008;**40**:1156.
39 Gingras D, Boivin D, Deckers C, Gendron S, Barthomeuf C, Beliveau R. *Anticancer Drugs* 2003;**14**:91.
40 Karam A, Dorigo O. *Med Chem Anticancer Agents* 2012;**12**:764.
41 Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, et al. *Science* 2001;**292**:929.
42 Overall CM, Kleifeld O. *Br J Cancer* 2006;**94**:941.
43 For a review, see Wickström M, Larsson R, Nygren P, Gullbo J. *Cancer Sci* 2011;**102**:501.
44 Guzmán-Rojas L, Rangel R, Salameh A, Edwards JK, Dondossola E, Kim Y-G, et al. *Proc Natl Acad Sci U S A* 2012;**109**:1637.
45 (a) Ichinose Y, Genka K, Koike T, Kato H, Watanabe Y, Mori T, et al. *J Natl Cancer Inst* 2003;**95**:605; (b) For a review, see Wickström M, Larsson R, Nygren P, Gullbo J. *Cancer Sci* 2011;**102**:501.
46 Feng J, Jin K, Zhu H, Zhang X, Zhang L, Liu J, et al. *Bioorg Med Chem Lett* 2012;**22**:5863.
47 Van Herpen CML, Eskens FALM, de Jonge M, Desar I, Hooftman L, Bone EA, et al. *Br J Cancer* 2010;**103**:1362.
48 Matarrese P, Ascione B, Ciarlo L, Vona R, Leonetti C, Scarsella M, et al. *Mol Cancer* 2010;**9**:207.
49 Lankelma JM, Voorend DM, Barwari T, Koetsveld J, Van der Spek AH, De Porto APNA, et al. *Life Sci* 2010;**86**:225.
50 Kumar GDK, Chavarria GE, Charlton-Sevcik AK, Yoo KG, Song J, Strecker TE, et al. *Bioorg Med Chem Lett* 2010;**20**:6610.
51 Sudhan DR, Siemann DW. *Mol Cancer Ther* 2011;**10**:1535.
52 Chavarria GE, Horsman MR, Arispe WM, Kishore Kumar GD, Chen S-E, Strecker TE, et al. *Eur J Med Chem* 2012;**58**:568.
53 Yosef S, Brodsky M, Sredni B, Albeck A, Albeck M. *ChemMedChem* 2007;**2**:1601.
54 Montaser M, Lalmanach G, Mach L. *Biol Chem* 2002;**383**:1305.
55 Okada Y, Yamada S, Toyoshima M, Dong J, Nakajima M, Sugahara K. *J Biol Chem* 2002;**277**:42488.
56 Hulett MD, Hornby JR, Ohms SJ, Zuegg J, Freeman C, Gready JE, et al. *Biochemistry* 2000;**39**:15659.
57 Ferro V, Hammond E, Fairweather JK. *Mini Rev Med Chem* 2004;**4**:693.
58 Miao H-Q, Liu H, Navarro E, Kussie P, Zhu Z. *Curr Med Chem* 2006;**13**:2101.
59 Basche M, Gustafson DL, Holden SN, O'Bryant CL, Gore L, Witta S, et al. *Clin Cancer Res* 2006;**12**:5471.
60 Khasraw M, Pavlakis N, McCowatt S, Underhill C, Begbie S, de Souza P, et al. *Ann Oncol* 2010;**21**:1302.
61 (a) Marchetti D, Reiland J, Erwin B, Roy M. *Int J Cancer* 2003;**104**:167; (b) Nadir Y, Vlodavsky I, Brenner B. *Semin Thromb Hemost* 2008;**34**:187.

62 Lillelund VH, Jensen HH, Liang X, Bols M. *Chem Rev* 2002;**102**:515.
63 Nishimura Y. *J Antibiot* 2009;**62**:407.
64 Barash U, Arvatz G, Farfara R, Naroditsky I, Doweck I, Feld S, et al. *PLoS One* 2012;**7**:e51494.
65 Serini G, Valdembri B, Bussolino F. *Exp Cell Res* 2006;**312**:651.
66 Niland S, Eble A. *J Oncol* 2012;**2012**:article ID 125278.
67 For reviews, see. (a) Desgrosellier JS, Cheresh DA. *Nature Rev Cancer* 2010;**10**:9; (b) Millard M, Odde S, Neamati N. *Theranostics* 2011;**1**:154.
68 Sredni B, Albeck M, Tichler T, Shani A, Shapira J, Bruderman I, et al. *J Clin Oncol* 1995;**13**:2342.
69 Kalich-Philosoph L, Roness H, Carmely A, Fishel-Bartal M, Ligumsky H, Paglin S, et al. *Sci Transl Med* 2013;**5**:185ra62.
70 Mas-Moruno C, Rechenmacher F, Kessler H. *Anticancer Agents Med Chem* 2010;**10**:753.
71 Kumar CC, Malkowski M, Yin Z, Tanghetti E, Yaremko B, Nechuta T, et al. *Cancer Res* 2001;**61**:2232.
72 Funahashi Y, Sugi NH, Semba T, Yamamoto Y, Hamaoka S, Tsukahara-Tamai N, et al. *Cancer Res* 2002;**62**:6116.
73 Mita M, Kelly KR, Mita A, Ricart AD, Romero O, Tolcher A, et al. *Clin Cancer Res* 2011;**17**:193.
74 Davies SL, Serradell N, Bolós J, Bayés M. *Drugs Future* 2007;**32**:123.
75 Wickström SA, Alitalo K, Keski-Oja J. *J Biol Chem* 2004;**279**:20178.
76 Kulke MH, Bergsland EK, Ryan DP, Enzinger PC, Lynch TJ, Zhu AX, et al. *J Clin Oncol* 2006;**24**:3555.
77 Ling Y, Yang Y, Lu N, You QD, Wang S, Gao Y, et al. *Biochem Biophys Res Commun* 2007;**361**:79.
78 Ma X, Yao Y, Yuan D, Liu H, Wang S, Zou Ch, et al. *PLoS One* 2012;**7**:e53449.
79 http://www.clinicaltrials.gov/ct/gui/show/NCT00049790; jsessionid=91EE48B7C2F7D935B96F12E31D5609E3?order=1.
80 Mirochnik Y, Kwiatek A, Volpert OV. *Curr Drug Targets* 2008;**9**:851.
81 Haviv F, Bradley MF, Kalvin DM, Schneider AJ, Davidson DJ, Majest SM, et al. *J Med Chem* 2005;**48**:2838.
82 De Vos FY, Hoekstra R, Eskens FALM, De Vries EG, Van der Gaast A, Groen HJM, et al. *J Clin Oncol* 2004;**22**:3077.
83 Markovic SN, Suman VJ, Rao RA, Ingle JN, Kaur JS, Erickson LA, et al. *Am J Clin Oncol* 2007;**30**:303.
84 Garside SA, Henkin J, Morris KD, Norvell SM, Thomas FH, Fraser HM. *Endocrinology* 2010;**151**:5905.
85 Folkman J. *Exp Cell Res* 2006;**312**:594.
86 Schiffmann S, Maier TJ, Wobst I, Janssen A, Corban-Wilhelm H, Angioni C, et al. *Biochem Pharmacol* 2008;**76**:179.
87 (a) Venkatesan P, Puvvada N, Dash R, Prashanth Kumar BN, Sarkar D, Azab B, et al. *Biomaterials* 2011;**32**:3794; (b) Alonso MJ, Csaba NS. *Nanostructured biomaterials for overcoming biological barriers. RSC drug discovery series*. London: Royal Society of Chemistry; 2012.
88 Herbst RS, Hammond LA, Carbone DP, Tran HT, Holroyd KJ, Desai A, et al. *Clin Cancer Res* 2003;**9**:4108.
89 Akhter S, Nath SK, Tse CM, Williams J, Zasloff M, Donowitz M. *Am J Physiol Cell Physiol* 1999;**45**:C136.
90 Pietras RJ, Weinberg OK. *Evid Based Complement Alternat Med* 2005;**2**:49.
91 Richardson P, Anderson K. *J Clin Oncol* 2004;**22**:3212.
92 Saunders G. *J Oncol Pharm Practice* 2005;**11**:83.
93 Kumar S, Witzig TE, Dispenzieri A, Lacy MQ, Wellik LE, Fonseca R, et al. *Leukemia* 2004;**18**:624.
94 García-Sanz R, González-Porras JR, Hernández JM, Polo-Zarzuela M, Sureda A, Barrenetxea C, et al. *Leukemia* 2004;**18**:856.
95 Stewart AK, Trudel S, Bahlis NJ, White D, Sabry W, Belch A, et al. *Blood* 2013;**121**:1517.
96 Dredge K, Dalgleish AG, Marriott JB. *Anticancer Drugs* 2003;**14**:331.
97 Bauer KS, Dixon SC, Figg WD. *Biochem Pharmacol* 1998;**55**:1827.
98 Maier SK, Hammond JM. *Ann Pharmacother* 2006;**40**:286.
99 Streetly MJ, Gyertson K, Daniel Y, Zeldis JB, Kazmi M, Schey SA. *Br J Haematol* 2008;**141**:41.

100 Lacy MQ, McCurdy AR. *Blood* 2013;**122**:2305.
101 Teo SK, Chen Y, Muller GW, Chen RS, Thomas SD, Stirling DI, et al. *Chirality* 2003;**15**:348.
102 Liekens S, De Clerq E, Neyts J. *Biochem Pharmacol* 2001;**61**:253.
103 Liu S, Widom J, Kemp CW, Crews CM, Clardy J. *Science* 1998;**282**:1324.
104 Kim S, LaMontagne K, Sabio M, Sharma S, Versace RW, Yusuff N, et al. *Cancer Res* 2004;**64**:2984.
105 (a) Wernert N, Stamjek A, Kiriakidis S, Hügel A, Jha HC, Mazitschek R, et al. *Angew Chem Int Ed* 1999;**38**:3228; (b) For a review, see Dittmer J. *Mol Cancer* 2003;**2**:29.
106 Logothetis CJ, Wu KK, Finn LD, Daliani D, Figg W, Ghaddar H, et al. *Clin Cancer Res* 2001;**7**:1198.
107 Connell RD, Beebe JS. *Expert Opin Ther Patents* 2001;**11**:77.
108 Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, et al. *Nature Med* 2004;**10**:255.
109 Benny O, Fainaru O, Adini A, Cassiola F, Bazinet L, Adini I, et al. *Nature Biotechnol* 2008;**26**:799.
110 Wang J, Sheppard GS, Lou P, Kawai M, BaMaung N, Erickson SA, et al. *Cancer Res* 2003;**63**:7861.
111 Miranda-Lorenzo I, Dorado J, Lonardo E, Alcalá S, Serrano AG, Clausell-Tormos J, et al. *Nat Methods* 2014; http://dx.doi.org/10.1038/nmeth.3112.
112 Takahashi-Yanaga F, Kahn M. *Clin Cancer Res* 2010;**16**:3153.
113 For a review, see Hu Y, Fu L. *Am J Cancer Res* 2012;**2**:340.
114 For a review, see Curtin JC, Lorenzi MV. *Oncotarget* 2010;**1**:552.
115 Palmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. *J Cell Biol* 2001;**154**:369.
116 So JY, Lee HJ, Smolarek AK, Paul S, Wang CX, Maehr H, et al. *Mol Pharmacol* 2011;**79**:360.
117 Park CH, Hahm ER, Park S, Kim HK, Yang CH. *FEBS Lett* 2005;**579**:2965.
118 El-Khoueiry AB, Ning Y, Yang D, Cole S, Kahn M, Zoghbi M, et al. *J Clin Oncol* 2013;**31**(Suppl.)abstr 2501.
119 Lum L, Clevers H. *Science* 2012;**337**:922.
120 Luu H. *Curr Cancer Drug Targets* 2004;**4**:653.
121 For a review of the role of Notch signaling in tumorigenesis, see Leong KG, Karsan A. *Blood* 2006;**107**:2223.
122 Paganin M, Ferrando A. *Blood Rev* 2011;**25**:83.
123 Roti G, Carlton A, Ross KN, Markstein M, Pajcini K, Su AH, et al. *Cancer Cell* 2013;**18**:39.
124 For a review, see Shih I-M, Wang T-L. *Cancer Res* 2007;**67**:1879.
125 Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. *Blood* 2007;**110**:278.
126 Al-Hussaini H, Subramanyam D, Reedijk M, Sridhar SS. *Mol Cancer Ther* 2011;**10**:9.
127 Wei P, Walls M, Qiu M, Ding R, Denlinger RH, Wong A, et al. *Mol Cancer Ther* 2010;**9**:1618.
128 (a) Zhang CC, Pavlicek A, Zhang Q, Lira ME, Painter CL, Yan Z, et al. *Clin Cancer Res* 2012;**18**:2008; (b) Zhang CC, Yan Z, Zong Q, Fang DD, Painter C, Zhang Q, et al. *Stem Cells Trans Med* 2013;**2**:233.
129 Patent WO/2012/129353; Int. App. PCT/US2012/030021.
130 Tolcher AW, Messersmith WA, Mikulski SM, Papadopoulos KP, Kwak EL, Gibbon DG, et al. *J Clin Oncol* 2012;**30**:2348.
131 Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, et al. *Nature* 2009;**462**:182.
132 Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, et al. *Nature* 2010;**464**:1052.
133 Ridgway J, Zhang G, Wu Y, Stawicki S, Liang W-C, Chanthery Y, et al. *Nature* 2006;**444**:1083.
134 Pasca di Magliano M, Hebrok M. *Nature* 2003;**3**:903.
135 Tremblay MR, McGovern K, Read MA, Castro AC. *Curr Opin Chem Biol* 2010;**14**:428.
136 Robarge KD, Brunton SA, Castanedo GM, Cui Y, Dina MS, Goldsmith R, et al. *Bioorg Med Chem Lett* 2009;**19**:5576.
137 Rudin CM. *Clin Cancer Res* 2012;**18**:1.
138 Peukert S, He F, Dai M, Zhang R, Sun Y, Miller-Moslin K, et al. *ChemMedChem* 2013;**8**:1261.
139 Dwyer RM, Khan S, Barry FP, O'Brien T, Kerin MJ. *Stem Cell Res Ther* 2010;**1**:25.
140 Whitty A, Kumaravel G. *Nature Chem Biol* 2006;**2**:112.
141 Sun J. Zhao Z. *BMC Genomics* 2010;**11**(Suppl. 3):S5.

142 Hanahan D, Weinberg RA. *Cell* 2011;**144**:646.
143 Davis JM, Tsou LK, Hamilton AD. *Chem Soc Rev* 2007;**36**:326.
144 For representative reviews, see. (a) Ivanov AA, Khuri FR, Fu H. *Trends Pharmacol Sci* 2013;**34**:393 [review]; (b) Cummings CG, Hamilton AD. *Curr Opin Chem Biol* 2010;**14**:341.
145 Harris CC. *Proc Natl Acad Sci U S A* 2006;**103**:1659.
146 For representative reviews, see. (a) Wells JA, McClendon CL. *Nature* 2007;**450**:1001; (b) Meireles LM, Mustata G. *Curr Top Med Chem* 2011;**11**:248; (c) Nero TL, Morton CJ, Holien JK, Wielens J, Parker MW. *Nature Rev Cancer* 2014;**14**:248.
147 Tzakos AG, Fokas D, Johannes Ch, Moussis V, Hatzimichael E, Briasoulis E. *Molecules* 2011;**16**:4408.
148 Valkov E, Sharpe T, Marsh M, Greive S, Hyvönen M. *Top Curr Chem* 2012;**317**:145.
149 Wu B, Zhang Z, Noberini R, Barile E, Giulianotti M, Pinilla C, et al. *Chem Biol* 2013;**20**:19.
150 Sun Q, Burke JP, Phan J, Burns MC, Olejniczak ET, Waterson AG, et al. *Angew Chem Int Ed Engl* 2012;**51**:6140.
151 Scheper J, Guerra-Rebollo M, Sanclimens G, Moure A, Masip I, González-Ruiz D, et al. *PLoS One* 2010;**5**: e11403.
152 Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, et al. *Cell Prolif* 2012;**45**:487.
153 Petak I, Houghton JA, Kopper L. *Curr Signal Transduct Ther* 2006;**1**:113.
154 Elmore SW, Oost TK, Park C-M. *Annu Rep Med Chem* 2005;**40**:245.
155 O'Brien MA, Kirby R. *J Vet Emerg Crit Care* 2008;**18**:572.
156 (a) Fulda S, Galluzzi L, Kroemer G. *Nature Rev Drug Discov* 2010;**9**:447; (b) Wen S, Zhu D, Huang P. *Future Med Chem* 2013;**5**:53.
157 Fulda S. *Int J Cell Biol* 2010;**2010**, article 370835.
158 Avery-Kiejda KA, Bowden NA, Croft AJ, Scurr LL, Kairupan CF, Ashton KA, et al. *BMC Cancer* 2011;**11**:203.
159 Krepela E, Dankova P, Moravcikova E, Krepelova A, Prochazka J, Cermak J, et al. *Int J Oncol* 2009;**35**:1449.
160 Huang X, Wu Z, Mei Y, Wu M. *EMBO J* 2013;**32**:2204.
161 Mobahat M, Narendran A, Riabowol K. *Int J Mol Sci* 2014;**15**:2494.
162 Végran F, Mary R, Gibeaud A, Mirjolet C, Collin B, Oudot A, et al. *Cancer Res* 2013;**73**:5391.
163 (a) For a review of apoptosis-based therapies, see Fischer U, Schulze-Osthoff K. *Cell Death Differ* 2005;**12**:942; (b) For a review of apoptosis-based therapies in cancer, see Wong RSY. *J Exp Clin Cancer Res* 2011;**30**:87.
164 Bellail AC, Qi L, Mulligan P, Chhabra V, Hao C. *Rev Recent Clin Trials* 2009;**4**:34.
165 Villa-Morales M, Fernández-Piqueras J. *Expert Opin Ther Targets* 2012;**16**:85.
166 Ashkenazi A. *Nature Rev Drug Discov* 2008;**7**:1001.
167 See, for instance Soria JC, Smit E, Khayat D, Besse B, Yang X, Hsu CP, et al. *J Clin Oncol* 2010;**28**:1527.
168 Day CL, Chen L, Richardson SJ, Harrison PJ, Huang DC, Hinds MG. *J Biol Chem* 2005;**280**:4738.
169 Wang JL, Liu D, Zhang Z-J, Shan S, Han X, Srinivasula SM, et al. *Proc Natl Acad Sci U S A* 2000;**97**:7124.
170 Hartman ML, Czyz M. *Anticancer Agents Med Chem* 2012;**12**:966.
171 Kang MH, Reynolds CP. *Clin Cancer Res* 2009;**15**:1126.
172 Mukherjee P, Desai P, Zhou YD, Avery M. *J Chem Inf Model* 2010;**50**:906.
173 Tagscherer KE, Fassl A, Campos B, Farhadi M, Kraemer A, Böck BC, et al. *Oncogene* 2008;**27**:6646.
174 Albershardt TC, Salerni BL, Soderquist RS, Bates DJ, Pletnev AA, Kisselev AF, et al. *J Biol Chem* 2011;**286**:24882.
175 Gandhi L, Bahleda R, Tolaney SM, Kwak EL, Cleary JM, Pandya SS, et al. *J Clin Oncol* 2011;**29**:909.
176 Rudin CM, Hann CL, Garon EB, Ribeiro de Oliveira M, Bonomi PD, Camidge DR, et al. *Clin Cancer Res* 2012;**18**:3163.

177 (a) O'Brien SM, Claxton DF, Crump M, Faderl S, Kipps T, Keating MJ, et al. *Blood* 2009;**113**:299; (b) Schimmer AD, O'Brien S, Kantarjian H, Brandwein J, Cheson BD, Minden MD, et al. *Clin Cancer Res* 2008;**14**:8295.
178 Pérez-Tomés R, Viñas M. *Curr Med Chem* 2010;**17**:2222.
179 (a) Trudel S, Li ZH, Rauw J, Tiedemann RE, Wen XY, Stewart AK. *Blood* 2007;**109**:5430; (b) Joudeh J, Claxton D. *Expert Opin Invest Drugs* 2012;**21**:363.
180 Bodur C, Basaga H. *Curr Med Chem* 2012;**19**:1804.
181 Rehman K, Tariq M, Akash MSH, Gillani Z, Qazi MH. *Chem Biol Drug Design* 2014;**83**:317.
182 Tzung SP, Kim KM, Basañez G, Giedt CD, Simon J, Zimmerberg J, et al. *Nature Cell Biol* 2001;**3**:183.
183 James DF, Castro JE, Loria O, Prada CE, Aguillon RA, Kipps TJ. *J Clin Oncol* 2006;**24**:6605.
184 Melnikova I, Golden J. *Nature Rev Drug Discov* 2004;**3**:905.
185 (a) Rai KR, Moore J, Wu J, Novick SC, O'Brien SM. *J Clin Oncol* 2008;**26**:7008; (b) Abou-Nassar K, Brown JR. *Clin Adv Haematol Oncol* 2010;**8**:886.
186 Jansen B, Wacheck V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C. Lucas T, et al. *Lancet* 2000;**356** (9243):1728.
187 Chanan-Khan AA, Niesvizky R, Hohl RJ, Zimmerman TM, Christiansen NP, Schiller GJ, et al. *Leuk Lymphoma* 2009;**50**:559.
188 Kotsinas A, Aggarwal V, Tan E-J, Gorgoulis VG. *Cancer Lett* 2011;**327**:97.
189 For a review, see Khoo KH, Verma CS, Lane DP. *Nature Rev Drug Discov* 2014;**13**:217.
190 Huang CL, Yokomise H, Miyatake A. *Future Oncol* 2007;**3**:83.
191 Boeckler FM, Joerger AC, Jaggi G, Rutherford TJ, Veprintsev DB, Fersht AR. *Proc Natl Acad Sci U S A* 2008;**105**:10360.
192 Carry J-C, García-Echeverria C. *Bioorg Med Chem* 2013;**23**:2480.
193 Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, et al. *Science* 1996;**274**:948.
194 García-Echeverría C, Chène P, Blommers MJJ, Furet P. *J Med Chem* 2000;**43**:3205.
195 Sharma SK, Ramsey TM, Blair KW. *Curr Med Chem Anticancer Agents* 2002;**2**:311.
196 Cummings MD, Schubert C, Parks DJ, Calvo RR, LaFrance LV, Lattanze J, et al. *Chem Biol Drug Design* 2006;**67**:201.
197 Shangary S, Wang S. *Annu Rev Pharmacol Toxicol* 2008;**49**:223.
198 Secchiero P, Bosco R, Celeghini C, Zauli G. *Curr Pharm Des* 2011;**17**:569.
199 Ray-Coquard L, Blay JY, Italiano A, Le Cesne A, Penel N, Zhi J, et al. *Lancet Oncol* 2012;**13**:1133.
200 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. *Science* 2004;**303**:844.
201 Anil B, Riedinger C, Endicott JA, Noble ME. *Acta Crystallogr D* 2013;**69**:1358.
202 Zhuang C, Miao Z, Zhu L, Dong G, Guo Z, Wang S, et al. *J Med Chem* 2012;**55**:9630.
203 Popowicz G, Czarna A, Wolf S, Wang K, Wang W, Dömling A, et al. *Cell Cycle* 2010;**9**:1104.
204 Zheng M, Yang J, Xu X, Sebolt JT, Wang S, Sun Y. *Anticancer Res* 2010;**30**:3321.
205 Bishop MR, Iversen PL, Bayever E, Sharp JG, Greiner TC, Copple BL, et al. *J Clin Oncol* 1996;**14**: 1320.
206 Suzuki K, Matusubara H. *J Biomed Biotechnol* 2011;**2011**:978312.
207 Gabrilovich DI. *Expert Opin Biol Ther* 2006;**6**:823.
208 For a review, see Vermeij R, Leffers N, van der Burg SH, Melief CJ, Daemen T, Nijman HW. *J Biomed Biotechnol* 2011;**2011**:702146.
209 Essmann F, Schulze-Osthoff K. *Br J Pharmacol* 2012;**165**:328.
210 Li X, Fan R, Zou X, Gao L, Jin H, Du R, et al. *Biochem Biophys Res Commun* 2007;**358**:489.
211 Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. *Cell* 2005;**120**:649.
212 Jing H, Kase J, Dörr JR, Milanovic M, Lenze D, Grau M, et al. *Genes Dev* 2011;**25**:2137.
213 Gao X, Deeb D, Liu Y, Arbab AS, Divine GW, Dulchavsky SA, et al. *Cancers (Basel)* 2011;**3**:3353.

214 Hovstadius P, Larsson R, Jonsson E, Skov T, Kissmeyer AM, Krasilnikoff K, et al. *Clin Cancer Res* 2002;**8**:2850.
215 Karin M, Yamamoto Y, Wang QM. *Nature Rev Drug Discov* 2004;**3**:17.
216 Liu S, Chen ZJ. *Cell Res* 2011;**21**:6.
217 Tandle A, Hanna E, Lorang D, Hajitou A, Moya CA, Pasqualini R, et al. *Cancer* 2009;**115**:128.
218 Chen B, Shanshan C, Zhang Y, Wang X, Liu J, Hui X, et al. *BMC Cell Biol* 2009;**10**:63.
219 Owens TW, Gilmore AP, Streuli CH, Foster FM. *J Carcinogen Mutagen* 2013;**S14**:004.
220 Du C, Fang M, Li Y, Li L, Wang X. *Cell* 2000;**102**:33.
221 Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. *Nature* 2000;**406**:855.
222 Wang H, Guo Q, Jiang Y, Liu E, Zhao Y, Wang H, et al. *Adv Funct Mater* 2013;**23**:6068.
223 Krepler C, Chunduru SK, Halloran MB, He X, Xiao M, Vultur A, et al. *Clin Cancer Res* 2013;**19**:1784.
224 See the pipeline of Novartis Oncology at http://www.novartis.com. Novartis, 2011.
225 Petrucci E, Pasquini L, Bernabei M, Saulle E, Biffoni M, Accarpio F, et al. *PLoS One* 2012;**7**:e35073.
226 Cai Q, Sun H, Peng Y, Lu J, Nikolovska-Coleska Z, McEachern D, et al. *J Med Chem* 2011;**54**:2714.
227 Lu J, McEachern D, Sun H, Bai L, Peng Y, Qiu S, et al. *Mol Cancer Ther* 2011;**10**:902.
228 Giaccone G, Zatloukal P, Roubec J, Floor K, Musil J, Kuta M, et al. *J Clin Oncol* 2009;**27**:4461.
229 Mita AC, Mita MM, Nawrocki ST, Giles FJ. *Clin Cancer Res* 2008;**14**:5000.
230 Fulda S, Vucic D. *Nature Rev Drug Discov* 2012;**11**:109.
231 Cao C, Mu Y, Hallahan DE, Lu B. *Oncogene* 2004;**23**:7047.
232 Du ZX, Zhang HY, Gao DX, Wang HQ, Li YJ, Liu GL. *Exp Mol Med* 2006;**38**:230.
233 Park E, Gang EJ, Hsieh YT, Schaefer P, Chae S, Klemm L, et al. *Blood* 2011;**118**:2191.
234 Ohnishi K, Scuric Z, Schiesti RH, Okamoto N, Takahashi A, Ohnishi T. *Radiat Res* 2006;**166**:454.
235 Pennati M, Folini M, Zaffaroni N. *Carcinogenesis* 2007;**28**:1133.
236 Chaudhury S, Welch TR, Blagg BS. *ChemMedChem* 2006;**1**:1331.
237 Giaccia A, Siim BG, Johnson RS. *Nature Rev Drug Discov* 2003;**2**:1.
238 Ali MMU, Roe SM, Vaughan CK, Meyer P, Panaretou B, Piper PW, et al. *Nature* 2006;**440**:1013.
239 Kitson RS, Chang C-H, Xiong R, Williams HEL, Davis AL, Lewis W, et al. *Nature Chem* 2013;**5**:307.
240 Dimopoulos MA, Mitsiades CS, Anderson KC, Richardson PG. *Clinical lymphoma, myeloma & leukemia* 2011;**11**:17.
241 Burger AM, Fiebig HH, Stinson SF, Sausville EA. *Anticancer Drugs* 2004;**15**:377.
242 Sequist LV, Gettinger S, Senzer NN, Martins RG, Jänne PA, Lilenbaum R, et al. *J Clin Oncol* 2010;**28**:4953.
243 Chiosis G, Lopes EC, Solit D. *Curr Opin Invest Drugs* 2006;**7**:534.
244 Dickson MA, Okuno SH, Keohan ML, Maki RG, D'Adamo DR, Akhurst TJ, et al. *Ann Oncol* 2013;**24**:252.
245 Sharp SY, Prodromou Ch, Boxall K, Powers MV, Holmes GB, Thomas PM, et al. *Mol Cancer Ther* 2007;**6**:1198.
246 Eccles SA, Massey A, Raynaud FI, Sharp SY, Box G, Valenti M, et al. *Cancer Res* 2008;**68**:2850.
247 Brough PA, Aherne W, Barril X, Borgognoni J, Boxall K, Cansfield JE, et al. *J Med Chem* 2008;**51**:196.
248 Ying W, Du Z, Sun L, Foley KP, Proia DA, Blackman RK, et al. *Mol Cancer Ther* 2012;**11**:475.
249 (a) Murray CW, Carr MG, Callaghan O, Chessari G, Congreve M, Cowan S, et al. *J Med Chem* 2010;**53**:5942; (b) Woodhead AJ, Angove H, Carr MG, Chessari G, Congreve M, Coyle JE, et al. *J Med Chem* 2010;**53**:5956.
250 Menezes DL, Taverna P, Jensen MR, Abrams T, Stuart D, Yu GK, et al. *Mol Cancer Ther* 2012;**11**:730.
251 Schultz CR, Golembieski WA, King DA, Brown SL, Brodie C, Rempel SA. *Mol Cancer* 2012;**11**:20.
252 Zielinski R, Chi KN. *Future Oncol* 2012;**8**:1239.
253 Caudill MM, Li Z. *Expert Opin Biol Ther* 2001;**1**:539.

CHAPTER

BIOLOGICAL THERAPY OF CANCER

12

CONTENTS

1 INTRODUCTION

This chapter focuses on the medicinal chemistry aspects of biological approaches to cancer therapy—that is, the use of living organisms or substances derived from them (including synthetic versions of such

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00012-0

substances) to treat cancer. Biological therapies include monoclonal antibodies, immunotoxins, therapeutic vaccines, cancer-killing microorganisms, gene therapy, and adoptive T-cell transfer. The last strategy involves the isolation, ex vivo expansion and infusion into patients of tumor specific T cells to achieve greater number of these cells than could be obtained by vaccination alone. Most of the targets and mechanisms included in this chapter are related to immune responses.

2 MONOCLONAL ANTIBODIES AGAINST CANCER CELLS

The advent of monoclonal antibody (mAb) technology[1] led to great expectations for their potential to provide effective targeted therapy for cancer. During the late 1980s and early 1990s, many clinical results were disappointing, but mAbs are now widely recognized as therapeutic molecules and many of them have been approved with great therapeutic and commercial successes, especially in hematologic malignancies. Malignant cells present some surface antigens that are not found in normal cells and are therefore excellent targets for the binding of specific antibodies, which normally remain bound to the cell surface but in some cases are endocytosed. The development of specific mAbs targeted at these antigens is a field of anticancer therapy that is undergoing very fast growth[2] because these treatments are generally very well tolerated. Unfortunately, none of these are able to cure cancer as a single agent, they still have secondary effects and may also generate resistances.

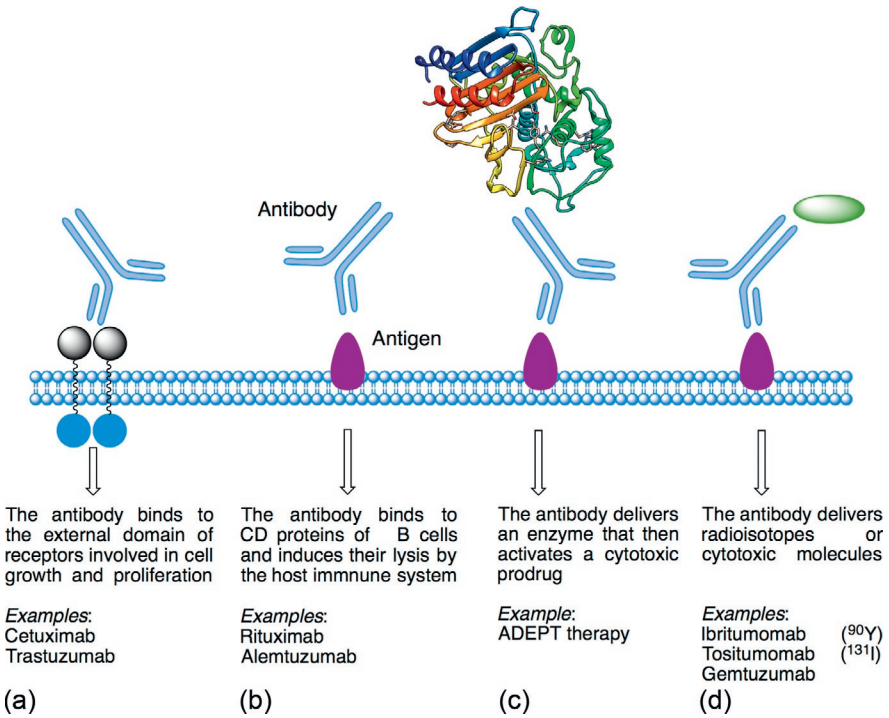


FIGURE 12.1

The various roles of antibodies in anticancer therapy.

An antibody can be used in anticancer therapy in different ways (Figure 12.1).[3] As single entities, mAbs may be targeted to bind to the external domain of membrane receptors of signal transduction pathways involved in cell growth and proliferation (Figure 12.1a). Trastuzumab (Herceptin®), bevacizumab (Avastin®), and Cetuximab (Erbitux®) are examples of this approach that have been included in the appropriate sections of Chapter 10. In a second strategy, mAbs are used to enhance the immunogenesis by recruiting the body's own immune system to destroy cancer cells (Figure 12.1b). Examples that are discussed later are rituximab (Rituxan®) and alemtuzumab (Campath®). A third strategy involves the use of antibodies to guide a prodrug/drug-releasing enzyme to the tumor cells (Figure 12.1c), as in the "antibody-directed enzyme prodrug therapy" (ADEPT) that is discussed in Section 2.4 of Chapter 13. Finally, selective targeted mAbs are used as drug carriers that guide linked radioisotopes or cytotoxic molecules to the tumor cells to reduce the exposure of sensitive organs and tissues to drugs while enhancing the exposure of the tumor and metastatic foci (Figure 12.1d). Ibritumomab tiuxetan (^{90}Y) (Zevalin®), ^{131}I-tositumomab (Bexxar®), and gemtuzumab ozogamicin (Mylotarg®) are examples.

Examples of mAbs that are used in the clinic or have entered clinical trials are shown in Table 12.1.

Table 12.1 Examples of mAbs Used as Single Entities in the Clinic or under Clinical Trials

Target	Chapter/Section	mAb
EGFR (HER-1)	10/4.1.2	Cetuximab (IMC-C225, Erbitux®)
	10/4.1.2	Panitumumab (ABX-EGF, Vectibix®)
	10/4.1.2	Matuzumab (EMD-72000)
	10/4.1.2	Nimotuzumab
	10/4.1.2	MDX-447
HER-2 (ErbB2)	10/4.2	Trastuzumab (Herceptin®)
	10/4.2	Pertuzumab (2C4, Perjeta®)
HER-2 and CD64	12/2.3	MDX-210
HER-2 and CD3	12/2.3	Ertumaxomab (Rexomun®)
TCR and CD3	12/2.3	Blinatumomab (AMG103, Blincyto®)
HER-3	10/4.3	MM-121
	10/4.3	U3-1287
	10/4.3	LJM716
VEGF, VEGFR	10/4.6.2	Bevacizumab (Avastin®)
	12/2.1	Ramucirumab (IMC-1121B, Cyramza®)
FGFR	10/4.7.4	PRO-001
PRLR	10/4.11	LFA102
EpCAM	12/2.2	Adecatumumab (MT201)
	12/2.2	Edrecolomab (Panorex®)
EpCAM and CD3	12/2.3	Catumaxomab (Removab®)
αvβ3 integrin	12/2.1	Etaracizumab (Vitaxin®, Abegrin®)
α5β1 integrin	12/2.1	Volociximab (M-200)
CTLA-4	12/2.2	Ipilimumab (Yervoy®)
	12/2.2	Tremelimumab (ticilimumab)
PD-1	12/2.2	Nivolumab (BMS936559, Opdivo®)

Continued

Table 12.1 Examples of mAbs Used as Single Entities in the Clinic or under Clinical Trials—cont'd

Target	Chapter/Section	mAb
	12/2.2	Pembrolizumab (lambrolizumab, MK-3475, Keytruda®)
CD-20	12/2.1	Rituximab (Rituxan®)
	12/2.1	^{131}I-tositumomab (Bexxar®)
	12/2.1	Obinutuzumab (Gazyva®)
	12/2.1	Ofatumumab (HuMax-CD20, Arzerra®)
CD-40	12/2.1	Lucatumumab (HCD122)
CD-52	12/2.1	Alemtuzumab (Campath®)
CD-38	12/2.1	Daratumumab
TGF-β/ALK-1	10/7	Fresolimumab (GC-1008)
	12/2.1	PF-03446962
CD-227	12/2.1	huHMFG1 (Therex®)
	12/2.1	GT-MAB 2.5-GEX (PankoMab®)
DKK-1	12/2.1	BHQ880

2.1 mAbs TARGETING SPECIFIC ANTIGENS

Among different surface cell molecules that are targets for immunophenotyping, the cluster of differentiation (CD) is formed by proteins that may act in signal cascades as receptors or ligands and are implied in different functions such as cell adhesion. The CD classification is used for the identification of the corresponding antibodies, which allows the nomenclature of mAbs against epitopes at the surface of different cells. Rituximab (Rituxan®) is a chimeric human–murine monoclonal antibody against the B-lymphocyte antigen CD20 that revolutionized the treatment of B-cell malignancies. It was the first monoclonal antibody approved for cancer therapy in 1997, although tositumomab, which targets the same antigen, was discovered first. Rituximab appears to be the most important treatment leading to improved outcome in a range of B-cell lymphomas and, recently, in B-cell chronic lymphocytic leukemia (CLL). It binds to amino acids 170–173 and 182–185 of the CD20 antigen, destroying both normal and malignant B cells that have this antigen on their surfaces and thus allowing the development of a new population of healthy B cells from lymphoid stem cells. Tositumomab linked to the ^{131}I radioisotope [^{131}I-tositumomab (Bexxar®)] was approved by the U.S. Food and Drug Administration (FDA) in 2003. Obinutuzumab (Gazyva®), a third-generation type II anti-antibody, selectively binds to the extracellular domain of the human CD20 antigen on malignant human B cells and was approved in 2013 for the treatment of previously untreated CLL. Ofatumumab (HuMax-CD20, Arzerra®) is a fully human monoclonal antibody, also directed at CD20, that was approved by the FDA for treating CLL that is refractory to fludarabine and alemtuzumab.

Many other anti-CD mAbs have entered the clinic.[4] All of them eliminate their targets through a wide range of effector pathways and may induce an adaptive antitumor immune response or "vaccination" effect. Lucatumumab (HCD122), a fully humanized antibody against the antigen CD40, entered a phase I clinical trial for CLL.[5] Alemtuzumab (Campath®) is a humanized monoclonal antibody that binds to CD52 (an antigen expressed on B and T lymphocytes) that was approved in

2001 by the FDA for patients with B-cell CLL when other therapies have failed. Another interesting antigen is CD227 (MUC1 mucin), which is expressed in epithelial tissues, human blood dendritic cells (DCs), and T cells. MUC1 is a heavily *O*-glycosylated transmembrane protein that is overexpressed in 90% of breast cancers and also in prostate cancer. In tumor cells, MUC1 has a shorter glycosylation pattern and is present over the entire cell surface with new epitopes exposed, which allows specific interactions with antibodies such as huHMFG1 (Therex®)[6] and GT-MAB 2.5-GEX® (formerly Panko-Mab).[7] Both have been clinically developed for the treatment of breast and ovarian cancers and breast and lung cancers, respectively.

Another promising mAb is daratumumab, a humanized antibody directed at CD38 with potent multifaceted antitumor activity. CD38 is a transmembrane glycoprotein and the prototypic member of the adenosine ribose (ADPR) transfer enzymes known as NAD-glycohydrolases (NAD-ases). In addition to its role as a coenzyme, nicotinamide adenine dinucleotide (NAD^+) and its metabolites play important roles in cell signaling as substrates for nucleotide-metabolizing enzymes and by providing ligands for extra- and intracellular receptors.[8] CD38 has two enzyme activities: one as an ADP-ribosyl cyclase and another as a cyclic adenosine diphosphate ribose (cADPR) hydrolase. The ADP-ribosyl cyclase activity of CD38 catalyzes the synthesis of cADPR, a second messenger for Ca^{2+}. cADPR is a cyclic adenine nucleotide connected through the two phosphate groups linked to the 5′-OH of the adenosine to another ribose at the 5′ position, which closes the cycle by glycosidic bonding to the nitrogen 1 (N^1) of the adenine base (Figure 12.2).[9]

Daratumumab is in phase III clinical trials and seems to be a promising single agent for the treatment of multiple myeloma (MM). It may also have potential in other cancers on which CD38 is expressed, including diffuse large B-cell lymphoma, CLL, acute lymphoblastic leukemia, plasma cell leukemia, acute myeloid leukemia (AML), follicular lymphoma, and mantle cell lymphoma.[10] Daratumumab has been granted breakthrough therapy designation from the FDA for the treatment of patients with MM who have received at least three prior lines of therapy, including a proteasome inhibitor and an immunomodulatory agent.

Among antibodies targeted to integrins, the most important one is volociximab (M-200), which blocks the α5β1 integrin and is under clinical evaluation for patients with refractory solid tumors, especially renal cell carcinoma.[11] Etaracizumab (Vitaxin®, Abegrin®) is a humanized monoclonal

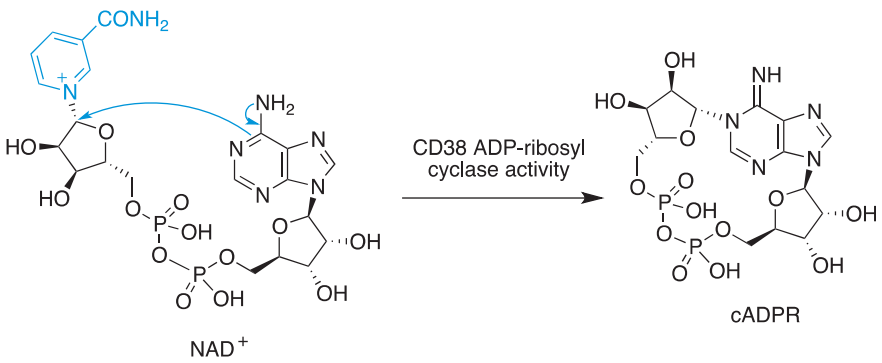


FIGURE 12.2

Reaction catalyzed by CD38 as an ADP-ribosyl cyclase.

antibody against the αvβ3 integrin that is being investigated for the treatment of metastatic melanoma and prostate, ovarian, and various other types of cancer.[12]

PF-03446962 targets activin receptor-like kinase-1 (ALK1), which is part of the transforming growth factor-β (TGF-β)/Smad signaling pathway (see Chapter 10, Section 7). ALK1 induces Smad1/5 phosphorylation, leading to an increase in endothelial cell proliferation and migration, whereas ALK5 promotes Smad2/3 activation and inhibits both processes. By shutting down ALK1, PF-03446962 inhibits the vascular endothelial growth factor (VEGF) pathway in a manner different from other antiangiogenic treatments on the market; for this reason, it is in preliminary clinical trials as a potential treatment for tumors resistant to VEGF therapy.[13] The anti-VEGF receptor (VEGFR) mAb ramucirumab (IMC-1121B, Cyramza®), has shown survival benefits in patients with advanced gastric or gastroesophageal junction adenocarcinomas progressing after first-line chemotherapy and was approved by the FDA in 2014 for this indication.[14]

BHQ880 is an antibody against DKK1 (dickkopf-related protein 1, see Chapter 11, Section 7.1) that has entered clinical trials in search for a myeloma first-line treatment in people with kidney problems to prevent bone damage and pain. Elevated levels of DKK1 in bone marrow, plasma, and peripheral blood are associated with the presence of osteolytic bone lesions in patients with MM, and its blocking promotes osteoblast activity.

Siltuximab (Sylvant®) is a chimeric mAb made from mouse and human proteins that targets interleukin-6 (IL-6). By interfering with IL-6 binding to its receptors, siltuximab prevents the IL-6-mediated secretion of VEGF and also the growth of B lymphocytes. It has been clinically investigated for several cancers, including metastatic renal cell cancer, prostate cancer, non-Hodgkin's lymphoma, and MM, and was approved by the FDA in 2014 for the treatment of Castleman's disease, an uncommon lymphoproliferative disorder.

2.2 SPECIFIC IMMUNOMODULATORY mAbs

In the context of cancer immunotherapy, mAbs may inhibit immunosuppressive molecules/cells or activate immunostimulatory molecules. The development of techniques for reengineering the T-cell receptor genes has yielded dramatic results in some patients, especially those with leukemia and melanoma, in which T cells allow a far better targeting than surgery, radiation, chemotherapy, or hormonal therapy.

The cytotoxic T-lymphocyte antigen 4 (CTLA-4), also known as CD152 (cluster of differentiation 152), is an inhibitory co-receptor that interferes with T-cell activation and proliferation; consequently, its inhibition by targeted antibodies has been studied in the treatment of several cancers.[15] The identification of this T-cell co-receptor in 1984[16] was an important breakthrough in understanding the human immune system, pioneering subsequent work in the genetics of immunology that has had a direct impact on personalized cancer medicine (see later).

The programmed cell death 1 (PD-1) receptor is another inhibitory receptor expressed by T cells whose primary ligand, programmed cell death 1 ligand (PD-L1; CD274), is frequently expressed within the tumor microenvironment. PD-1 signaling in tumors is required for both suppressing effector T cells and maintaining tumor regulatory T cells (Tregs). Consequently, its blockade augments tumor inhibition by increasing effector T-cell activity and attenuating Treg suppression. CD38, also known as cyclic ADP ribose hydrolase, is a glycoprotein that is expressed in most tissues and, in particular, on the surface of many immune cells, including $CD4^+$, $CD8^+$, B lymphocytes, and natural killer cells. It is a

powerful disease marker for human leukemias and myelomas. Because the products of NAD^+ and $NADP^+$ hydrolysis catalyzed by CD38 are essential for the regulation of intracellular Ca^{2+}, the most ancient and universal cell signaling pathway, this enzyme controls complex processes including immune responses. Loss of CD38 function is associated with impaired immune responses.[17]

Antibodies directed against CTLA-4, PD-1, and PD-L1 have entered clinical trials in patients with lung cancer and are being tested in phase III studies both as first-line (anti-CTLA-4) and later-line treatments (anti-PD-1). It has been recently reported that the dual blockade of PD-1 and CTLA-4, combined with a tumor vaccine, effectively restores T-cell rejection function in tumors.[18]

Ipilimumab (Yervoy®) binds to the CTLA-4 co-receptor and was approved by the FDA and the European Medicines Agency (EMA) in 2011 for the treatment of metastatic melanoma. It is currently undergoing clinical trials for other tumors.[19] As previously mentioned, in the antigen presentation process, antigen-presenting cells (APCs) display foreign antigens complexed with major histocompatibility complexes (MHCs) on their surfaces, and T cells recognize these complexes using their T-cell receptors (TCRs). Activation of T cells also requires a second signal—the interaction between its CD28 receptor with the APC's B7 protein (Figure 12.3a). After this T-cell activation process, the co-receptor CTLA-4 is recruited and triggers its inhibitory signal through its interaction with B7 (Figure 12.3b). The binding of ipilimumab to CTLA-4 blocks this inhibition effect (Figure 12.3c).

Another antibody against CTLA-4 that has been clinically studied in patients with metastatic melanoma is tremelimumab (formerly ticilimumab, CP-675,206). A phase III clinical trial did not demonstrate superiority to standard chemotherapy,[20] but further studies on prostate and bladder cancers are ongoing. Results with advanced hepatocellular carcinoma support further investigation for this application, although its use is associated with specific undesirable side effects, such as colitis and hypophysitis, that may be partially reduced with high doses of steroids. One mechanism by which cancer tissues limit the host immune response is via upregulation of PD-L1 and its ligation to PD-1 receptors on antigen-specific CD8(+) T cells.[21] The PD-1/PD-L1 blockade induces responses by inhibiting

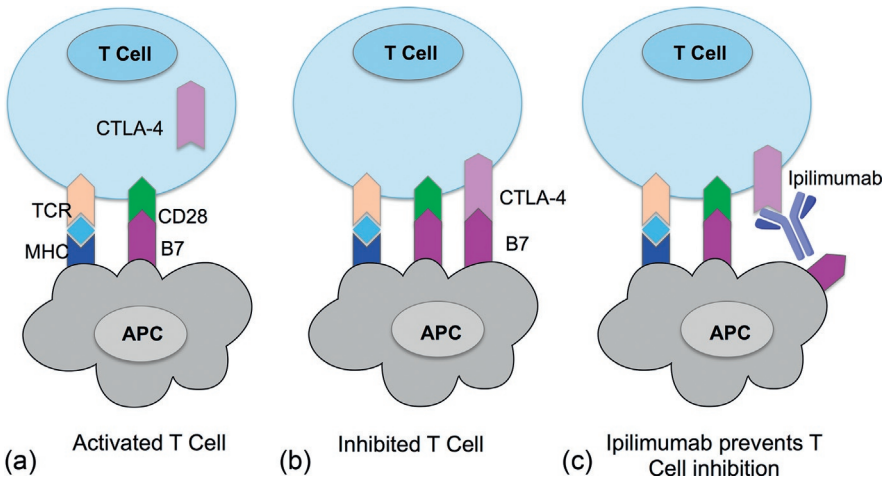


FIGURE 12.3

Mechanism of action of ipilimumab by preventing T-cell inhibition.

adaptive immune resistance and unprecedented rates of durable clinical responses in patients with various cancer types.[22]

The PD-1/PD-L1 axis was validated as a therapeutic target in two phase I clinical trials with the anti-PD-1 antibody nivolumab (Opdivo®, formerly known as BMS936558) and the anti-PD-L1 antibody BMS936559.[23] The FDA assigned a priority review designation to nivolumab as a treatment for pretreated patients with advanced melanoma, and it assigned a breakthrough designation for patients with Hodgkin's lymphoma. The EMA also granted an accelerated assessment to nivolumab.

Pembrolizumab (lambrolizumab, MK-3475, Keytruda®), which is a highly selective mAb against PD-1 receptor expressed by T cells, is being developed for potential use in metastatic melanoma (Figure 12.4).[24] It was approved by the FDA in 2014 for use following treatment with ipilimumab or after treatment with ipilimumab and a b-Raf inhibitor in patients who carry a b-Raf mutation.

MPDL3280A is an engineered anti-PD-L1 antibody designed to target PD-L1 expressed on tumor cells and tumor-infiltrating immune cells and also to prevent binding to PD-1 and B7.1 on the surface of T cells. This process, as well as the blocking of PD-1 receptors, may enable the activation of T cells as well as recruit other T cells to attack the tumor, thus empowering the body's own immune system to fight multiple types of cancer.[25] Phase I data for MPDL3280A in monotherapy or combination regimens are encouraging.[26]

Other monoclonal antibodies, including edrecolomab (Panorex®) and adecatumumab (MT201), target the Wnt receptor EpCAM (epithelial cell adhesion molecule) that is highly expressed on human carcinomas and is recognized as a target for immunotherapy of cancer. Both antibodies have shown interesting results in colorectal carcinoma,[27] but a phase III trial with edrecolomab in combination with 5-fluorouracil showed no statistically significant effect on overall survival.[28]

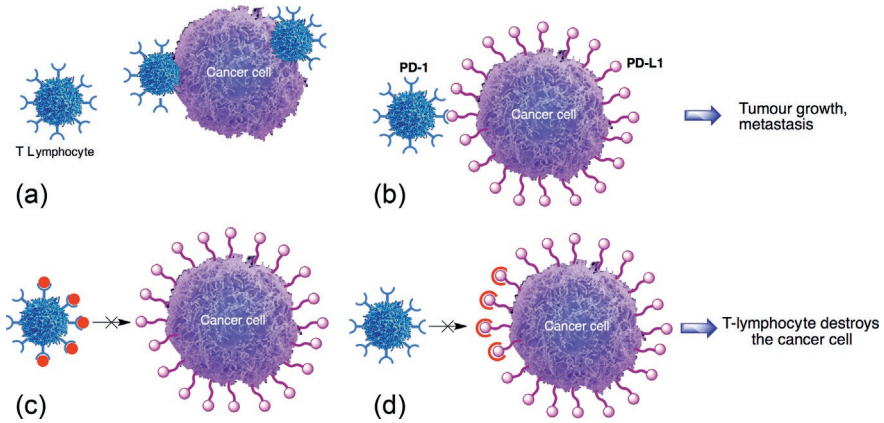


FIGURE 12.4

Mechanism of action of pembrolizumab. (a) T lymphocytes are able to destroy normal cancer cells. (b) Some cancer cells have in their membrane a protein (PD-L1) that protects them by recognizing a complementary protein at the surface of the T lymphocytes (PD-1). (c and d) Drugs able to block either PD-1 or PD-L1 restore the ability of T lymphocytes to recognize the cancer cells as a target for immune response. In this particular case, pembrolizumab acts by binding to PD-1 (mechanism C).

2.3 BISPECIFIC ANTIBODIES

Many mAb shortcomings could be overcome by creating bispecific antibodies (bsAbs)[29]—which are able to simultaneous bind to two different targets—because such molecules would have the possibility to target a large variety of payloads to cancer cells.

Catumaxomab (Removab®) is a trifunctional antibody that binds to EpCAM and to CD3 antigens and also to Fc receptors on APCs. It was approved in the European Union in 2009 for the intraperitoneal treatment of patients with malignant ascites, a condition that occurs in patients with metastasizing cancer,[30] and then by the FDA in 2011. It is also being studied for the treatment of peritoneal carcinomatosis in patients with gastric adenocarcinomas. Another bispecific antibody under clinical evaluation for breast or ovarian cancer that overexpresses the proto-oncogene HER-2 is MDX-210, which binds to HER-2 and CD64.[31]

Bispecific T-cell engagers (BiTEs®) are another class of bispecific antibodies, which are aimed at inducing the immune system to act against cancer cells by simultaneously binding to a cancer cell and T lymphocytes (Figure 12.5).

Ertumaxomab (Rexomun®) has two antigen-recognition sites, one for CD3 (an antigen expressed on mature T cells) and one for HER-2. In early clinical trials of patients with malignant ascites due to peritoneal carcinomatosis, administration of rather low doses of ertumaxomab led to the complete elimination of tumor cells and the disappearance of ascites accumulation in all patients.[32] Ertumaxomab has entered phase II trials for the treatment of metastatic breast cancer.

Blinatumomab (AMG103, Blincyto®) has an antigen-recognition site for the CD3 complex, a group of T-cell surface glycoproteins that bind to the TCR, and another for CD19, a tumor-associated antigen (TAA) overexpressed on the surface of B cells. Thus, blinatumomab allows T cells in the cancer patient to recognize malignant B cells. It was approved by the FDA in late 2014 for acute lymphoblastic leukemia.

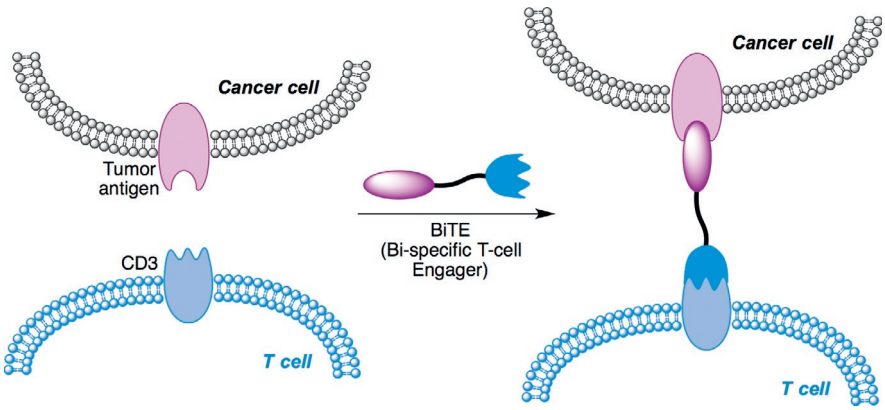


FIGURE 12.5

Mechanism of action of bispecific T-cell engagers (BiTEs®).

3 CANCER IMMUNOTHERAPY: GENERAL ASPECTS

As mentioned in Section 2.2, immune evasion is an emerging hallmark of cancer progression and activation of the immune system may be an effective treatment, but this system is extremely complex and has many regulatory mechanisms. Tumor progression is mainly restricted by effector T cells (cytotoxic T lymphocytes, CTLs) and facilitated by regulatory T cells (Tregs), formerly known as suppressor T cells, which are crucial for the maintenance of immunological tolerance. Immunomodulatory agents attempt to increase the efficacy of the CTLs, whose adequate presence in number and functionality is a prerequisite for the immune system to attack cancer cells.

There are three main requirements for cancer immunotherapy to be effective: (1) There must be enough high-affinity tumor-specific lymphocytes, (2) these lymphocytes must successfully infiltrate the tumor, and (3) the tumor-infiltrating lymphocytes must effectively kill tumor cells. The real situation is completely different because the potentially tumor-reactive T-lymphocyte population is represented by a small number of low-affinity T lymphocytes, the local tumor microenvironment is an important barrier for T-lymphocyte infiltration, and tumor cells develop several strategies to successfully evade antitumor immune response.

Helper T cells help to activate cytotoxic T cells, but they can function only when activated on the surface of APCs to become effector cells. Naive T cells require at least two signals for activation provided by an APC (usually a dendritic cell). One signal is provided by MHCs binding to TCRs, whereas the other signal is mainly provided by B7 co-stimulatory proteins binding to CD28 on the T-cell surface. If a T cell receives only one signal, it is usually deleted or inactivated (see Figure 12.3).

To date, very few clinical studies have combined conventional chemotherapies with anticancer vaccination and/or immunostimulatory regimens, and there is no assessment of the order in which cytotoxic therapies and tumor vaccines should be administered.[33] Nevertheless, immunotherapy is taking its first, highly promising steps, especially in lung cancer and metastatic treatments.[34] The final issue of the journal *Science* for 2013, devoted to the scientific advances of the year, regarded cancer immunotherapy as the most important breakthrough of the top 10 achievements of the year.[35]

The multiple approaches for cancer immunotherapy, many of which are in various stages of preclinical research, may be grouped into two categories—passive or active. Active approaches, or cancer vaccines, aim at inducing immune effector cells *in vivo* through the administration of immune mediators or cells capable of activating the immune system[36] or redirecting the normal T cells to recognize tumor antigens.[37] Passive immunotherapy uses *in vitro*-produced immunologic effectors capable of influencing effector T-cell responses through the administration of recombinant cytokines or mAbs. Each tumor mutation alters some amino acid sequences of the proteins encoded by the affected genes. Therefore, these mutant peptides are tumor-specific antigens foreign to the immune system and could be incorporated as vaccines by using viruses or DCs encoding or presenting the mutant peptides, antibodies, or T cells with reactivity against these mutant peptides.[38] However, because most proteins affected by mutations are intracellular, the mutant residues will not be visible to the immune system unless they are presented in the context of a human leukocyte antigen (HLA) protein.[39] A major additional problem is that tumors can lose immunogenicity through a variety of genetic alterations that preclude the presentation of epitopes.[40]

Due to the multiple mechanisms that cancer exploits to avoid immune-cell recognition and antitumor effector functions, immunotherapy strategies, including high-dose IL-2, interferon-α (IFN-α), and anti-CTLA-4 antibodies, are useful in only a low number of cancer patients.[41] Recombinant IFN-α-2b

(Intron A®) was approved by the FDA in 1997 for non-Hodgkin's lymphoma, and it was previously approved for hairy cell leukemia and AIDS-related Kaposi's sarcoma. The function of DCs, which are the most important APCs that initiate the primary antitumor effector T-cell response, is interfered by cytokines, chemokines, and metabolites produced by tumor cells. The tumor-induced hypoxia stimulates immunosuppressive cells such as macrophages to produce proangiogenic factors that cause numerical and functional defects of DCs with impaired capabilities for antigen uptake, diminished cell motility, and impaired ability to activate naive T cells. Direct interaction between tumor cells and tumor-infiltrating lymphocytes may result in an impaired TCR signaling that inhibits the lytic function of cytotoxic T lymphocytes. In addition, the expression by tumor cells of ligands such as the Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) may deliver death signals to activated T cells even at distant sites from the tumor.

Two types of immune cells with suppressive capacity on CTLs are currently known: Tregs and myeloid-derived suppressor cells (MDSCs).[42]

3.1 REGULATORY T CELLS

Tregs are a subpopulation of $CD4^+CD25^+$ T lymphocytes, considered the most powerful inhibitors of antitumor immunity, that confer cell growth and metastatic advantages. Their defects contribute to the induction of severe autoimmune diseases such as rheumatoid arthritis, whereas their stimulation induces the secretion of immunosuppressive cytokines that directly inhibit effector T cells and convert CDs into suppressive APCs. Treg depletion, suppression of Treg function, inhibition of its tumoral homing, and exploitation of T-cell plasticity are strategies to counter these effects.

Treg depletion may be achieved by chemical or radiation lymphoablation, mAbs directed against CD25 (such as those used to prevent rejection in organ transplantation) and immunotoxins.[43] Aldesleukin (Proleukin®) is a modified human IL-2[44] that was approved by the FDA in 1992. It was the first approved immunotherapy treatment for metastatic melanoma and is used in renal cell carcinoma.

The engineered protein denileukin diftitox (Ontak®), which combines IL-2 with the diphtheria toxin, received accelerated approval by the FDA for treatment of persistent or recurrent $CD25^+$ cutaneous T-cell lymphoma. Ontak® interacts with the high-affinity IL-2 receptor (CD25/CD122/CD132) on the cells surface and inhibits cellular protein synthesis.[45] Metronomic cyclophosphamide (metronomic refers to very low nontoxic doses of chemotherapy drugs delivered frequently for a prolonged period of time) may also induce Treg depletion.[46] A clinical trial involving pancreatic ductal adenocarcinoma (PDAC) patients, for which single-agent immunotherapies failed due in part to the barrier to immune infiltration and function that provides the tumor microenvironment, has shown that the allogeneic vaccine GVAX, in combination with metronomic cyclophosphamide, induces the formation of intratumoral tertiary lymphoid aggregates, resulting in the upregulation of immunosuppressive mechanisms including the PD-1/PD-L1 pathway. This study provides the first example of immune-based therapy converting a "nonimmunogenic" neoplasm into an "immunogenic" neoplasm by inducing infiltration of T cells and development of tertiary lymphoid structures in the tumor microenvironment.[47]

Suppression of Treg function may be achieved with anti-CTLA4 antibodies such as ipilimumab or tremelimumab, the antibody DTA-1, directed against the glucocorticoid-induced tumor necrosis factor receptor,[48] and the anti-RankL monoclonal antibody denosumab (Xgeva®, Prolia®), used for the treatment of osteoporosis. RankL is a member of the tumor necrosis factor (TNF) cytokine family that

functions as a key factor for osteoclast differentiation and activation. It is also important in the development of certain cancers. In fact, denosumab has been approved for unresectable giant cell bone tumor and targets bone metastases by increasing bone growth.

The Treg function may also be suppressed by activation of receptors such as the Toll-like receptor (TLR) or the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (see Section 3.3). This activation induces secretion of proinflammatory cytokines, stimulation of the member of the TNFR superfamily of receptors OX40 (CD134), or inhibition of CD73 (also known as 5′-ectonucleotidase, NT5E). CD73 is a membrane-anchored protein highly expressed on the surfaces of many types of cancer cells that converts AMP into adenosine and free phosphate, an activity associated with immunosuppression and prometastatic effects. The high concentration of adenosine produced by the CD73 on glioblastoma multiforme triggers an adenosine signaling that induces the multidrug resistance phenotype characteristic of this tumor.[49]

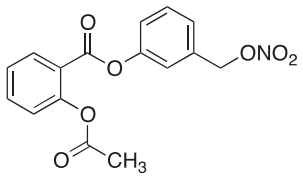
Inhibition of Treg tumoral homing may be achieved by blocking the selective recruitment and retention of Tregs at tumor sites through the interaction of some chemokine receptors and the protein CCL22, which is secreted by DCs and macrophages. Finally, exploitation of T-cell plasticity may be achieved by modulating IL-6, TGF-β, and PGE2 expression, as occurs with the COX-2 inhibitor celecoxib.[50]

3.2 MYELOID-DERIVED SUPPRESSOR CELLS

The second immune cells with suppressive capacity on CTLs are MDSCs. In healthy individuals, MDSCs generated in bone marrow quickly differentiate into monocytes, granulocytes, or DCs; in cancer (and also in patients with trauma, sepsis, or autoimmune diseases), this differentiation is partially blocked, which results in expansion and release of MDSCs.[51] These cells inhibit T-cell activation and enhance gene expression of cancer stem cells by removing the repression mediated by the transcriptional co-repressor C-terminal-binding protein 2 (CtBP2).[52]

MDSCs represent a significant obstacle to successful immunotherapy,[53] especially in non-small cell lung cancer (NSCLC). Their inhibition may be achieved with 5-fluorouracil, gemcitabine (Gemzar®), or VEGF/c-kit blockers such as sunitinib, imatinib, or dasatinib.[54] Other MDSC-suppressing or differentiation-inducing agents are 5-aza-2′-deoxycytidine, IL-10, anti-IL-4R aptamers (oligonucleic acid or peptide molecules that bind to a specific target molecule),[55] curcumin, and vitamin D_3.

Decreased T cell responsiveness promoted by MDSCs, involves nitric oxide synthase (NOS)2 and arginase (ARG1), to generate NO (that blocks the signal cascade from the IL-2 receptor) and induce L-arginine depletion (that modifies the T cell receptor with impairment of the its signaling properties). The NO releaser nitroaspirin (NCX-4016), besides its antiangiogenic activity,[56] normalizes the immune status of tumor-bearing hosts and promotes tumor eradication by cancer vaccination by interfering with the inhibitory activities of these two enzymes in myeloid cells.[57]



Nitroaspirin (NCX-4016)

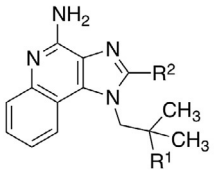
3.3 TOLL-LIKE AND NOD2 RECEPTORS

Nucleotide-binding and leucine rich repeat domain-containing proteins (NLR) are central to the formation of many inflammasome complexes. Toll-like receptors (TLRs) are transmembrane glycoproteins with an extracellular domain (ectodomain) that contains leucine-rich repeats responsible for mediating ligand recognition; a single transmembrane helix; and an intracellular Toll-like/IL-1 receptor domain responsible for downstream signaling.

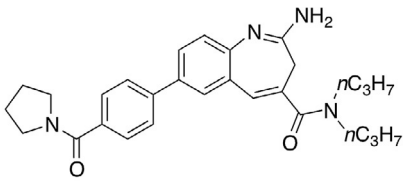
TLRs have a key role in host defense against pathogens by recognizing a variety of pathogen-associated molecular patterns. They are closely connected to inflammatory responses and are involved in the initiation of both innate and adaptive immune responses. Because functional TLRs are expressed not only on immune cells but also on cancer cells, they have a role in cancer by regulating cell proliferation and survival. TRL ligands have been described[58] as a "double-edged sword" in cancer because, on the one hand, uncontrolled TLR signaling creates a microenvironment that allows tumor cells to evade the immune response and proliferate, but on the other hand, TLRs can induce an antitumor immune response.

Some imidazoquinolines, such as imiquimod (R-837) and resiquimod (R-848), have been identified as agonists of TLRs, especially TLR-7, with subsequent secretion of cytokines, particularly IFN-α, IL-6, and TNF-α. Topical imiquimod (Zyclara®, Aldara®) is used to treat genital warts and certain skin cancers such as basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, superficial malignant melanomas, and actinic keratosis, generally following surgery. Resiquimod is also used for the treatment of several types of skin lesions.

Motolimod is an agonist of TLR-8 that is able to activate myeloid DCs, monocytes, and natural killer cells, resulting in the liberation of mediators that integrate the innate and adaptive antitumor responses to a number of cancers. As a consequence, the combination of motolimod (VTX-2337) with small-molecule chemotherapeutic agents or mAbs increases their antitumor response. Phase II trials of some such combinations in solid tumors, including ovarian and head and neck cancer, are in progress.[59] In 2014, the FDA granted fast track designation to the motolimod-pegylated liposomal doxorubicin combination for the treatment of women with relapsed ovarian cancer after platinum-based chemotherapy.



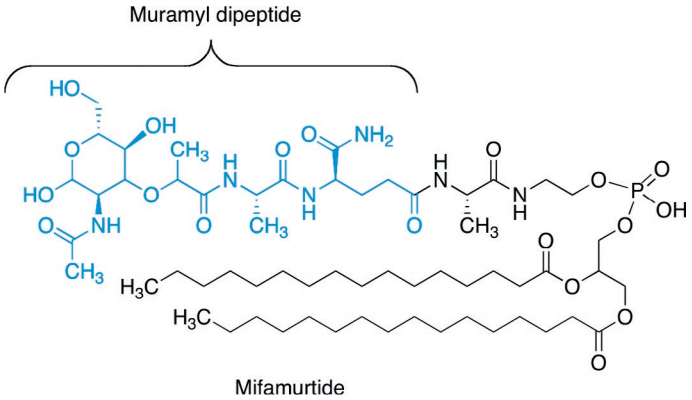
Imiquimod $R^1 = R^2 = H$
Resequimod $R^1 = CH_2OH$; $R^2 = CH_2OEt$



Motilimod

Besides TLRs, the intracellular receptor of innate immunity NOD2 (nucleotide-binding oligomerization domain-containing protein 2) is another interesting immunity-related target. Although the molecular mechanisms underlying this signal transduction pathway remain largely unknown,[60] its activation by muramyl dipeptide (MDP), a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria, stimulates an immune reaction mediated by monocytes and macrophages. Mifamurtide (liposomal muramyl tripeptide phosphatidyl ethanolamine, MTP-PE, Mepact®), a derivative of MDP, has similar effects, stimulating an immune reaction against cancer cells[61] with the advantage of having a longer half-life in plasma. Encapsulated into liposomes, where due to its phospholipid nature is

accumulated in the lipid bilayer, is indicated in the EU and some other countries for the treatment of high-grade, resectable, non-metastatic osteosarcoma after macroscopically complete surgical resection.[62]



Mifamurtide was discovered in the early 1980s and achieved the orphan drug status by the FDA and the EMA in 2001 and 2004, respectively. In 2007 the FDA denied its approval for the treatment of osteosarcoma, but it received the EMA marketing authorization in 2009.

The PD-1/PD-L1 axis was validated as a therapeutic target in two phase I clinical trials with the anti-PD-1 antibody nivolumab (Opdivo®, formerly known as BMS936558) and the anti—PD-L1 antibody BMS936559.[63] The European Medicines Agency (EMA) granted an accelerated assessment to nivolumab and the FDA gave it a breakthrough designation for patients with Hodgkin lymphoma and approved it in late 2014 for the treatment for patients with unresectable or metastatin melanoma.

Adiciones AL 14

INO-1001[64] enhances the antitumor effects of doxorubicin in p53 deficient breast cancer and is in clinical trials for several types of cancer in combination with temozolomide.[65] Iniparib (BSI-201) is not really a PARP inhibitor, although it was firstly considered as such. Its development was discontinued in 2011 after the disappointing results obtained from a phase III clinical study. Rucaparib (AG014699, PF-01367338), an analogue of AG14361, was the first clinically studied PARP inhibitor in combination with temozolomide.[66] This combination is well tolerated and PARP inhibition was observed at all dose levels studied, with increased SSBs in all patients with melanoma, pancreas and prostate cancer, among other tumors. Based on these results, a phase II study was conducted in patients with metastatic malignant melanoma.[67] Olaparib (AZD-2281, Lynparza®) is an orally active PARP inhibitor lethal for BRCA-defficient cells that has been tested in women with advanced ovarian cancer that showed mutation in BRCA1 or BRCA2 genes.[68] After giving very good result in clinical trials, it was approved by the FDA in December 2014 for previously treated BCRA-mutated ovarian cancers. Veliparib (ABT-888)

is another potent PARP inhibitor[69] that enhances the temozolomide effects in different cancer models, being a radiosensitizer in acute hypoxia conditions.[70] Unfortunately, it causes important myelosuppression. Another clinically evaluated compound is CEP-9722,[71] a prodrug that attenuated *in vivo* PARP activity and resulted in significant chemosensitization of temozolomide and irinotecan.[72]

4 CANCER VACCINES

As previously mentioned, cancer vaccines are active immunotherapy approaches aiming to induce immune effector cells through the administration of immune mediators or cells capable of activating the immune system or redirecting the normal T cells to recognize tumor associated antigens that otherwise escape immunologic surveillance.

In addition to their profound impact on public health through treatment of infectious diseases, vaccination strategies for cancer seem to hold clinical promise based on the expanded understanding of the interactions among tumor cells, their infiltrating microenvironment, and the host immune response. During the past several decades, major research efforts have been focused on the development of cancer vaccines, which are intended either to treat existing cancers (therapeutic vaccines) or to prevent the development of cancer (prophylactic vaccines). Some of these have been approved or have reached advanced clinical trials,[73,74] although many promising preclinical studies have failed to translate into meaningful clinical results.[75] Clinical results of first-generation vaccines, which were based on whole-cell preparations or tumor lysates derived from autologous or allogeneic tumors, established the feasibility of immunizing cancer patients against their own tumors. Results of the second-generation vaccines, which were designed to target well-characterized tumor-associated antigens, showed that they may be safe without inducing unacceptable clinical signs of autoimmunity. Technological advances in vaccine development and manufacture and improved regulatory review have substantially minimized the risk of harm from cancer vaccines. However, safety is still a matter of concern in cancer preventive vaccination.

Although cancer vaccination is a promising novel approach by itself, its combination with additional therapies can produce synergistic effects.[76]

4.1 TUMOR CELL VACCINES AND VACCINATION ANTIGENS

Cancer treatment vaccines are intended to delay or stop cancer cell growth, to cause tumor shrinkage, to prevent cancer from returning, or to eliminate cancer cells that have not been killed by other forms of treatment or by the immune system. The strategies developed to stimulate the immunoresponse to cancer are based on the existence of cancer cell antigens that are rarely present on normal cells or on the use of their modified versions to make them more clearly foreign.

Cancer treatment vaccines may use weakened or dead cancer cells that carry specific cancer-associated antigens or immune cells that are modified to express such antigens. These cells can come from the patients themselves (autologous vaccines) or from other patients (allogeneic vaccines). For instance, genetically modified T cells have been tested in the treatment of patients with advanced MM. In this case, APCs such as dendritic cells are previously stimulated with the patient's own cancer antigens and are reinjected into the patient, with the expectation that the activated T cells, encoding an enhanced version of the killer TCR, will attack tumor cells that express those antigens (Figure 12.6).

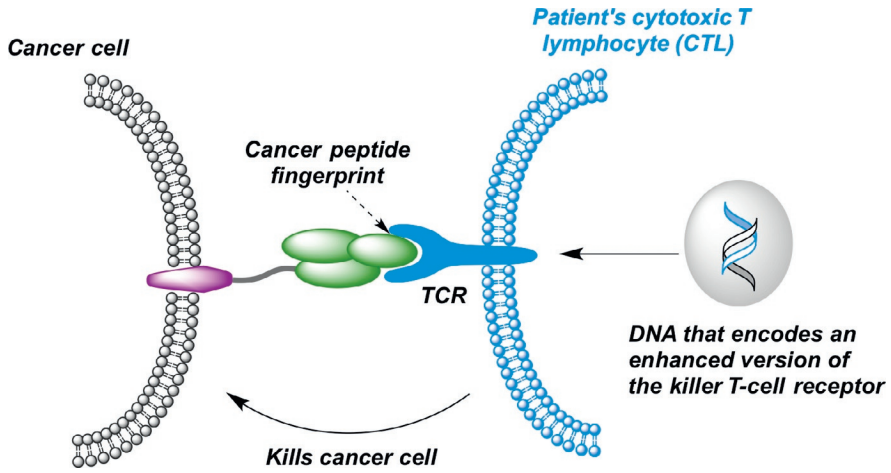


FIGURE 12.6

Cancer treatment with cells modified to prompt an immune response.

4.2 DNA AND RNA CANCER VACCINES

Vaccines for cancer treatment may also use DNA or RNA molecules that encode cancer-associated antigens and are injected into a patient as "naked nucleic acids" or incorporated into harmless viruses. A drawback of DNA vaccines is the potential of the DNA to integrate into the genome of the cell that takes it up, thus potentially promoting malignancy. This integration problem may be avoided in RNA vaccines,[77] but their efficacy can be compromised by degradation by RNAases.

Among the whole-cell-based autologous cell (personalized) vaccines[78] is sipuleucel-T (Provenge®), the first cancer treatment vaccine approved by the FDA in 2010 for patients who have advanced to the late stage of hormone-refractory prostate cancer.[79] This autologous vaccine was designed to stimulate an immune response to prostatic acid phosphatase (PAP), an antigen that is found on most prostate cancer cells. Unlike some other cancer treatment vaccines under development, sipuleucel-T is customized to each patient through a rather complex process. First, the patient's white blood cells, primarily DCs, are extracted in a leukapheresis procedure. This blood product is sent to a factory and incubated with a fusion protein consisting of PAP and an immune signaling factor granulocyte–macrophage colony-stimulating factor (GM-CSF) to help the DCs mature. Finally, this activated blood product is reinfused into the patient, who receives three treatments, usually 2 weeks apart, with each round of treatment requiring the same manufacturing process. Although the precise mechanism of action of sipuleucel-T is not known, it appears that the APCs that have taken up PAP–GM-CSF stimulate T cells of the immune system to kill tumor cells that express PAP. Provenge® is one of the most expensive cancer treatments, and its benefit has been controversial[80] because the vaccine does not cure prostate cancer but helps extend patients' lives by several months on average. Studies to determine if this vaccine can help men with less advanced prostate cancer are in progress.

4.3 CARBOHYDRATE VACCINES

Cell surfaces are covered by a complex array of glycoproteins, and these oligosaccharides have a crucial role in modulating cell–cell and cell–matrix interactions. Changes in this glycosylation pattern are a universal feature of tumor cells; therefore, these carbohydrate structures may serve as antigens for the elaboration of vaccines.[81] Several families of carbohydrates are expressed at higher levels in malignant cells; these tumor-associated carbohydrate antigens (TACAs) can be classified as follows:

1. Glycolipids, which contain a carbohydrate linked to a ceramide lipid that anchors the structure to the lipid bilayer of the cell membrane. They are further subdivided into several families, including the gangliosides (e.g., GM1), the globo- series (e.g., Globo-H), and the lacto- and neolacto- series (e.g., Lewis^y or Le^y).
2. Glycoproteins, including Tn, TF, and STn, in which the carbohydrate is covalently linked to the hydroxyl group of serine or threonine residues in the protein.

TACAs have several advantages for the preparation of vaccines. Because they are the most common antigens on the surface of cancer cells, their presence correlates very well with cancer progression, and they are shared by many cancer cell types. Furthermore, there is strong experimental evidence that anti-TACA immune responses increase the survival rate of cancer patients. The main obstacle to the development of anti-TACA vaccines has been problems with their isolation and purification from natural sources, which has required the development of methodology allowing their preparation by total synthesis.[82]

The first generation of synthetic anticancer vaccines to be evaluated on humans were monomeric because they were constructed from a single carbohydrate antigen conjugated to a carrier protein, most often the keyhole limpet hemocyanin (KLH) protein. The first antigen to be studied was Globo-H, a hexasaccharide that is overexpressed in the surfaces of several types of tumors, including colon, lung, ovary, and prostate, followed by Lewis^y (Le^y) and fucosyl GM1 (Figure 12.7). The most advanced of these compounds is the Globo-H–KLH construct shown in Figure 12.8, which is in phase II/III clinical trials for breast cancer.

The mucins are a group of glycoproteins that are overexpressed on tumor cell surfaces, and they show clusters of several carbohydrate domains. In an effort to achieve a resemblance to these structures, a second generation of monomeric anticancer vaccines was designed, which contained several units of mono- and disaccharides present in the mucins. These compounds are exemplified by the structures shown in Figure 12.9, and many of them are under clinical evaluation.

Monovalent vaccines have the disadvantage that they do not account for the presence of multiple carbohydrate antigens on the surface of tumor cells. One approach to solving this problem is the simultaneous administration of several antigens that have been previously shown to be associated to a particular type of cancer. Although this *polyvalent monomeric approach* has been shown to be successful in clinical trials, it does have some shortcomings, including the following:

1. The use of increased amounts of the carrier protein.
2. The need to carry out as many bioconjugation steps as antigens are present in the vaccine because each antigen is conjugated to a protein. These steps are low yielding and difficult to reproduce, and they constitute the bottleneck of the synthetic process.
3. The need for regulatory validation of each component of the mixture of antigens.

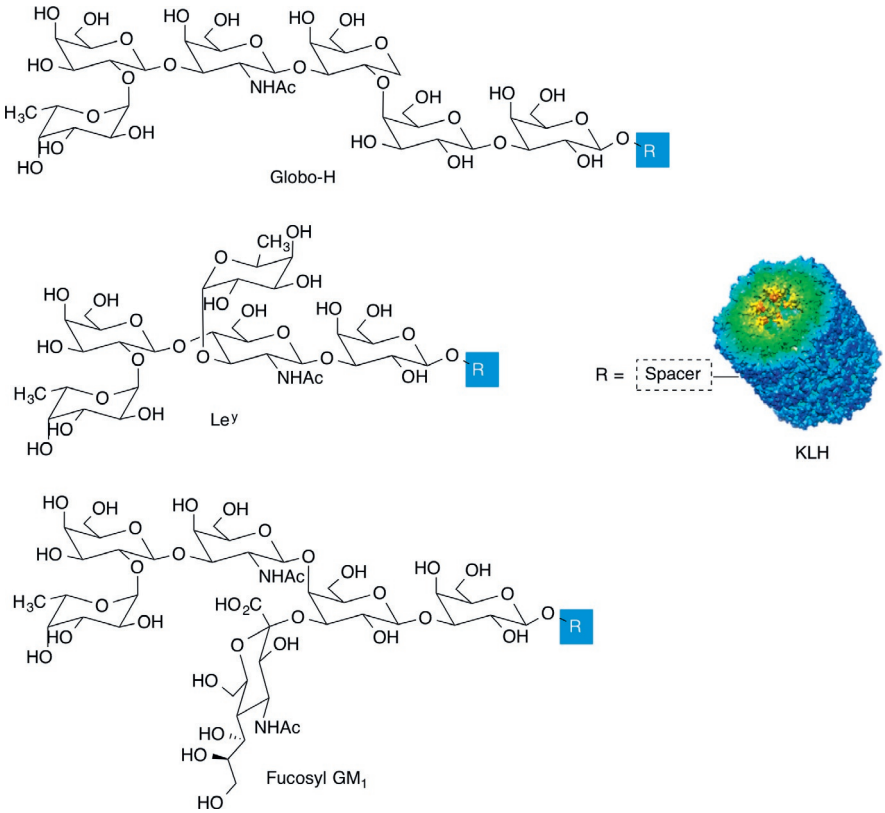


FIGURE 12.7

Representative synthetic monomeric anticancer vaccines.

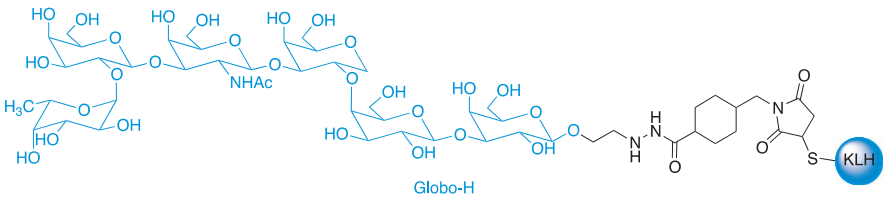


FIGURE 12.8

The structure of Globo-H–KLH.

The realization of these pitfalls led to the introduction of *unimolecular multivalent vaccines*, in which several carbohydrate antigens are incorporated into a single peptide backbone. Some of these have entered phase I clinical trials. Two examples of such constructs are shown in Figure 12.10.

Racotumomab (Vaxira®) is a therapeutic cancer vaccine that is being clinically studied for the treatment of solid tumors and induces an immune response against *N*-glycolyl-GM3 (NGcGM3), a ganglioside found on the cell surface in a variety of tumors, including lung, breast, and melanoma.

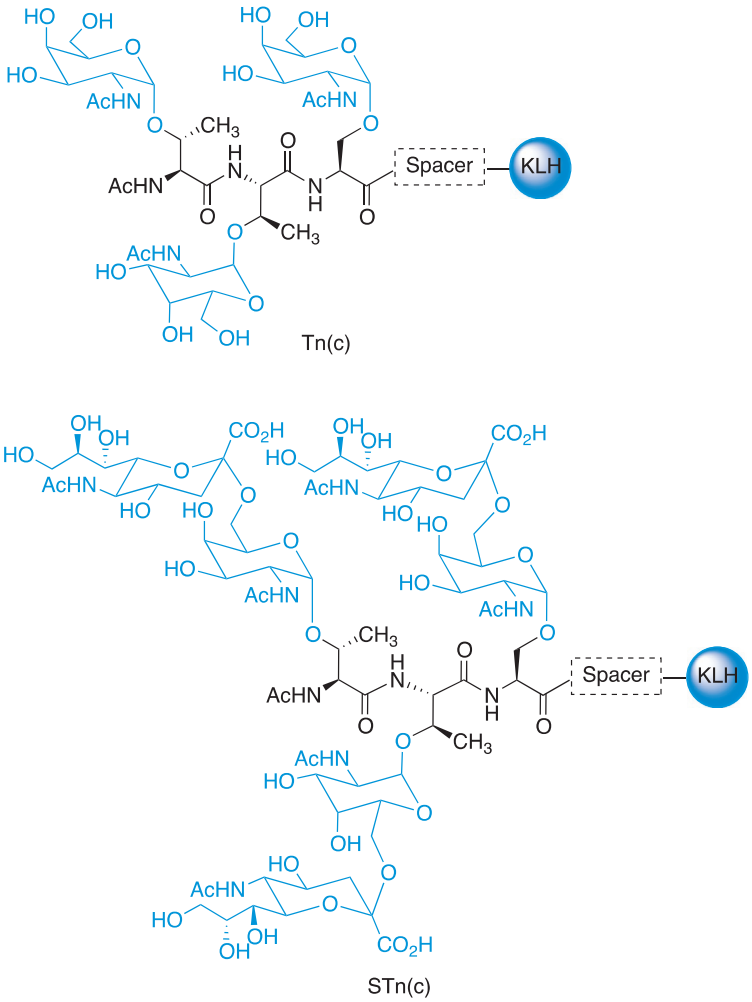


FIGURE 12.9

Monomeric cancer vaccines derived from the mucins.

4.4 PEPTIDE VACCINES

Many clinical trials of peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1 (MAGE1)-derived peptide-based vaccine was reported.[83] The earlier generations of peptide vaccines were composed of one to several human leukocyte antigen class I-restricted CTL-epitope peptides of a single human leukocyte antigen type. Currently, various types of next-generation peptide vaccines are under development.[84]

The development of a vaccine directed against the tumor-specific antigen MAGE-A3 for the treatment of lung cancer has recently been interrupted, although an ongoing phase III trial in melanoma is still in progress. This vaccine is a fusion protein of MAGE-A3 and *Haemophilus influenzae* protein D, combined with a proprietary immunoadjuvant.[85]

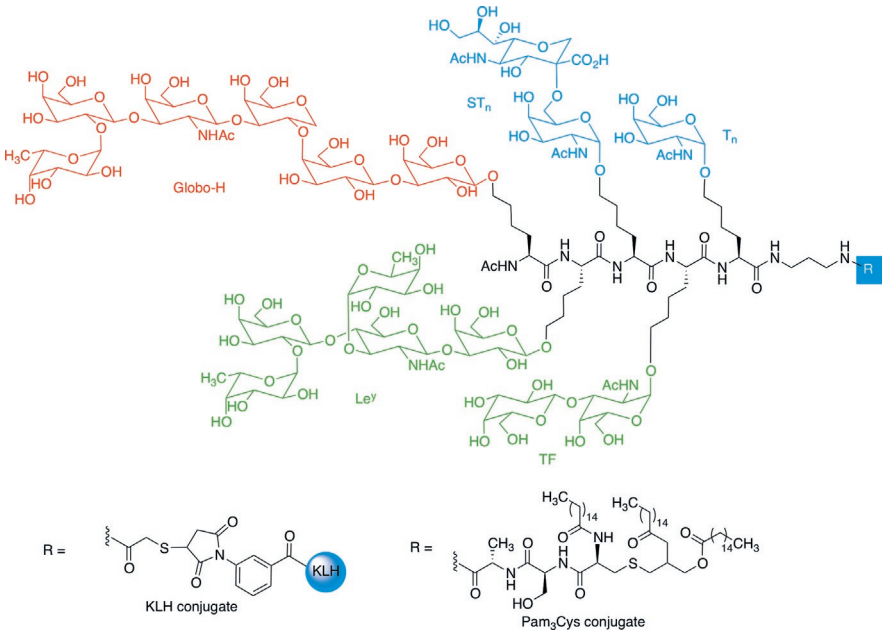


FIGURE 12.10

Two examples of unimolecular multivalent cancer vaccines.

Heat shock proteins (Hsps) are among the most ubiquitous soluble intracellular proteins and have a key role in immunologic phenomena, allowing their use for cancer immunotherapy. The autologous Hsp–peptide vaccine HSPPC-96 (Vitespen®, formerly Oncophage®) is a protein peptide complex consisting of GP96, a 96-kDa Hsp, and an array of GP96-associated cellular peptides.[86] Immunization with HSPPC-96 entered a phase II multicenter clinical trial, which showed that it may be clinically beneficial on recurrent glioblastoma multiforme (GBM) patients because it induces T-cell-specific immunity against those peptides.[87]

NeuVax is another peptide-based vaccine that has begun a phase III trial aimed at preventing or delaying the recurrence of breast cancer. It works by harnessing the patient's own immune system to seek out and attack any cells that express HER2/neu, a protein associated with tumors in breast, ovarian, pancreatic, colon, bladder, and prostate cancers. It consists of an E75 synthetic peptide initially isolated from HER2/neu proto-oncogene combined with the immune adjuvant, GM-CSF (rhGM-CSF from yeast).[88] Another group of interesting candidates as vaccination antigens are antiapoptotic proteins such as inhibitors of apoptotic signals (IAPs) and Bcl-2 because they enhance the survival of cancer cells and facilitate their escape from cytotoxic therapies.[89] The transmembrane protein mucin 1 (MUC1), a glycoprotein that is widely overexpressed in lung, breast, prostate, and colorectal cancers, has been targeted in different vaccines against NSCLC that have been clinically studied. Tecemotide (emepepimut-S, BLP25) is a synthetic lipopeptide formed by 27 amino acids, the first 25 of which are derived from the MUC1 sequence. This lipopeptide is used as the antigen in a liposomal therapeutic cancer vaccine known as Stimuvax (liposomal BLP25 vaccine,

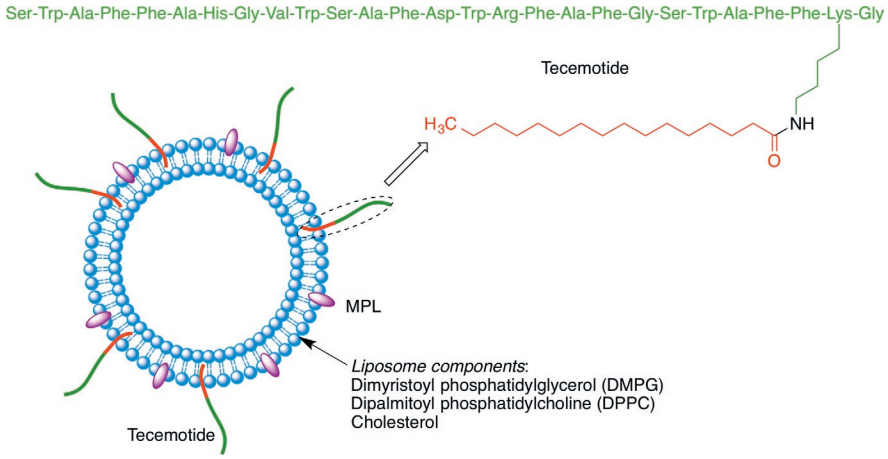


FIGURE 12.11

Structure of the Stimuvax liposomal vaccine.

L-BLP25), which is designed to induce a cellular immune response to cancer cells that overexpress MUC1. For the preparation of the vaccine, tacemotide and the adjuvant 3-*O*-deacyl-4′-monophosphoryl lipid A (MPL), derived from a molecule found in the membrane of gram-negative bacteria known as lipid A, are anchored in the liposomal membrane (Figure 12.11). This vaccine underwent several years of clinical studies against NSCLC,[90] but this clinical program was discontinued in 2014.

Another cancer vaccine related to the MUC1 protein is MUC1-SP-L (ImMucin, VXL100), a synthetic vaccine formed by the complete signal peptide domain of MUC1[91] that was shown to promiscuously bind multiple MHC alleles[92] (see Section 2.2) and has successfully completed phase I/II clinical testing in MM patients.

Met-Thr-Pro-Gly-Thr-Gln-Ser-Pro-Phe-Phe-Leu-Leu-Leu-Leu-Leu-Thr-Val-Leu-Thr-Vasl-Val-NH_2

MUC1-SP-L (ImMucin, VXL100)

4.5 TELOMERASE-TARGETED VACCINES

Telomerase reverse transcriptase (hTERT) represents an attractive target for cancer immunotherapy because it is expressed in very low levels in normal cells and is reactivated in most human tumors.[93] Nearly 30 different hTERT peptide components, acting as antigens, cause an expansion of telomerase-specific $CD8^+$ CTLs, directing the patient's own immune system to target and kill telomerase-positive tumor cells. Several of them are being developed as vaccines and tested in patients with melanoma, lung, prostate, breast, and pancreatic cancers, generally producing a specific immune response. Vaccination with the peptide $hTERT_{540–548}$ showed functional antitumor responses in prostate, breast, and melanoma patients.[94] The injectable vaccine GV1001, formed by the peptide $hTERT_{611–626}$, entered phase I/II clinical trials for several cancers and could become the first approved anti-telomerase-based

cancer therapy, although results seem to be disappointing.[95] GRNVAC1, a vaccine that has completed phase II clinical trials in patients with acute myelogenous leukemia and metastatic prostate cancer,[96] uses autologous immature DCs that are transduced *ex vivo* with mRNAs encoding a near full-length hTERT protein. After these DCs are matured, they are returned back to patients to elicit a polyclonal anti-hTERT T-cell response.

4.6 VACCINES AGAINST ONCOGENIC VIRUSES

Approximately 15–20% of cancers are associated with viral infections. HTLV-1 (adult T-cell leukemia, ATL), human papillomavirus (HPV; cervical, head, neck, and other cancers), HHV-8 (Kaposi's sarcoma), hepatitis B virus (HBV) and hepatitis C virus (HCV) (hepatocellular carcinoma), and Epstein–Barr virus (EBV; Burkitt's lymphoma) are examples of oncogenic viruses.[97] Currently, vaccines against HPV and against HBV are available, and scientific endeavor continues for six other cancer-associated infections, mostly viruses (other infectious agents such as bacteria and parasites are also associated with some types of cancer). These vaccines are based on viral antigens that are modified to make "virus-like particles" that are not infectious and therefore cannot cause disease. Synthetic versions of antigens that modify their chemical structure to stimulate immune responses are also being created in the laboratory for use in cancer preventive vaccines.

The first cancer preventive vaccine, approved by the FDA in 1981, was directed against HBV, an infection that can lead to liver cancer. The Papanicolaou test, developed in the 1920s, was introduced clinically in the 1940s. After its widespread implementation by collecting cells from the cervix to evaluate changes in cellular morphology consistent with preneoplasia or cancer, deaths from cervical cancer declined rapidly. In 1983, it was established that several HPV strains that can be transmitted sexually have oncogenic potential and produce cervical cancers, and also some vaginal, vulvar, anal, penile, and oropharyngeal cancers.[98] The search for vaccines to protect against these HPV infections led to FDA approval in 2006 of Gardasil® and Cervarix®, which protect against 4 and 2 HPV types, respectively. These vaccines cannot prevent the development of all cervical cancers, but they may reduce their incidence by 70%.[99]

5 GENE THERAPY

Gene therapy is based on the insertion of a functional gene into the somatic cells of a patient to correct an inborn metabolic error, to repair an acquired genetic abnormality, or to provide a new function to a cell. The main problem associated with gene therapy is the lack of efficient and selective vectors to deliver the genes. Ideally, a gene therapy vector would target a specific tissue with high transduction efficiency, sustaining a stable and regulated gene expression without any side effects or immunogenic responses. These criteria are not yet fulfilled.

Viruses are the most commonly employed vectors used in gene therapy, although they are not ideal because they trigger an immunological response. Nonviral vectors are safer but less efficient. The most promising of these are the synthetic cationic liposomes formed by positively charged amphiphilic molecules in which the positive charges interact electrostatically with negative charges in DNA phosphate groups, forming complexes that can enter the cells. Unfortunately, due to the low efficiency of DNA delivery by these systems, the amount of liposome currently required is too large to allow clinical use.

Efforts to treat cancer through immunization or deliberate infection with natural oncolytic viruses began in the mid-twentieth century, when the technology for creating a custom virus did not exist. These treatments induced significant morbidity and mortality, and the very frequent development of an immune response destroyed the virus, thus preventing its oncolytic function. For these reasons, this strategy was nearly abandoned for a time. Today, the technology required to modify viruses has been thoroughly developed, and systemic oncolytic viral therapy is a plausible alternative.[100] Adenoviruses have a low pathogenicity in humans and are relatively easy to manipulate using recombinant DNA techniques. Furthermore, their genome does not undergo rearrangement at a high rate, and the inserted foreign genes are maintained through multiple rounds of viral replication. During vector development, the viral surface proteins may be modified, removed, or replaced.[101] Once internalized into the cells, oncolytic viruses or viral vectors use a vesicular transport in which the viral envelope is metabolized and their content is released to the cytoplasm, enters the nucleus through a nuclear pore, and viral transgene expression occurs.

To improve patient safety and increase the gene transfer efficiency of viral vectors, the target cells may be removed from the patient, transduced with viral vectors, and reintroduced into the patient. However, this method is limited to cells available either by extraction or by growing from the stem cells *in vitro*. Excluding this *ex vivo* approach, the vectors need to be directly injected into the patient or delivered and retained in target areas with vector reservoirs. Among other administration methods, the inclusion of the antibody into paramagnetic particles enables the physical concentration of viral vectors by applying a local magnetic field.[102] Several potential strategies for cancer therapy based on gene therapy that are being explored in clinical trials are summarized here.

5.1 REPLACEMENT OF DEFICIENT OR ABSENT TUMOR SUPPRESSOR GENES: ONCOLYTIC VIRUSES

Oncolytic virus therapy is based on the concept of using live viruses to selectively replicate in cancer cells, with minimal destruction of normal tissue. Replication amplifies the input dose of the oncolytic virus and helps spread the agent to adjacent tumor cells. This strategy is very important in cancer therapy because tumor suppressor gene-inactivating mutations predominate over oncogene-activating mutations in the most common solid tumors. The first gene-based products were Gendicine® and Oncorine®, which entered the Chinese market in 2003 and 2006, respectively. Gendicine® is a recombinant human adenovirus vector containing the therapeutic *P53* gene. It is used in China for the treatment of various cancers,[103] and its combination with radiotherapy favors the control of nasopharyngeal carcinoma. Oncorine® is a genetically modified oncolytic adenovirus that was approved in China for the treatment of head and neck cancer. Oncorine® and the very similar ONYX-015 have been engineered to remove a viral defense mechanism that involves the human gene *P53*. Many viruses exploit the defects of cancer cells in the p53 tumor suppressor pathways to replicate, package its genome, lyse the cell, and spread to new cells. To do so, they produce E1B proteins that bind to and degrade p53 transcription factors, preventing cell apoptosis. In the ONYX-015 and Oncorine® adenoviruses, the *E1B* gene has been knocked out and the infected cells are unable to block the p53 function. If ONYX-015 or Oncorine® infect a normal cell with a functioning p53 gene, their multiplication will be prevented by the action of the p53 transcription factor, but if they infect a p53-deficient cell, they should be able to survive and replicate, resulting in selective destruction of cancer cells.

DNX-2401 (also known as Ad5 Delta-24-RGD-4C) is an oncolytic adenovirus that selectively replicates in cancer cells defective in the Rb/p16 tumor suppressor pathway. The *Rb* gene product and the cyclin-dependent kinase inhibitor p16 are integral components of the late G_1 restriction point. DNX-2401 has an RGD-4C peptide motif, inserted into the adenoviral fiber, that allows it to anchor directly to integrins in a receptor-independent infection of tumor cells where its active replication may induce oncolysis.[104] It is in phase I/II trials for recurrent GBM. When combined with temozolamide, it is much more effective than any other treatment against GBM stem cells, which supports the initiation of clinical studies for this combination.

Talimogene laherparepvec (T-VEC, OncoVEX®) is an oncolytic virus engineered from the herpes simplex-1 virus that has undergone phase III clinical study for the treatment of melanoma as a single therapy, although it failed to significantly improve survival rates.[105] A talimogene laherparepvec–ipilimumab combination is also under study, and phase I data have shown good tolerability at the doses administered.

JX-594 is a multitargeted oncolytic poxvirus that was tested in phase II in patients with hepatocellular carcinoma and demonstrated increased survival as compared to patients treated with sorafenib.

Pelareorep (Reolysin®) is a proprietary formulation of the human reovirus that is under development for the treatment of various cancers. A phase II study has been completed in patients with sarcomas metastatic to the lung.

5.2 GENE TRANSFER (SUICIDE GENE) THERAPY

As previously mentioned, viral vectors that selectively infect dividing tumor cells can be modified to carry a gene for an enzyme that activates an antitumor prodrug so that after its administration the prodrug is preferentially bioactivated in the tumor cells. For this reason, this approach is known as virus-directed enzyme prodrug therapy (VDEPT). For instance, sitimagene ceradenovec (Cerepro®) uses the adenoviral vector Ad5 to introduce the gene that causes tumor cells to express the herpes simplex virus thymidine kinase (TK) and activate ganciclovir to its triphosphate. Because of the absence of the 3′-OH deoxyribose group, when this compound is incorporated into DNA, it behaves as a chain terminator, blocking DNA synthesis and killing the cell (Figure 12.12).[106]

The combination of Cerepro® and ganciclovir entered clinical trials for treatment of the malignant brain tumor glioblastoma multiforme. Following the standard surgery to remove the solid tumor mass, Cerepro® was injected into the surrounding healthy brain tissue and ganciclovir was administered 5 days after surgery. Based on the results of three clinical trials performed from 1998 to 2004, it

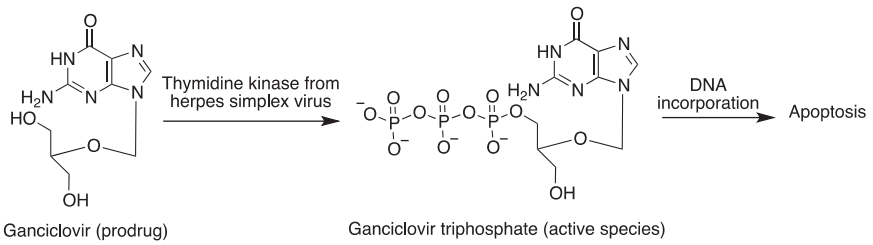


FIGURE 12.12

Activation of ganciclovir by the thymidine kinase from herpes simplex virus.

was designated as an orphan drug by the EMA and the FDA. However, after a further trial was required by the EMA, Ark Therapeutics removed its application in 2010.

For further discussion of the VDEPT technique, see Section 2.3 of Chapter 13.

5.3 TRANSFER OF RESISTANCE GENES FOR CHEMOPROTECTION OF HEMATOPOIETIC STEM/PROGENITOR CELLS

Although dose-limiting toxicity of chemotherapeutic agents can limit their effectiveness, protection of hematopoietic stem/progenitor cells by transfer of drug-resistance genes provides the possibility to escalate antitumor drug doses and, consequently, to improve the therapeutic index. In this approach, before a patient receives chemotherapy, bone marrow cells are withdrawn, transduced *in vitro* with genes responsible for drug resistance, and then given back to the patient.[107]

5.4 IMMUNOMODULATORY GENE THERAPY

This is a new approach to cancer immunotherapy that can be achieved by *ex vivo* genetic modification of cells involved in the immune response. These cells are taken from the tumor, transduced with a viral vector containing immunoregulatory cytokine genes, and reimplanted in the tumor, where they produce the cytokines without the toxicity associated with its systemic administration. Alternatively, T cells can be genetically modified to express artificial (chimeric) antigen receptors of tumor-associated antigens. It is supposed that these modified cells destroy tumor cells expressing these antigens, and they remain persistent in the body to guard it against residual or recurring malignant disease. For instance, T cells collected from chemotherapy refractory CLL patients were transduced with a lentiviral vector encoding the anti-CD19 chimeric antigen receptor and, after being expanded *ex vivo*, were infused into the patients, inducing a rapid and potent antitumor effect.[108,109] This chimeric antigen receptor has one extracellular domain containing some fragments that bind to the antigen CD19 (Figure 12.13).

There are several treatments for prostate cancer in clinical trials that are based on the immunomodulatory gene therapy principle. One of these is rilimogene galvacirepvec (Prostvac®), which is in phase III clinical trials and involves the use of a recombinant vaccinia vector for primary vaccination, followed by booster vaccinations employing a recombinant fowlpox vector. These vectors contain genes for prostate-specific antigen (PSA) and multiple T-cell costimulatory molecules (TRICOM). When these PSA–TRICOM vaccines infect APCs, they generate proteins that are expressed on their surface as part of the immune response, thus inducing the interaction of these APCs with T cells, followed by immune response and tumor cell destruction.[110]

The use of the VDEPT method (discussed in detail in Section 2.3 of Chapter 13) to activate valaciclovir (AdV-tk valaciclovir, ProstAtak®) led to the discovery of a systemic immune response that allows attacking residual tumor cells and occult micrometastases. This response is due to the combined effects of tumor antigens released following tumor cell death, signals associated with virion injection, recruitment of APCs secondary to acute inflammation, and stimulation of T-cell proliferation driven by the antigen properties of the viral thymidine kinase (TK) protein (Figure 12.14). Based on the fact that it is able to induce an immune response, this approach is called gene-mediated cytotoxic immunotherapy (GMCT).

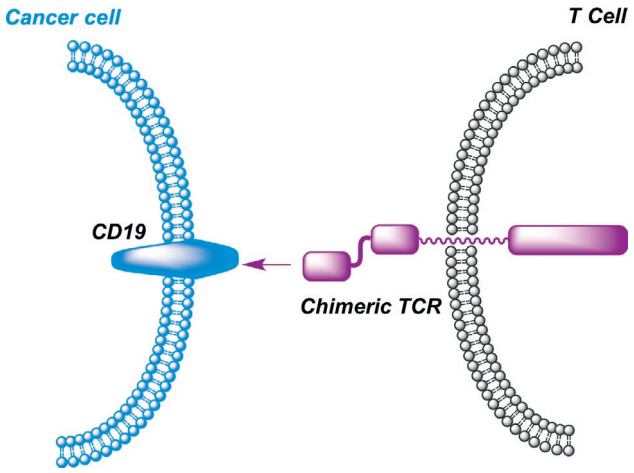


FIGURE 12.13

Action on cancer cells of T cells transduced with a lentiviral vector encoding the anti-CD19 chimeric antigen receptor.

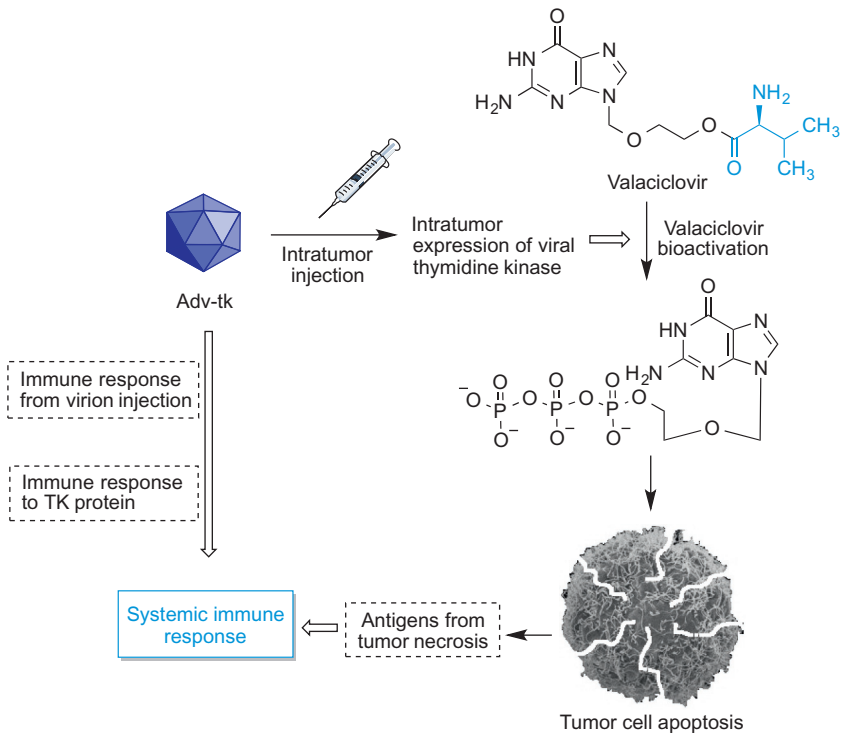


FIGURE 12.14

Mechanism of anticancer action of valaciclovir AdV-tk: the gene-mediated cytotoxic immunotherapy (GMCT) approach.

6 ANTISENSE OLIGONUCLEOTIDES IN CANCER TREATMENT

Antisense oligonucleotides (ASOs) are short synthetic stretches of DNA that hybridize with specific mRNA strands that correspond to target genes. Because ribosomes cannot translate double-stranded RNA, the translation of a given mRNA can be inhibited by a segment of its complementary sequence, the corresponding antisense RNA. This results in blocking the translation of the RNA message to generate a specific protein, as is shown in Figure 12.15, and in the degradation of the mRNA strand by ribonuclease H (RNAse H).

Because overexpression or mutation of oncogenes causes cancer, downregulation of their expression offers the possibility of a selective tumor ablation. To achieve this goal, it is necessary for the oligonucleotides not only to have a high and selective affinity toward the target mRNA sequence but also to elude the action of nucleases, which rapidly degrade native oligonucleotides in cells and body fluids and thus prevent them from reaching their targets.[111,112] The first generation of ASOs intended for clinical use were characterized by having one of the phosphate nonbridging oxygens of the phosphodiester linkages replaced by sulfur (**12.1**). Phosphorothioate oligonucleotides have acceptable physical and chemical properties and show reasonable resistance to nucleases. New generations of these phosphorothioate oligonucleotides that contain 2′-modified nucleoside building blocks to enhance RNA binding affinity and decrease indirect toxic effects have been developed. Other common structures are phosphoroamidates (**12.2**) and phosphorothioamidates (**12.3**) and their 2′-methoxy- and 2′-(2-methoxy)ethoxy derivatives (**12.4** and **12.5**), which have an increased affinity for their specific targets. Peptide nucleic acids (**12.6**) are structures in which the antisense bases are connected to various peptide backbones, a structural feature that improves their half-lives and enhances their hybridization properties but implies a poor cellular penetration and bad pharmacokinetic properties. The design of an antisense oligonucleotide first requires the identification of the sites on a given mRNA that are accessible and do not show sequence homologies with other genes of importance.[113]

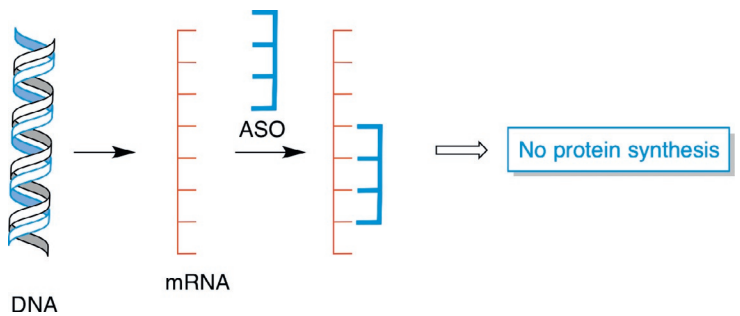
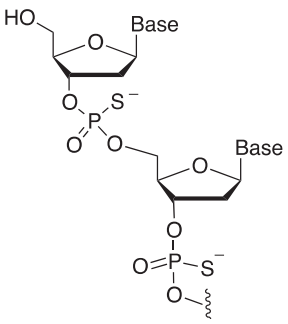
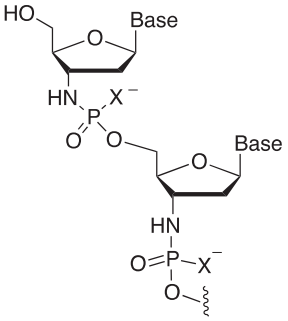


FIGURE 12.15

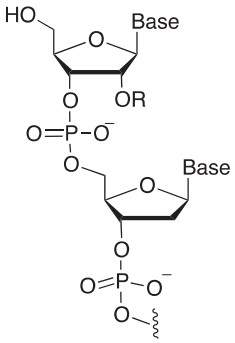
Mechanism of action of antisense oligonucleotides (ASOs).



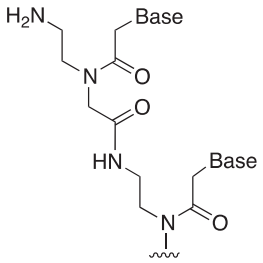
Phosphorothioates (**12.1**)



X = O Phosphoroamidates (**12.2**)
X = S Phosphorothioamidates (**12.3**)



12.4 R = CH_3
12.5 R = CH_3-O-$(CH_2)_2$



Peptide nucleic acids (**12.6**)

No antisense oligonucleotide cancer drugs have been approved to date, but several clinical trials with ASOs against different targets for different cancer types have been performed. Representative examples are summarized in Table 12.2, including many that have been mentioned previously. Here, two additional examples are studied. First, GTI-2040, which is directed against the ribonucleotide reductase M2 gene (*RRM2*) that encodes the reductase that catalyzes the formation of deoxyribonucleotides from ribonucleotides. It entered phase I/II trials in combination with docetaxel and prednisone for the treatment of patients with castration-resistant prostate cancer (CRPC).[115] Second, the GD2-targeted stabilized immunoliposome LR/INX-3001, developed for CML,[115] which contains an oligonucleotide that suppresses the expression of the c-Myb protein[116] and inhibits cell growth.[117] The disialoganglioside GD2 is an antigen found on malignant cells and is an attractive target for immunoliposomal therapy of tumors of neuroectodermal origin, and the c-Myb protein is a proto-oncogene that functions as a downstream target of PDGF-mediated survival signal.[118] Antisense nucleotide delivery from stabilized immunoliposomes with cell surface-directed antibodies is a way to overcome their low cellular uptake.

Table 12.2 Examples of Antisense Oligonucleotides That Have Entered Clinical Trials

mRNA Target	Antisense Oligonucleotide (Chapter/Section)	Evaluated Tumors
PKC	ISIS-3521 (LY-900003, Affinitac®) (10/5.4)	NSCLC, solid tumors
RAS	ISIS-2503 (10/6.1)	Solid tumors
c-RAF	ISIS-5132 (CGP-69846A) (10/6.5.1)	Solid tumors
BCL-2	Oblimersen (Genasense®) (11/8.1.3)	Melanoma, MM, CLL, NSCLC
DNMT	MG-98 (8/2.2)	Solid tumors
hTR	Imetelstat (GRN-163 L) (7/8.3)	MM, solid tumors
Clusterin	OGX-011 (Custirsen®) (11/8.2)	Lung and prostate cancers
P53	OL(1)p53 (11/8.1.4)	AML, MDS
RRM2	GTI-2040 (12/6)	CRPC
c-MYB	LR/INX-3001 (12/6)	CML

7 BACTERIA AND BACTERIAL TOXINS IN CANCER THERAPY

A variety of natural and genetically modified nonpathogenic bacterial species are being explored as potential antitumor agents. The use of bacterial toxins is another aspect of the application of microorganisms to cancer therapy.[119] One example of a clinical trial involving the use of bacteria is the use of VNP20009, a genetically modified strain of *Salmonella typhimurium*, for advanced or metastatic solid tumors. Furthermore, intravesical bacillus Calmette–Guérin (BCG), an attenuated mycobacterium developed from the *Mycobacterium bovis* strain used for tuberculosis vaccination, has become the treatment of choice for high-risk superficial bladder cancer in most countries because it provides superior protection from tumor recurrence and even reduction of disease progression.[120] Patients treated with intravesical BCG plus interferon have a 60–70% chance of a complete and durable cure. Although its precise mechanism of action has not been clearly determined, the intense local immune activation after BCG correlates with pronounced infiltration of the bladder wall by immunocompetent cells together with the secretion of cytokines.

Bacterial toxins have also entered clinical trials as anticancer agents. Examples are Tf-CRM 107 and IL-4–PE (IL-4–*Pseudomonas* exotoxin), a transferrin–diphtheria toxin conjugate assayed for brain tumors, and IL-13–PE –(IL-13–*Pseudomonas* exotoxin), which is under assay for several kinds of tumors.

REFERENCES

1 Kohler G, Milstein C. *Nature* 1975;**256**:495.
2 Segota E, Bukowski RM. *Cleveland Clin J Med* 2004;**71**:551.
3 Weiner LM, Adams GP, von Mehren M. In: DeVita VT, Hellman S, Rosenberg SA, editors. *Cancer: principles & practice of oncology*. 6th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001. p. 495.
4 (a) Lim SH, Beers SA, French RR, Johnson PWM, Glennie MJ, Cragg MS. *Haematologica* 2010;**95**:135; (b) For a review, see Alduaij W, Illidge TM. *Blood* 2011;**117**:2993.

5 Byrd JC, Kipps TJ, Flinn IW, Cooper M, Odenike O, Bendiske J, et al. *Leuk Lymphoma* 2012;**53**:2136.
6 Royer B, Yin W, Pegram M, Ibrahim N, Villanueva C, Mir D, et al. *Br J Cancer* 2010;**102**:827.
7 Danielczyk A, Stahn R, Faulstich D, Löffler A, Märten A, Karsten U, et al. *Cancer Immunol Immunother* 2006;**55**:1337.
8 Billington RA, Bruzzone S, De Flora A, Genazzani AA, Koch-Nolte F, Ziegler M, et al. *Mol Med* 2006;**12**:324.
9 Takasawa S, Tohgo A, Noguchi N, Koguma T, Nata K, Sugimoto T, et al. *J Biol Chem* 1993;**268**:26052.
10 Van der Veer MS, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. *Blood Cancer J* 2011;**1**:e41.
11 http://clinicaltrials-nccs.nlm.nih.gov/ct/show/NCT00401570;jsessionid=A8186BC084BE2C690ACBDDE0B44F8330?order=25.
12 Posey JA, Khazaeli MB, Del Grosso A, Saleh MN, Lin CY, Huse W, et al. *Cancer Biother Radiopharm* 2001;**16**:125.
13 Cunha SI, Pietras K. *Blood* 2011;**117**:6999.
14 Fuchs ChS, Tomasek J, Yong ChJ, Dumitru F, Passalacqua R, Goswami Ch, et al. *Lancet* 2014;**383**:31.
15 Leach DR, Krummel MF, Allison JP. *Science* 1996;**271**:1734.
16 Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. *Nature* 1984;**308**:145.
17 For a review, see Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, et al. *Physiol Rev* 2008;**88**:841.
18 Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. *Cancer Res* 2013;**73**:3591.
19 Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. *N Engl J Med* 2010;**363**:711; (b) Robert C, Thomas L, Bondarenko I, O'Day S, Garbe C, Lebbe C, et al. *N Engl J Med* 2011;**364**:2517.
20 Ribas A, Kefford R, Marshall MA, Punt CJA, Haanen JB, Marmol M, et al. *J Clin Oncol* 2013;**31**:616.
21 Chen DS, Irving BA, Hodi FS. *Clin Cancer Res* 2012;**18**:6580.
22 Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. *Nature* 2014;**515**:568.
23 Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. *N Engl J Med* 2012;**366**:2455.
24 Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. *N Engl J Med* 2013;**369**:134.
25 Chen DS, Mellman I. *Immunity* 2013;**39**:1.
26 See, for instance Powles T, Vogelzang NJ, Fine GD, Eder JP, Braiteh FS, Loriot Y, et al. *J Clin Oncol* 2014;**32**:5s.
27 Münz M, Murr A, Kvesic M, Rau D, Mangold S, Pflanz S, et al. *Cancer Cell Int* 2010;**10**:44.
28 Fields ALA, Keller A, Schwartzberg L, Bernard S, Kardinal C, Cohen A, et al. *J Clin Oncol* 2009;**27**:1941.
29 Chames P, Baty D. *MAbs* 2009;**1**:539.
30 Chelius D, Ruf P, Gruber P, Plöscher M, Liedtke R, Gansberger E, et al. *MAbs* 2010;**2**:309.
31 Pullarkat V, Deo Y, Link J, Spears L, Marty V, Curnow R, et al. *Cancer Immunol Immunother* 1999;**48**:9.
32 Heiss MM, Strohlein MA, Jager M, Kimmig R, Burges A, Schoberth A, et al. *Int J Cancer* 2005;**117**:435.
33 Zitvogel L, Apetoh L, Ghiringhelli F, André F, Tesniere A, Kroemer G. *J Clin Invest* 2008;**118**:1991.
34 Madan RA, Gulley JL. *Immunotherapy* 2011;**3**:27.
35 Couzin-Frankel J. *Science* 2013;**342**:1432.
36 Sun Z, Shi L, Zhang H, Shao Y, Wang Y, Lin Y, et al. *Clin Immunol* 2011;**138**:23.
37 (a) Chinnasamy N, Wargo JA, Yu Z, Rao M, Frankel TL, Riley JP, et al. *J Immunol* 2011;**186**:685. (b) Zhang L, Morgan RA. *Adv Drug Deliv Rev* 2012;**64**:756.
38 Kirkwood JM, Butterfield LH, Tarhini AA, Zarour H, Kalinski P, Ferrone S. *CA Cancer J Clin* 2012;**62**:309.
39 Segal NH, Parsons DW, Peggs KS, Velculescu V, Kinzler KW, Vogelstein B, et al. *Cancer Res* 2008;**68**:889.
40 Challa-Malladi M, Lieu YK, Califano O, Holmes AB, Bhagat G, Murty VV, et al. *Cancer Cell* 2011;**20**:728.
41 Pardoll DM. *Nature Rev Cancer* 2012;**12**:252.
42 Aerts JG, Hegmans JP. *Cancer Res* 2013;**73**:2381.

43 Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, et al. *J Clin Oncol* 2000;**18**:1622.
44 Winkelhake JL, Gauny SS. *Pharmacol Rev* 1990;**42**:1.
45 For a review, see Turturro F. *Expert Rev Anticancer Ther* 2007;**7**:11.
46 Gebbia V, Boussen H, Valerio MR. *Anticancer Res* 2012;**32**:529.
47 Lutz ER, Wu AA, Bigelow E, Sharma R, Mo G, Soares K, et al. *Cancer Immunol Res* 2014;**2**:1.
48 Cohen AD, Schaer DA, Liu C, Li Y, Hirschhorn-Cymmerman D, Kim SC, et al. *PLoS One* 2010;**5**:e10436.
49 Quezada C, Garrido W, Oyarzún C, Fernández K, Segura R, Melo R, et al. *J Cell Physiol* 2013;**228**:602.
50 Byrne WL, Mills KH, Lederer JA, O'Sullivan GC. *Cancer Res* 2011;**71**:6915.
51 (a) Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP, et al. *Gastroenterology* 2008;**135**:234; (b) Pan PY, Ma G, Weber KJ, Ozao-Choy J, Wang G, Yin B, et al. *Cancer Res* 2010;**70**:99.
52 Cui TX, Kryczek I, Zhao L, Zhao E, Kuick R, Roh MH, et al. *Immunity* 2013;**39**:611.
53 Gabrilovich DI, Nagaraj S. *Nature Rev Immunol* 2009;**9**:162.
54 Apetoh L, Vegran F, Ladoire S, Ghiringhelli F. *Curr Mol Med* 2011;**11**:365.
55 (a) Tu SP, Jin H, Shi JD, Zhu LM, Suo Y, Lu G, et al. *Cancer Prev Res* 2012;**5**:205; (b) Roth F, De La Fuente AC, Vella JL, Zoso A, Inverardi L, Serafini P. *Cancer Res* 2012;**72**:1373.
56 Parinandi NL, Sharma A, Eubank TD, Kaufman BF, Kutala VK, Marsh CB, et al. *Antioxid Redox Signal* 2007;**9**:1837.
57 De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, et al. *Proc Natl Acad Sci U S A* 2005;**102**:4185.
58 Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. *Arch Pharm Res* 2012;**35**:1297.
59 https://clinicaltrials.gov/ct2/results?term=vtx-2337&Search=Search.
60 Mo J, Duncan JA. *Methods Mol. Biol* 2013;**1040**:153.
61 (a) Kufer TA, Banks DJ, Philpott DJ. *Ann. N. Y. Acad. Sci* 2006;**1072**:19. (b) Meshcheryakova E, Makarov E, Philpott D, Andranova T, Ivanov V. *Vaccine* 2007;**25**:4515.
62 Frampton JE. *Pediatric Drugs* 2010;**12**:141.
63 Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. *N. Engl. J. Med* 2012;**366**:2455.
64 (a) Wang C, Bedikian A, Kim K, Papadopoulos N, Hwu W, Hwu P. *J. Clin. Oncol* 2006;**24**:12015. (b) Mason KA, Valdecanas D, Hunter NR, Milas L. *Invest. New Drugs* 2008;**26**:1.
65 Bedikian AY, Papadopoulos NE, Kim KB, Hwu WJ, Homsi J, Glass MR, Cain S, Rudewicz P, Vernillet L, Hwu P. *Cancer Invest* 2009;**27**:756.
66 Plummer R, Lorigan P, Evans J, Steven N, Middleton M, Wilson R, Snow K, Dewji R, Calvert H. *J. Clin. Oncol* 2006;**24**:A8013.
67 Wang YF. *Drugs Future* 2009;**34**:177.
68 (a) Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, Friedlander M, Arun B, Loman N, Schmutzler RK, Wardley A, Mitchell G, Earl H, Wickens M, Carmichael J. *Lancet* 2010;**376**:205. (b) Chan SL, Mok T. *Lancet* 2010;**376**:211.
69 Penning TD, Zhu GD, Gandhi VB, Gong J, Liu X, Shi Y, Klinghofer V, Johnson EF, Donawho CK, Frost DJ, Bontcheva-Diaz V, Bouska JJ, Osterling DJ, Olson AM, Marsh KC, Luo Y, Giranda VL. *J. Med. Chem* 2009;**52**:514.
70 Liu SK, Coackley C, Krause M, Jalali F, Chan N, Bristow RG. *Radiother. Oncol* 2008;**88**:258.
71 Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. *Nature Rev. Cancer* 2010;**10**:293.
72 Miknyoczki S, Chang H, Grobelny J, Pritchard S, Worrell C, McGann N, Ator M, Husten J, Deibold J, Hudkins R, Zulli A, Parchment R, Ruggeri B. *Mol. Cancer Ther* 2007;**6**:2290.

73 For select reviews, see; (a) Moingeon P. *Vaccine* 2001;**19**:1305; (b) Tabi Z, Man S. *Adv Drug Deliv Rev* 2006;**58**:902; (c) Emens LA. *Expert Opin Emerg Drugs* 2008;**13**:295
74 http://www.cancer.gov/cancertopics/factsheet/cancervaccine.
75 Klebanoff CA, Acquavella N, Yu Z, Restifo NP. *Immunol Rev* 2011;**239**:27.
76 For a review, see Tetsuro S, Nobukazu K, Shigetaka S, Akira Y, Masanori N, Kyoto I. *Eur J Cancer* 2010;**46**:1514.
77 Boczkowski D, Fair SK, Zinder D, Gilboa E. *J Exp Med* 1996;**184**:465.
78 For a review, see Menaria J, Kitawat S, Verma V. *Sch J Appl Med Sci* 2013;**1**:161.
79 Plosker GL. *Drugs* 2011;**71**:101.
80 Huber ML, Haynes L, Parker C, Iversen P. *J Natl Cancer Inst* 2012;**104**:273.
81 For representative reviews of carbohydrate cancer vaccines, see; (a) Buskas T, Thompson P, Boons J-G. *Chem Commun* 2009;5335; (b) Zhu J, Warren JD, Danishefsky SJ. *Expert Rev Vaccines* 2009;**8**:1399; (c) Yin Z, Huang X. *J Carbohydr Chem* 2012;**31**:143; (d) Fernández-Tejada A, Danishefsky SJ. *Carbohydr Chem* 2014;**40**:506.
82 (a) Keding SJ, Danishefsky SJ. *Proc Natl Acad Sci U S A* 2004;**101**:11937.
83 Hu X, Chakraborty NG, Sport JR, Kurtzman SH, Ergin MT, Mukherji B. *Cancer Res* 1996;**56**:2479.
84 For a review, see Yamada A, Sasada T, Noguchi M, Itoh K. *Cancer Sci* 2013;**104**:15.
85 Brichard VG, Lehmann CMG, Louahed FFE, Louahed J. US Patent 20100008980 A1, 2008.
86 Caudill MM, Li Z. *Expert Opin Biol Ther* 2001;**1**:539.
87 For reviews, see; (a) Amato RJ. *Expert Opin Biol Ther* 2007;**7**:1267; (b) Di Pietro A, Tosti G, Ferrucci PF, Testori A. *Hum Vaccines* 2009;**5**:727.
88 Benavides LC, Sears AK, Gates JD, Clifton GT, Clive KS, Carmichael MG, et al. *Expert Rev Vacc* 2011;**10**:201.
89 Andersen MH, Becker JC, Straten P. *Nature Rev Drug Discov* 2005;**4**:399.
90 See the results of a phase III trial in Butts C, Socinski MA, Mitchell PL, Thatcher N, Havel L, Krzakowski M, et al. *Lancet Oncol* 2014;**15**:59.
91 Kovjazin R, Horn G, Smorodinsky NI, Shapira MY, Carmon L. *PLoS One* 2014;**9**:e85400.
92 Kovjazin R, Volovitz I, Kundel Y, Rosenbaum E, Medalia G, Horn G, et al. *Vaccine* 2011;**29**:4676.
93 Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, et al. *J Immunol* 2005;**174**:3798.
94 Wenandy L, Sørensen RB, Sengeløv L, Svane IM, Thor Straten P, Andersen MH. *Clin Cancer Res* 2008;**14**:4.
95 Schlapbach C, Yerly D, Daubner B, Yawalkar N, Hunger RE. *J Dermatol Sci* 2011;**62**:75.
96 Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, et al. *J Immunol* 2005;**174**:3798.
97 McLaughlin-Drubin ME, Munger K. *Biochim Biophys Acta* 2008;**1782**:127.
98 Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. *EMBO J* 1984;**3**:1151.
99 http://www.cdc.gov/vaccines/hcp/vis/vis-statements/hpv-gardasil.html.
100 For a review, see Ferguson MS, Lemoine NR, Wang Y. *Adv Virol* 2012. Article ID 805629.
101 Räty JK, Pikkarainen JT, Wirth T, Ylä-Herttuala S. *Curr Mol Pharmacol* 2008;**1**:13.
102 Hughes C, Galea-Lauri J, Farzaneh F, Darling D. *Mol Ther* 2001;**3**:623.
103 Pan J-J, Zhang S-W, Chen C-B, Xiao S-W, Sun Y, Liu C-Q, et al. *J Clin Oncol* 2009;**27**:799.
104 For a review, see Jiang H, Gómez-Manzano C, Lang FF, Alemany R, Fueyo J. *Curr Gene Ther* 2009;**9**:422.
105 Andtbacka RHI, Collichio FA, Amatruda T, Senzer NN, Chesney J, Delman KA, et al. *J Clin Oncol* 2013;**31** (Suppl.), abstr LBA9008.
106 Sandmair AM, Loimas S, Puranen P, Immonen A, Kossila M, Puranen M, et al. *Hum Gene Ther* 2000;**11**:2197.
107 Budak-Alpdogan T, Bertino JR. *Methods Mol Biol* 2009;**542**:661.
108 Porter DL, Kalos M, Zheng Z, Levine B, June C. *J Cancer* 2011;**2**:331.
109 Jin Z, Maiti S, Huls H, Singh H, Olivares S, Mátés L, et al. *Gene Ther* 2011;**18**:849.

110 Madan RA, Arlen PM, Mohebtash M, Hodge JW, Gulley JL. *Expert Opin Invest Drugs* 2009;**18**:1001.
111 For a review, see Crooke T. *Oncogene* 2000;**19**:6651.
112 For a review, see Jansen B, Zangemeister-Wittke U. *Lancet Oncol* 2002;**3**:672.
113 Khuri FR, Kurie JM. *Clin Cancer Res* 2000;**6**:1607.
114 (a) Orr RM. *Curr Opin Invest Drugs* 2001;**2**:1462; (b) Juhasz A, Vassilakos A, Chew HK, Gandara D, Yen Y. *Oncol Rep* 2006;**15**:1299.
115 Luger SM. PQD Clinical Trial Abs, Protocol IDs: UPCC-3492, NCI-H94-0532.
116 Chen Y, Xu H, Liu J, Zhang C, Leutz A, Mo X. *Biochem Biophys Res Commun* 2007;**360**:433.
117 Pagnan G, Stuart DD, Pastorino F, Raffaghello L, Montaldo PG, Allen TM, et al. *J Natl Cancer Inst* 2000;**92**:253.
118 Chen Y, Xu H, Liu J, Zhang C, Leutz A, Mo X. *Biochem Biophys Res Commun* 2007;**360**:433.
119 For a review of the use of bacteria in cancer therapy, see Patyar S, Joshi R, Prasad Byrav DS, Prakash A, Medhi B, Das BK. *J Biomed Sci* 2010;**17**:21.
120 Grossman HB, O'Donnell MA, Cookson MS, Greenberg RE, Keane TE. *Rev Urol* 2008;**10**:281.

CHAPTER

DRUG TARGETING IN ANTICANCER CHEMOTHERAPY 13

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00013-2

1 INTRODUCTION

More than 70% of anticancer drugs that have been approved by the U.S. Food and Drug Administration (FDA) in approximately the past decade are small molecules or monoclonal antibodies (mAbs) that inhibit specific targets that are upregulated in cancer (see Chapters 9–12). Nevertheless, despite their design as specific anticancer agents, cross-reactivity with molecular targets in healthy cells and intratumor heterogeneity diminish the therapeutic efficacy of many of these therapies.

Another approach to achieve specific targeted therapies is to deliver selectively the anticancer drugs into cancer cells while affecting as few healthy cells as possible, which allows the use of more potent cytotoxic compounds. This goal is pursued by the development of specific targeted therapies exploiting the unique molecular properties associated with progression of a particular tumor. This chapter discusses the techniques developed to achieve this end.

2 SMALL-MOLECULE PRODRUGS FOR ANTICANCER DRUG TARGETING

The specificity of cytotoxic compounds in cancer therapy may be much improved by using prodrugs that are selectively activated in tumor tissues.[1] This selective bioactivation may be based on the exploitation of some unique aspects of tumor physiology, such as selective expression of endogenous enzymes, hypoxia, and low extracellular pH. Other approaches are based on tumor-specific delivery techniques that allow the selective activation of prodrugs by exogenous enzymes, which are delivered into the tumor using mAbs (antibody-directed enzyme prodrug therapy, ADEPT) or generated in tumor cells from DNA constructs that contain the corresponding gene using nonviral (gene-directed enzyme prodrug therapy, GDEPT) or viral (virus-directed enzyme prodrug therapy, VDEPT) vectors (see Sections 2.3 and 2.4).

2.1 SELECTIVE ENZYME EXPRESSION IN TUMOR CELLS

To achieve a tumor-specific bioactivation, the enzyme responsible should be uniquely present in the tumor cell. There is much evidence of pathways involving enzymes that are aberrantly expressed in tumors, but the success of these approaches is variable because the differences between healthy and tumor tissues are not normally consistent across different species, individuals, or cancers. One example, previously discussed in Section 4.3 of Chapter 2, is the selective bioactivation in tumors of capecitabine, a 5-fluorouracil (5-FU) prodrug. This compound is rapidly absorbed after oral administration due to its lipophilicity, and it is metabolized by carboxylesterase and cytidine deaminase to 5′-deoxy-5-fluorouridine. The final bioactivation step, which involves the transformation of the latter intermediate into 5-FU by thymidine phosphorylase, takes place up to 10 times more efficiently in cancer cells than in normal cells (Figure 13.1).

Another enzyme that is overexpressed in several tumors, including ovarian, colon, pancreas, and non-small cell lung cancers, is the cytosolic glutathione-*S*-transferase of the π class (GST-π). The active site of this enzyme contains a tyrosine residue that deprotonates the mercapto group of glutathione in order to increase its nucleophilicity and to allow its reaction with electrophilic toxic metabolites (represented as E^+ in Figure 13.2).

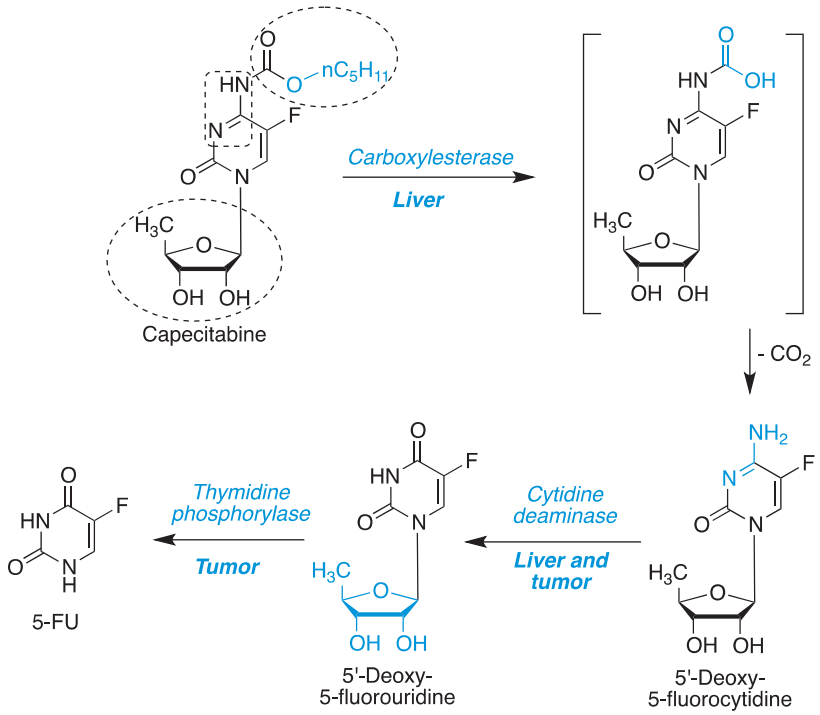


FIGURE 13.1

Selective bioactivation of capecitabine.

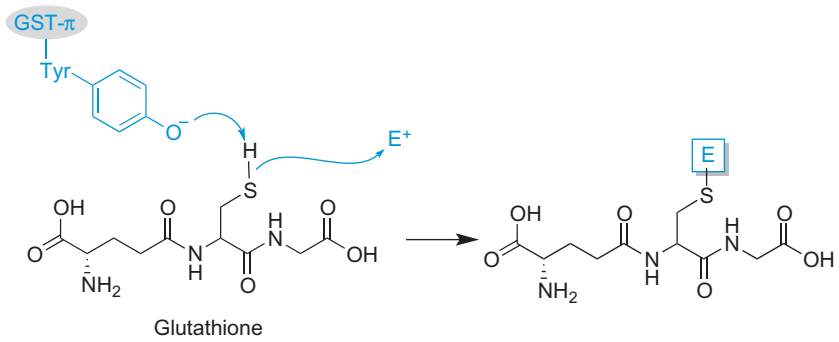


FIGURE 13.2

Activation of glutathione at the active site of GST-π.

The mustard prodrug canfosfamide (TLK-286, Telcyta®) contains a modified glutathione framework linked to an inactive phosphoramide mustard. The presence of a sulfone group in the linker portion was designed to enhance the acidity of its α-proton and thus facilitate a β-elimination reaction triggered by the basicity of the deprotonated tyrosine hydroxyl. The negative charge in the liberated phosphoramidate assists the intramolecular nucleophilic displacement reaction that leads to the alkylating aziridinium species (Figure 13.3).

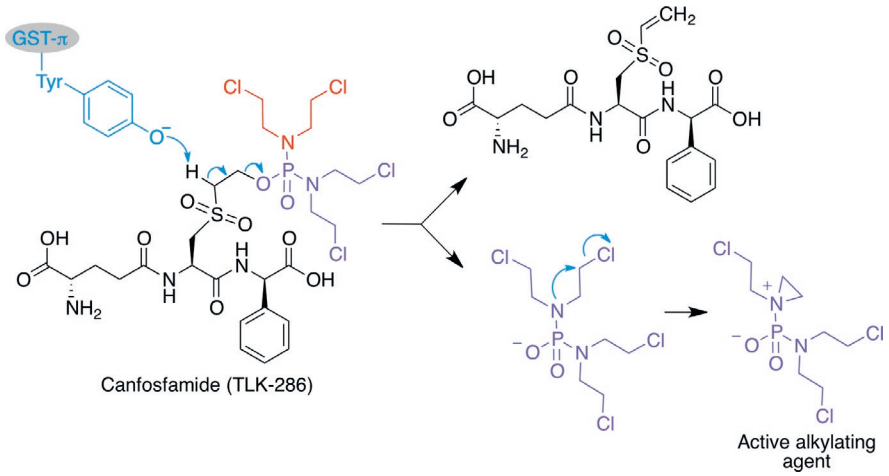


FIGURE 13.3

Selective bioactivation of TLK-286.

Although the hydrolytic activation of canfosfamide can occur spontaneously, the GST-π enzyme facilitates the kinetics of the process. This prodrug has been in multiple phase II and III trials in advanced cancer patients, and it has shown clinical activity for non-small cell lung and ovarian cancers[2] with a good safety profile.

A recent example of a tumor-specific bioactivated prodrug is G-202, which was designed to be an inactivated form of thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump (see Chapter 11, Section 7.2).

G-202 contains was designed as a prodrug of 12-ADT, an analog of thapsigargin, linked via a carboxyl group to a peptide containing four residues of glutamic acid. Upon intravenous administration, this nontoxic prodrug targets prostate-specific membrane antigen (PSMA), a type II membrane carboxypeptidase that catalyzes the sequential hydrolysis of glutamic acid residues. In this way, G-202 is converted into 12-ADT-Asp, an active analog of thapsigargin (Figure 13.4).

Because PSMA is overexpressed in prostate cancer cells and in the neovasculature of most solid tumors but not in normal blood vessels, G-202 is specifically activated in these cells, inhibiting tumor growth by preventing nutrient supply. Compared to thapsigargin alone, G-202 is able to achieve higher concentrations of the active agent at the tumor site without systemic toxicity and produced substantial tumor regression in a panel of human cancer xenografts. It has entered a phase I dose-escalation clinical trial in patients with advanced cancer.[3]

2.2 HYPOXIA-BASED STRATEGIES FOR TUMOR-SPECIFIC PRODRUG ACTIVATION

Hypoxia is a common and unique property of cells in solid tumors, and it is therefore a potential mechanism for specific prodrug activation.[4] The use of oxygen electrodes has allowed the accurate measurement of oxygen levels in human tumors, leading to the finding that they are highly heterogeneous. Many regions have very low levels, with partial pressures of oxygen approximately 5 mm Hg, which corresponds to approximately 0.7% O_2 in the gas phase or 7 μM in solution. These hypoxic cells are resistant

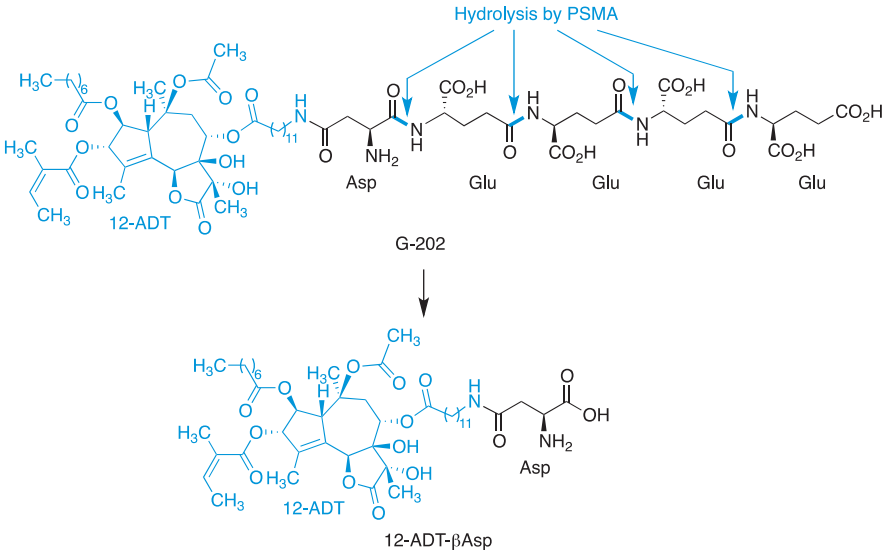


FIGURE 13.4

Hydrolytic activation of G-202.

to radiotherapy and also to most anticancer drugs because these are poorly diffused from the distant blood vessels. They have also lost sensitivity to p53-mediated apoptosis and show upregulation of genes involved in drug resistance. However, the existence of hypoxia and cell necrosis provides an opportunity for tumor-selective therapy, including the development of specifically activated bioreductive prodrugs.[5]

Discrimination between normal (oxygenated) and tumor (hypoxic) tissues can normally be achieved with prodrugs that contain a functional group susceptible to give an initial reduction that originates a prodrug radical that may act as a "trigger." When oxygen is present, as in the normal tissue, this reduction can be reverted by the transfer of one electron to oxygen, leading to a futile redox cycle that generates superoxide radical anions (Figure 13.5a). In the absence of oxygen, the prodrug radical is accumulated, generating the ultimate cytotoxic species, and hypoxia-selective cytotoxicity is achieved (Figure 13.5b). Obviously, for this approach to be useful in cancer therapy, the prodrug radical or its downstream products must have higher cytotoxicity than the superoxide radical anions arising from redox cycling in oxygenated cells.

The one-electron reduction potential of the functional group initially reduced is an important design parameter for hypoxia-selective tumor-activated prodrugs. Aromatic and aliphatic *N*-oxides, quinones, aromatic nitro groups, and cobalt complexes are common reductively activated prodrugs. Reductive prodrug bioactivation involves enzymes of the cytochrome P450 reductase family or nitroreductases.

2.2.1 N-*Oxides*

The best-known aromatic *N*-oxide used as an antitumor prodrug is tirapazamine (TPZ, Tirazone®), an orphan drug that is in phase III clinical trials for cisplatin-based chemoradiotherapy of head and neck cancer. It undergoes an enzymatic one-electron reduction, generating hydroxyl and benzotriazinyl

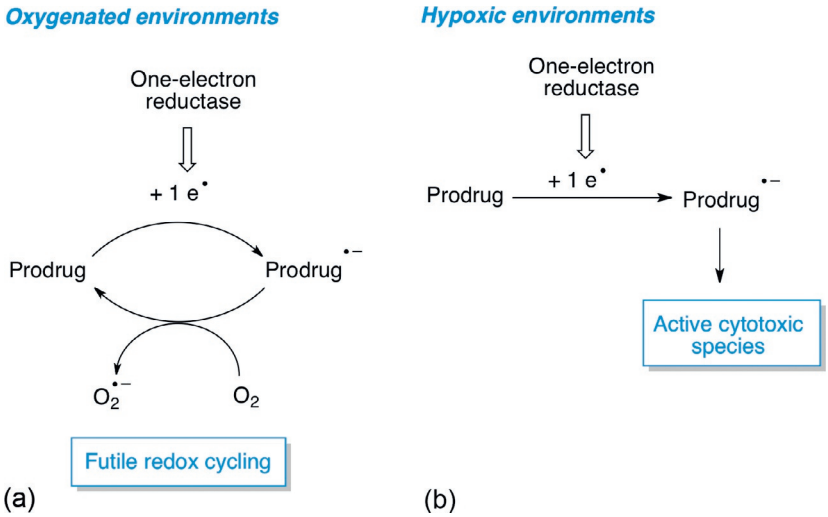


FIGURE 13.5

Chemical basis for hypoxia-selective cytotoxicity.

(BTZ) radicals that are DNA-damaging species. In normal cells, the TPZ radical reacts with oxygen to give back tirapazamine together with a superoxide radical (see Chapter 4, Section 9). However, in hypoxic environments, the TPZ radical undergoes two different types of fragmentation reactions, leading to hydroxyl and BTZ radicals, which cause DNA strand cleavage and topoisomerase II poisoning (Figure 13.6). TPZ is also a radiosensitizer because radiotherapy is based on the generation of hydroxyl radicals from the homolytic fragmentation of water molecules upon local application of ionizing radiation.

Aliphatic *N*-oxides are specific hypoxia-activated prodrugs of intercalating agents bearing side chains with basic tertiary amino groups because the negatively charged *N*-oxide oxygen atoms prevent their interaction with the anionic phosphate groups in DNA, which greatly decreases the affinity for this target.[6] The best known of these compounds is the anthraquinone banoxantrone (AQ4N), which has undergone clinical trials.[7] Its reduction followed by protonation furnishes the active species AQ4, which shows a tight binding to DNA by intercalation and subsequent interference with the topoisomerase II function (Figure 13.7). AQ4 is unusual among hypoxia-activated prodrugs in requiring a two-electron reduction. In humans, this reaction is effected mainly by the CYP3A members of the cytochrome P450 family, which are overexpressed in some tumors,[8] and it is inhibited by oxygen due to competition for the reduced heme group in the enzyme active site rather than from redox cycling.[9]

2.2.2 Quinones

Quinone derivatives were among the first compounds studied as hypoxia-selective tumor-activated prodrugs. They can undergo reduction by cytochrome P450 reductase, leading to captodative-stabilized semiquinone radical anions that can be back-oxidized by molecular oxygen in normal, well-oxygenated cells (see Chapter 4, Section 4). Quinones are also good substrates for two-electron reductases, particularly DT-diaphorase (Figure 13.8).

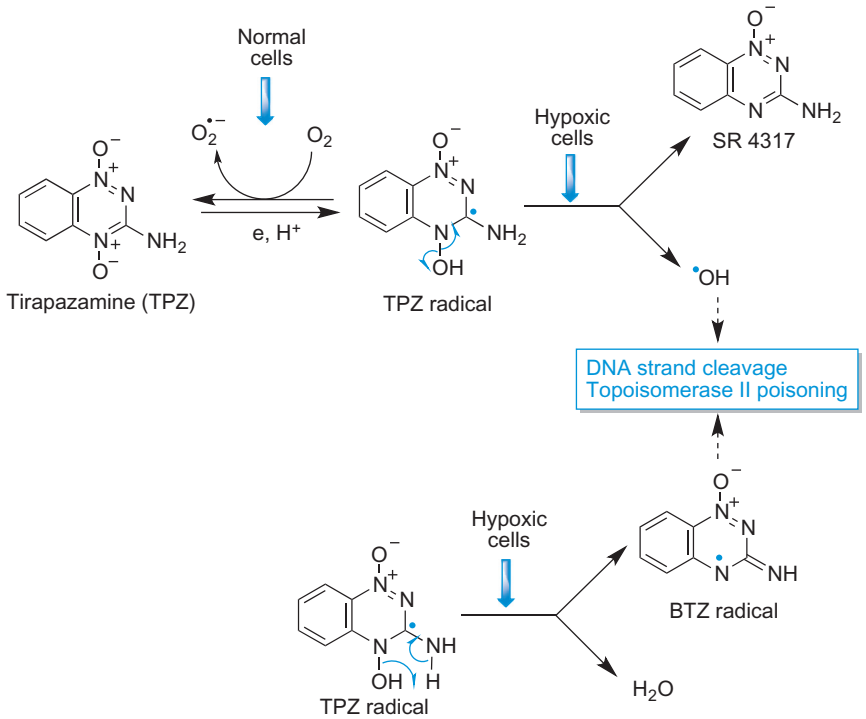


FIGURE 13.6

Reactions of the tirapazamine radical relevant to its anticancer activity.

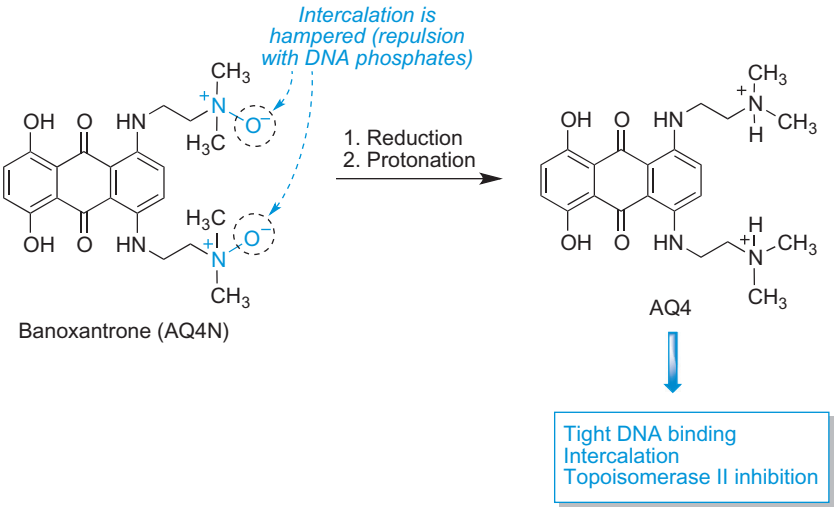


FIGURE 13.7

Hypoxia-related activation of the anthraquinone prodrug AQ4N.

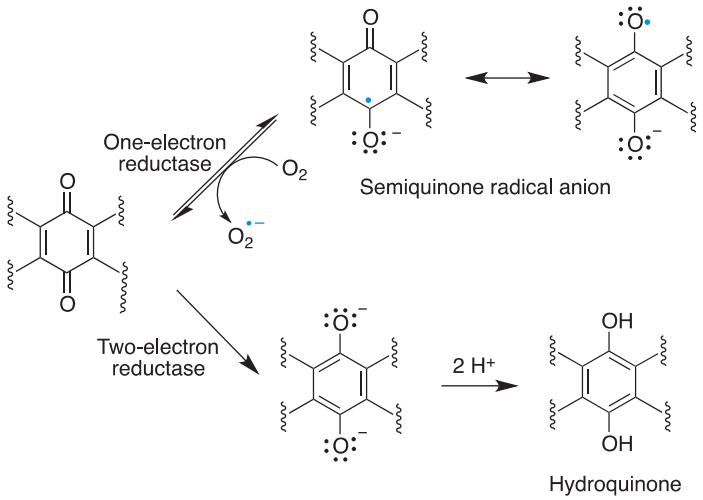


FIGURE 13.8

One-electron and two-electron quinone reduction.

The first examples of reductively activated quinones were compounds having a good leaving group at the α position of a side chain placed at the quinone C-2 carbon. After reduction, these compounds generate quinone methides that are highly reactive because their Michael additions allow re-aromatization of the benzene ring and that therefore behave as DNA alkylators (Figure 13.9).

Two simple examples of prodrugs designed using this strategy are quinones **13.1**[10] and **13.2**,[11] although they show only a marginal hypoxic selectivity. The natural products mitomycin C (Mitozytrex®) and porfiromycin (Promycin®) are also activated by bioreductive mechanisms (see Chapter 6, Section 3).

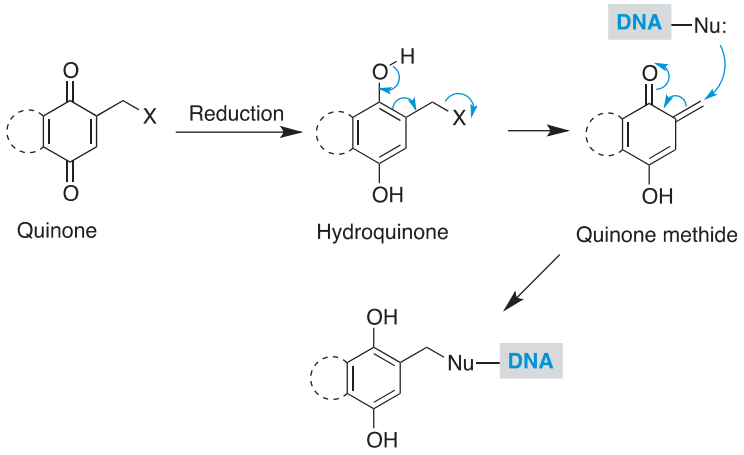
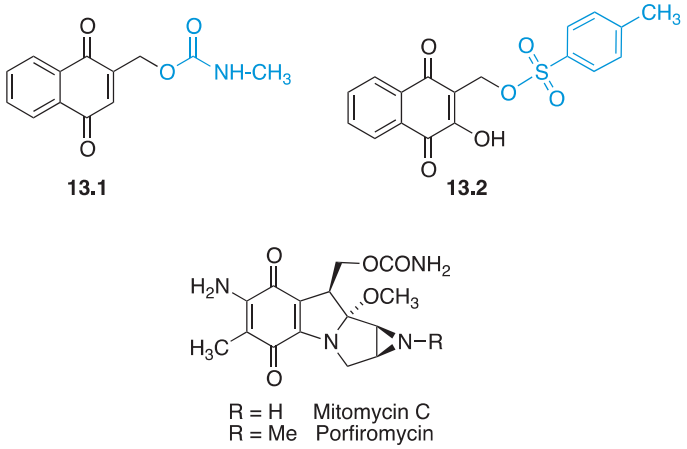


FIGURE 13.9

Reductive generation of electrophilic quinone methides from quinones.



Aziridinylquinones, represented by diaziquone (AZQ) and apaziquone (EO-9), are bioactivated by two-electron reductases, particularly DT-diaphorase, an enzyme that is overexpressed in many tumors (Figure 13.10). Apaziquone has been granted "fast track" status by the FDA for the treatment of superficial bladder cancer (i.e., a cancer not invasive of the muscle). For further discussion of aziridinylquinones as alkylating agents, see Section 3 of Chapter 5.

The bioreductive activation of quinones may also be used to trigger the release of alkylating species, especially nitrogen mustards. One example is compound **13.3**, which liberates a molecule of melphalan upon lactonization of its reduced hydroquinone form. In the case of **13.4**, reduction to hydroquinone is followed by C–N bond cleavage to release the aliphatic mustard **13.5**. This reaction is not possible prior

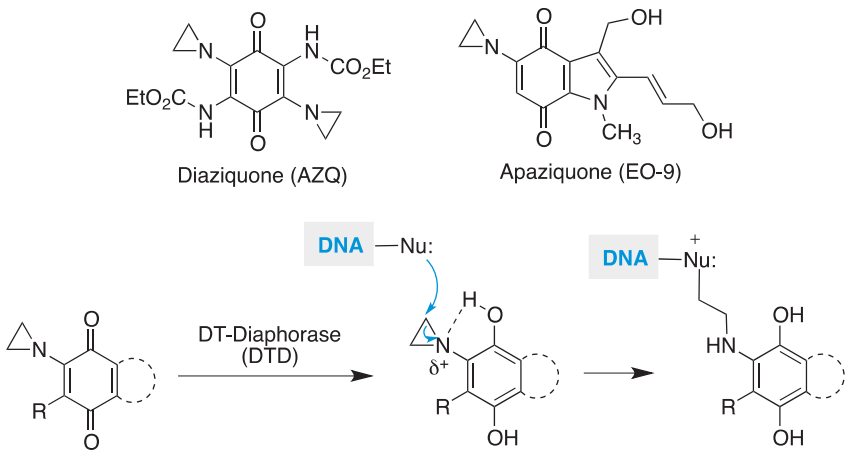


FIGURE 13.10

Reductive bioactivation of aziridinylquinones.

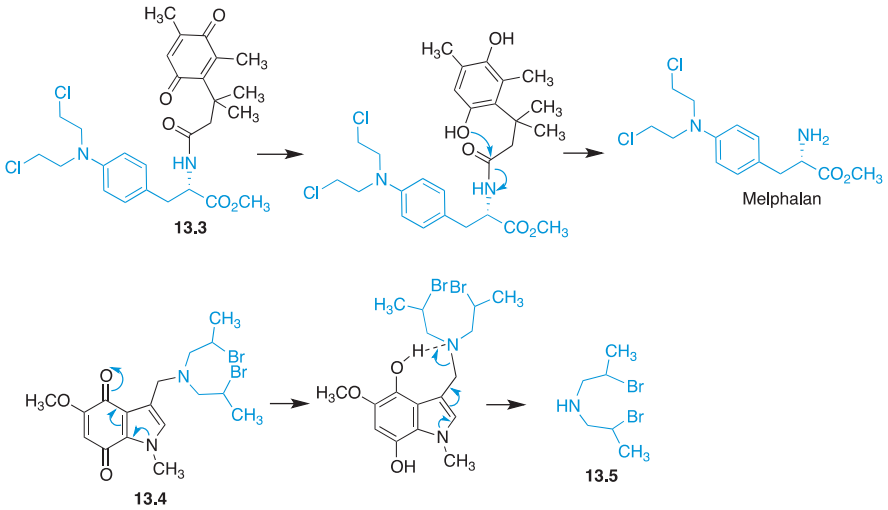


FIGURE 13.11

Release of alkylating species through reductive bioactivation of quinones.

to the reduction step because of the electron-withdrawing effect of the quinone moiety on the indole nitrogen (Figure 13.11).

2.2.3 Aromatic Nitro Derivatives

Nitroaromatic compounds are reduced by several nitroreductases, which are flavoprotein enzymes that catalyze the stepwise addition of up to six electrons. The major metabolite is normally the hydroxylamine, which is formed by addition of four electrons. Its stability is due to the very low reduction potential required for its further reduction to an amine. In nitroaromatic compounds with suitable reduction potentials (approximately −330 to −450 mV), the first radical anion formed by one-electron addition can be scavenged efficiently by molecular oxygen, and consequently its formation is restricted to hypoxic cells. Compounds with reduction potentials outside this range are less useful, normally for one of the following reasons: They are too easily reduced and therefore they show little selectivity for hypoxic tissues (reduction potentials higher than −330 mV), or they are too difficult to activate (reduction potentials below −450 mV).

The main nitroaromatic compounds used in cancer chemotherapy were nitroimidazoles. Although they were first introduced as radiosensitizers, it was later shown that they are able to induce cell death in hypoxic environments in the absence of radiation. This cytotoxic activity is mainly due to the formation of nitro radical anion **13.6** and hydroxylamine (**13.8**) metabolites. Compounds **13.6**, or their protonated derivatives **13.7**, can oxidize DNA chains, whereas the *O*-acetyl derivative of the hydroxylamine derivatives **13.8** may give covalent DNA adducts (Figure 13.12).[12]

The observation of cytotoxic activity in the absence of radiation normally requires concentrations of the nitro derivative that are too high to be found in clinical situations, but the presence of two alkylating moieties can lead to improved activity. For instance, RSU-1069 behaves as a DNA monoalkylator in

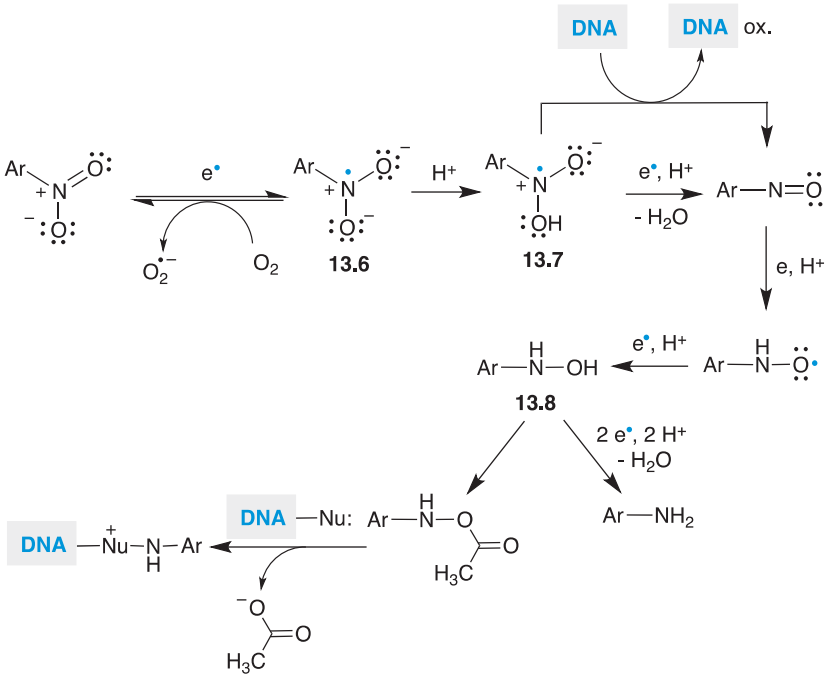


FIGURE 13.12

Reductive bioactivation of cytotoxic nitroaromatic compounds.

normal tissues and as a bis-alkylator under hypoxic environments, in which it is 50–100 times more cytotoxic (Figure 13.13). RSU-1069 entered clinical trials, but gastrointestinal toxicity limited its utility.

Nitracrine is another nitro derivative with selective cytotoxicity in hypoxic cell cultures that, in addition to its DNA intercalating properties, is able to alkylate DNA following reduction by thioles or enzymes. The nature of the electrophilic metabolites generated in this bioreductive process is still debated, and its hypoxia selectivity has not been observed in solid tumors probably because its high reduction potential (−303 mV) and its tight DNA binding slow its diffusion into hypoxic areas.

Because the alkylating reactivity of aromatic mustards is greatly determined by the electron density in the mustard nitrogen, enzymatic reduction of an aromatic nitro group to a hydroxylamine derivative can result in a higher potency as DNA alkylating agents. The simplest such prodrug, the nitrophenyl mustard (**13.9**), shows only a modest hypoxic selectivity because, due to its low reduction potential of approximately −515 mV, it is too difficult to activate. In its 2,4-dinitro analog SN-23862, the electron-attracting properties of the second nitro group induce a higher reduction potential, and it shows a higher hypoxic selectivity. Overall, this strategy is not very useful because the presence of two or more electron-deficient groups on the benzene ring of a nitrophenyl mustard to ensure a high enough reduction potential results in a low cytotoxicity even after reductive activation of part of these groups. This is also true for analogs based on heteroaromatic mustards such as **13.10**.

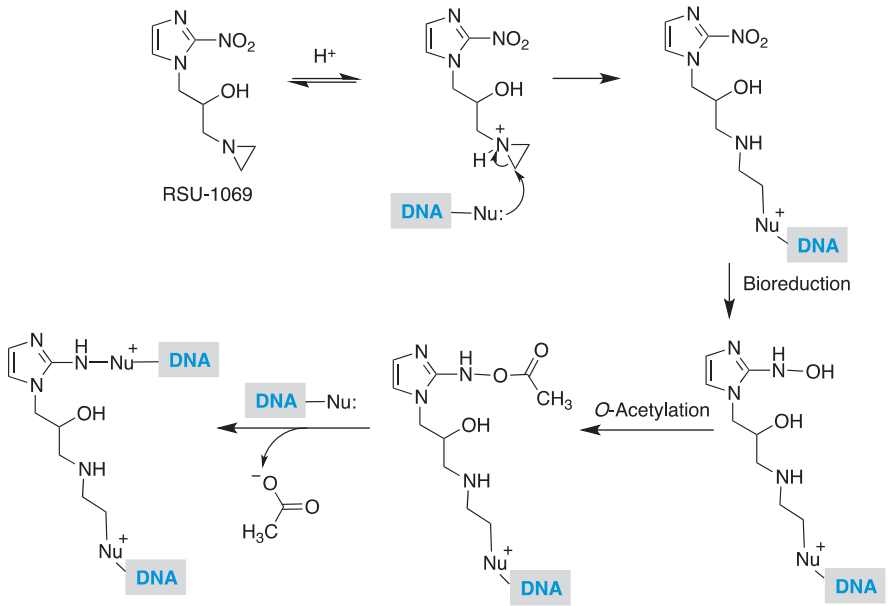
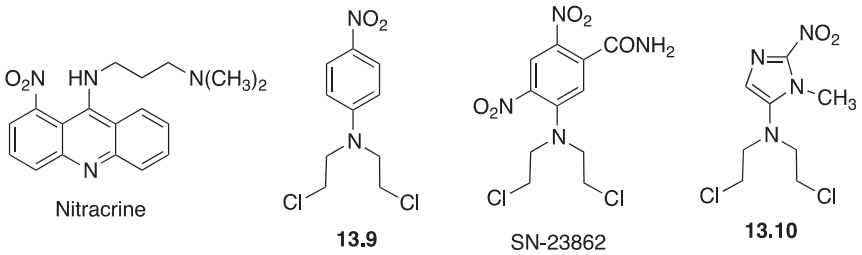


FIGURE 13.13

Enhanced cytotoxicity of RSU-1069 in hypoxic environments.



The problems associated with the presence of several nitro groups attached to the benzene ring of a nitrogen mustard are due to metabolic side reactions that contribute to its deactivation. Thus, reduction of the nitro group *ortho* to a mustard moiety may result in intramolecular alkylation, which is considered to be an inactivation pathway. For instance, the reductive metabolism of SN-23862 affords a mixture of hydroxylamino (**13.11**) and amino (**13.12**) *ortho*-reduced metabolites, which undergo a fast intramolecular cyclization to tetrahydroquinoxaline derivatives **13.13**,[13] where most antitumor activity has been lost (Figure 13.14).

However, reduction of the nitro group *para* to the mustard moiety normally generates potential DNA cross-linking cytotoxins, as is the case of the 3,5-dinitrobenzamide mustard PR-104, a hypoxia-activated DNA cross-linking agent with marked activity against human tumor xenografts,

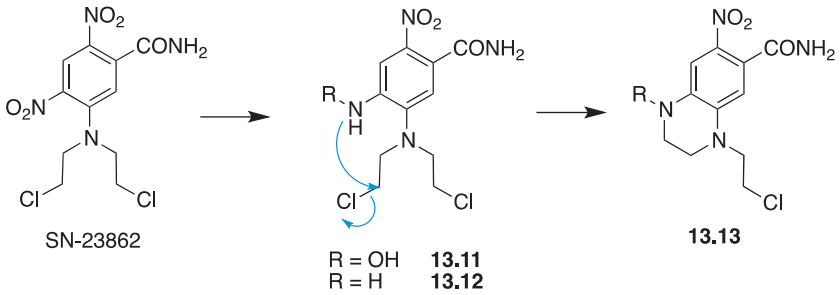


FIGURE 13.14

Inactivation of SN-23862 by reductive metabolism.

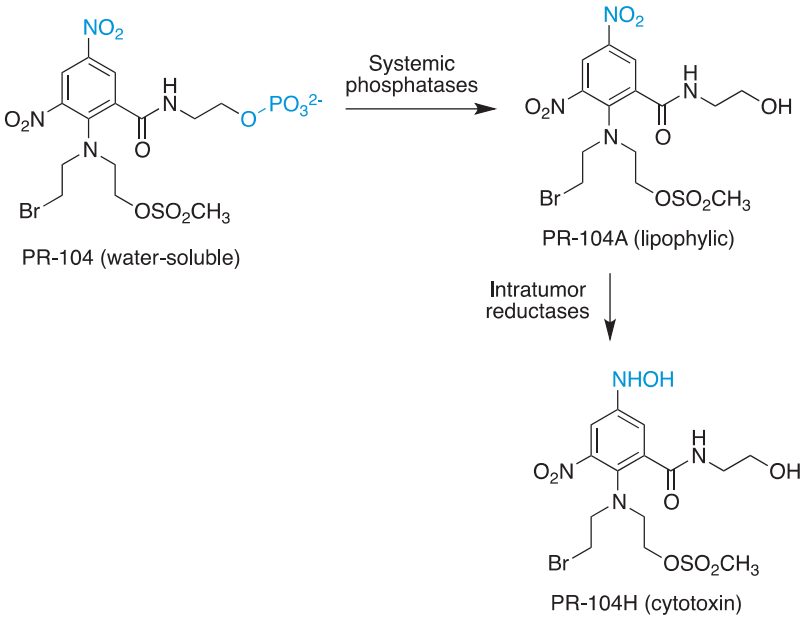


FIGURE 13.15

Bioactivation of the water-soluble PR-104 prodrug by sequential hydrolytic and reductive processes.

both as monotherapy and combined with radiotherapy and chemotherapy.[14] Upon intravenous administration, PR-104 is converted by systemic phosphatases to the alcohol intermediate PR-104A. PR-104A is intracellularly reduced under hypoxic conditions to the hydroxylamine PR-104H, which specifically cross-links hypoxic tumor cell DNA (Figure 13.15). This prodrug has entered clinical trials for advanced solid tumors.[15]

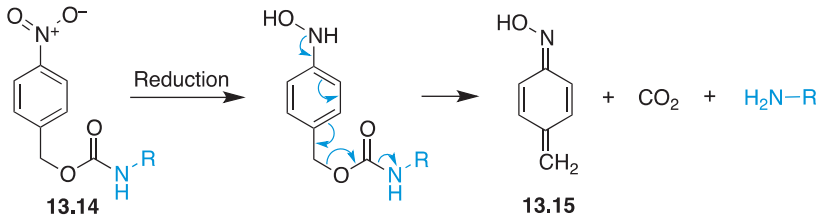


FIGURE 13.16

Reductive bioactivation of nitrobenzyl carbamates.

Similarly, the reduction of *p*-nitrobenzyl carbamates such as **13.14** to its hydroxylamino metabolite generates the electrophilic quinoneimine methide **13.15** together with amine R-NH_2. Compound **13.15** is a DNA cytotoxin, but because the reduction potential of the prodrug **13.14** is too low, this bioreduction is inefficient (Figure 13.16).

The same problem, related to a value that is too low for the reduction potential, has been shown in the bioreductive activation of the fluorouracil prodrug **13.16**, which has been designed to generate the active species together with the electrophilic quinoneimine methide by a "through-space" cyclization-extrusion process in the reduced metabolite **13.17** (Figure 13.17).

2.2.4 Cobalt Complexes

As mentioned in Section 2.4 of Chapter 5, another strategy to design hypoxic selective nitrogen mustards is the complexation of both nitrogen atoms from bidentate mustards with transition metals such as cobalt. Complexes in the low-spin Co(III) oxidation state, such as SN-24771, are very stable and have appropriate reduction potential values to be reduced by cellular reductases. This reduction is competitively inhibited by oxygen, but under hypoxia, the unstable high-spin Co(II) species resulting from reduction rapidly releases its ligands to coordinate with water molecules forming stable hexaaquo Co(II) species (Figure 13.18).[16] The limited activity shown *in vivo* by this prodrug discouraged its further development.

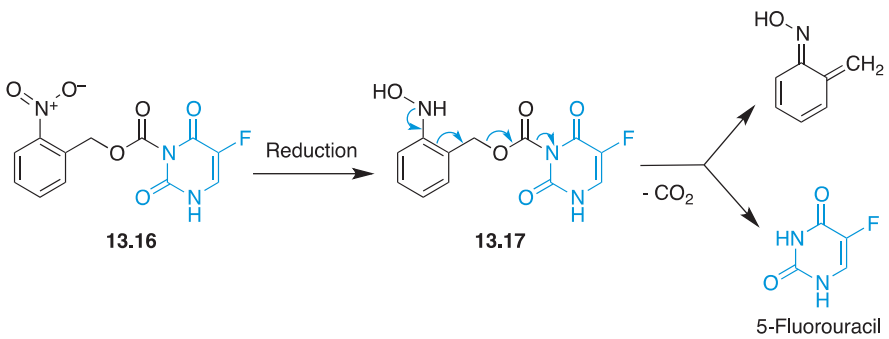


FIGURE 13.17

Reductive bioactivation of a 5-fluorouracil prodrug.

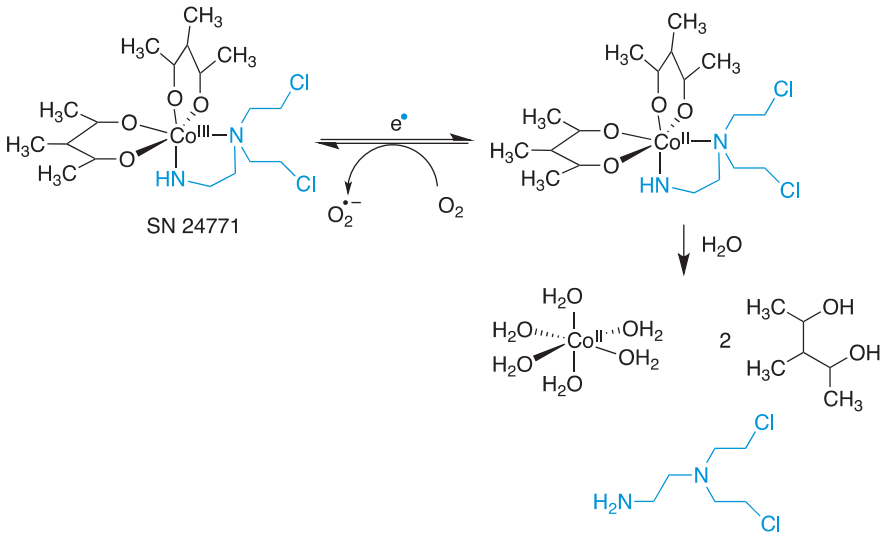


FIGURE 13.18

Hypoxia-selective activation of a Co(III)-complexed nitrogen mustard.

2.2.5 Prodrugs Activated by Therapeutic Radiation

Because severely hypoxic tissues are necrotic and therefore they lack the enzymes and cofactors required for the reductive activation of cytotoxic prodrugs, it may be useful to employ ionizing radiation for this purpose. Radiation-activated prodrugs (RAPs) are reduced by aquated/hydrated electrons (e_{aq}•) generated from the radiolysis of water. The one-electron reduction intermediates thus produced (RAPs•–) may be back-oxidized by O_2 or to scavenge more e_{aq}•. Because RAPs do not rely on enzymes for their bioreduction, activation can potentially occur in all hypoxic regions of a tumor, including necrotic regions. However, because a clinically relevant dose of radiation (2 Gy) generates only a small amount of reducing equivalents, the prodrugs need to be capable of releasing very potent cytotoxins. Some examples of prodrugs that can be activated by ionizing radiation under hypoxia are heterocyclic nitroarylmethyl quaternary ammonium salts,[17] OFU001,[18] and some Co(III) complexes, but they have not demonstrated sufficiently high activity. The uracil prodrug OFU001 is activated *in vitro* by hypoxic irradiation to release 5-FU, presumably via an intermediate species generated by incorporation of hydrated electrons into the antibonding σ* orbital of the C(1′)–N(1) bond, followed by its fragmentation (Figure 13.19).[19]

Another approach to ionization-induced prodrug activation is exemplified by the quaternary ammonium prodrug SN-25246, in which a 2-nitrobenzyl moiety is incorporated to a nitrogen mustard. The activation of such a species starts by the addition of one electron to the nitro group, giving a radical anion that, in the presence of oxygen, reverts back to the starting point. However, in hypoxic conditions, it fragments to give a resonance-stabilized *o*-nitrobenzyl radical and a molecule of the nitrogen mustard (Figure 13.20).[20]

Unfortunately, *in vivo* evaluation of OFU001 showed that the efficiency of the irradiation-activation approach seems insufficient to warrant further clinical studies.[21]

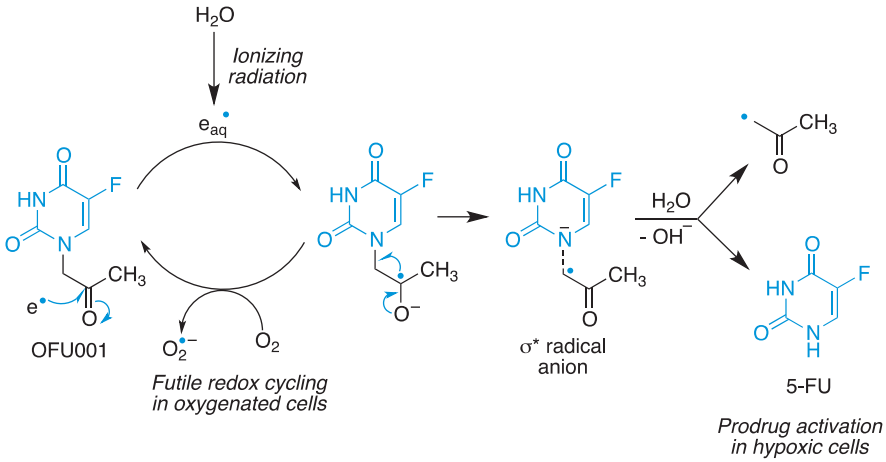


FIGURE 13.19

Activation of a 5-fluorouracil prodrug by ionizing radiation.

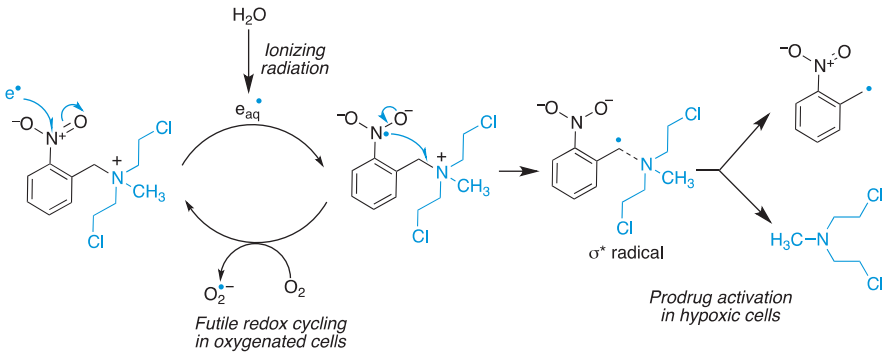


FIGURE 13.20

Ionizing radiation-induced activation of a nitrogen mustard by removal of an *o*-nitrobenzyl group.

2.3 GENE-DIRECTED ENZYME PRODRUG THERAPY AND VIRUS-DIRECTED ENZYME PRODRUG THERAPY

In these approaches, "suicide" genes encoding prodrug-activating enzymes are targeted to tumor cells, and this is followed by prodrug administration (see Chapter 12, Section 5.2).[22] Some examples of enzyme/prodrug combinations include herpes simplex virus thymidine kinase/ganciclovir, cytosine deaminase/5-fluorouracil (5-FC), cytochrome P450/cyclophosphamide, and horseradish peroxidase/indole-3-acetic acid. In gene-directed enzyme prodrug therapy (GDEPT), nonviral vectors, such as cationic lipids, peptides, or naked DNA, are used for gene targeting, whereas in virus-directed enzyme prodrug therapy (VDEPT), gene targeting is accomplished by using viral vectors, especially retroviruses and adenoviruses. VDEPT must not be confused with the use of oncolytic viruses as anticancer "drugs."[23]

In both GDEPT and VDEPT, the vector needs to be taken up by the target cells, the encoded enzyme must be expressed (transduction), and the prodrug must enter the target cells and be activated intracellularly. In addition, because it is not possible to target genes to every cell, the locally activated drug must also be able to kill cells that do not express the encoded enzyme through a phenomenon known as the "bystander effect" (Figure 13.21).

Some reductases from anaerobic bacteria are more efficient than human enzymes for the hypoxia-selective reductive activation of certain prodrugs. In these cases, it is possible to administer the prodrug associated with a viral vector that transports the gene responsible for the production of the required microbial enzyme. One example that entered clinical trials[24] is the association of the aziridine derivative CB-1954, related to the previously mentioned SN-23862, with a nonreplicating adenoviral vector that expresses the *nfsB* gene, corresponding to the *Escherichia coli* nitroreductase. This enzyme is a two-electron reductase that reduces either of the two nitro groups in CB-1954. The key metabolite seems to be the 2-hydroxylamino compound **13.18**, whose subsequent acetylation by acetyl-coenzyme

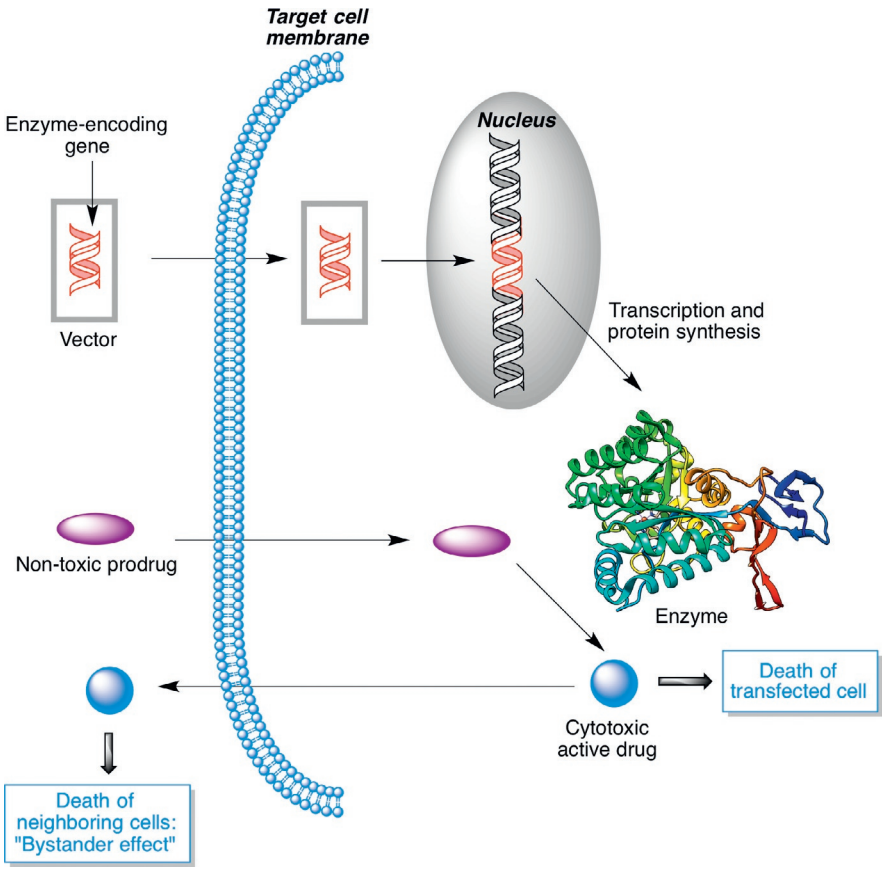


FIGURE 13.21

The basis of GDEPT and VDEPT therapies.

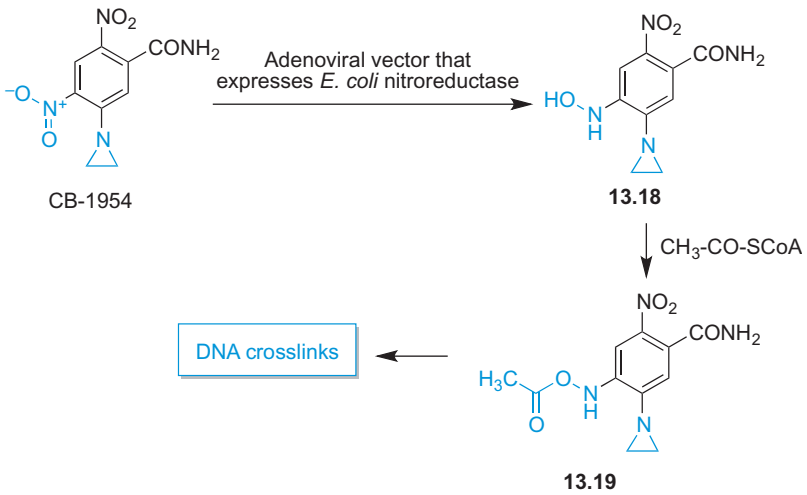


FIGURE 13.22

VDEPT-mediated bioactivation of the prodrug CB-1954.

A affords **13.19**, a potent DNA cross-linking agent (Figure 13.22).[25] Because the activated derivative of CB-1954 has a half-life of only a few seconds, very little of this toxic species is sufficiently stable to escape into the bloodstream and cause side effects. The clinical efficacy of this combination is limited by the low affinity and catalytic efficiency of the nitroreductase NfsB, despite several mutagenesis studies to improve the catalytic process.

It has been discovered that the activation of the prodrug CB-1954 by the enzyme quinone oxidoreductase 2 (NQO2) is dependent of the vitamin B-derived cofactor EP-0152R. This cofactor may travel through the blood, enter into cancer cells, and activate the enzyme NQO2 to efficiently reduce the prodrug CB-1954, allowing a greater than 10,000-fold increase in the prodrug cytotoxicity. This combination, considered as a possible treatment of hepatocellular cancer, has entered clinical trials.[26]

VDEPT approaches have also been used in the case of *N*-oxide bioreductive prodrugs by transfecting tumor cells with a mammalian expression vector, mainly adenovirus, containing the genes encoding for the enzymes necessary for their activation, namely CYP3A4 in the case of the previously discussed banoxantrone (AQ4N; Figure 13.23)[27] and cytochrome 450 reductase in the case of tirapazamine.[28]

VDEPT methodologies are not restricted to hypoxia-selective prodrug activation. For instance, *in situ* transduction by retroviral vectors of pancreatic tumor cells with the cytochrome P4502B1 (CYP2B1) suicide gene that encodes the enzyme responsible for activating cyclophosphamide increases the sensitivity of these cells to this drug. Redirecting adenoviruses to fibroblast growth factor receptors (FGFRs) localized to the plasma membrane of pancreatic tumor cells by using the FGF2-Ad-CYP2B/CPA system highly increases the potency of the CYP2B1/CPA suicide system.[29] In a related approach aimed at the treatment of gliomas, a mutant herpes simplex virus type 1 has been developed with insertion of two prodrug-activating genes—CYP2B1 and secreted human intestinal carboxylesterase. These enzymes can convert the inactive prodrugs cyclophosphamide and irinotecan (CPT-11) into their active metabolites.[30]

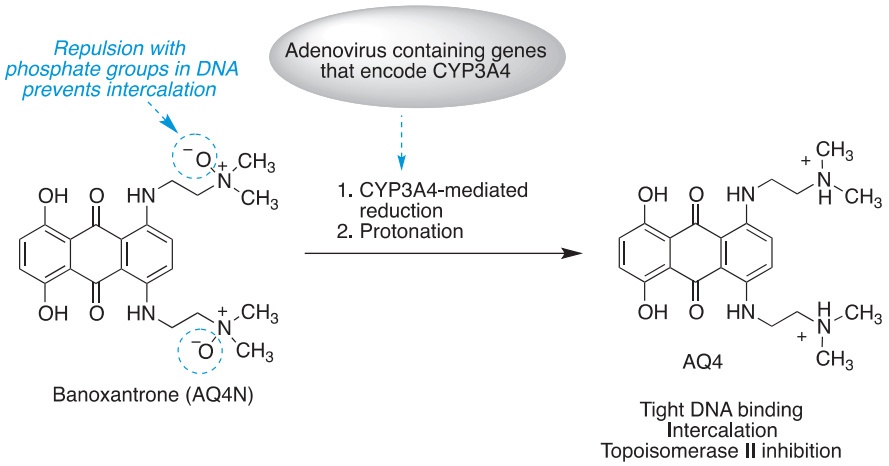


FIGURE 13.23

VDEPT-mediated reductive bioactivation of banoxantrone.

2.4 ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY

Another approach to achieve a high local concentration of antitumor drugs is the strategy known as Antibody-Directed Enzyme Prodrug Therapy (ADEPT), in which antigens expressed on tumor cells are used to target enzymes to the tumor site by means of suitable mAbs (see Chapter 12, Section 2). ADEPT therapy employs an immunoconjugate of the activating enzyme and an antibody specifically directed at a tumor antigen. After some time to allow the elimination from the general circulation of the nonbound enzyme, the corresponding prodrug is administered. Its activation takes place at the cell surface, and the active species is then uptaken into the tumor cells (Figure 13.24). One advantage of this approach is the possibility of using nonhuman enzymes, which may be more active for prodrug activation, and the main drawbacks are the scarcity of tumor-selective antigens, the possibility of immune reactions if nonhuman proteins are employed, and the need for the active species to cross the cell membrane (because activation occurs extracellularly). Bacterial enzymes have several properties that make them attractive for ADEPT therapy, including high catalytic rates and the fact that they allow the design of prodrugs that are highly selective because they are not converted in human tissues. On the other hand, they have a high likelihood of inducing immune reactions, but they can be engineered to reduce this problem.

ADEPT strategies appear to be promising to target glucuronidated prodrugs to tumor cells because β-glucuronidase activity is higher in inflammatory necrotic areas common in solid tumors and also because glucuronidated prodrugs are very poorly taken up by cells. One technique that has proven to have general value for this purpose is the use of β-glucuronyl self-immolative carbamate prodrugs. This technique is shown in Figure 13.25 for the case of the doxorubicin prodrug DOX-GA3 in combination with a mAb/β-glucuronidase conjugate, which has shown improved antitumor activity in mice compared with DOX-GA3 administration alone.[31] The enzyme immunoconjugate was prepared from the pancarcinoma Ep-CAM-specific mAb 323/A3 and β-glucuronidase. Prodrug activation can be assumed to take place by glucuronide hydrolysis to phenol **13.20**, followed by spontaneous loss of the *p*-hydroxybenzyl group through a 1,6-elimination reaction[32] and final decarboxylation of the carbamic acid **13.21** thus generated.

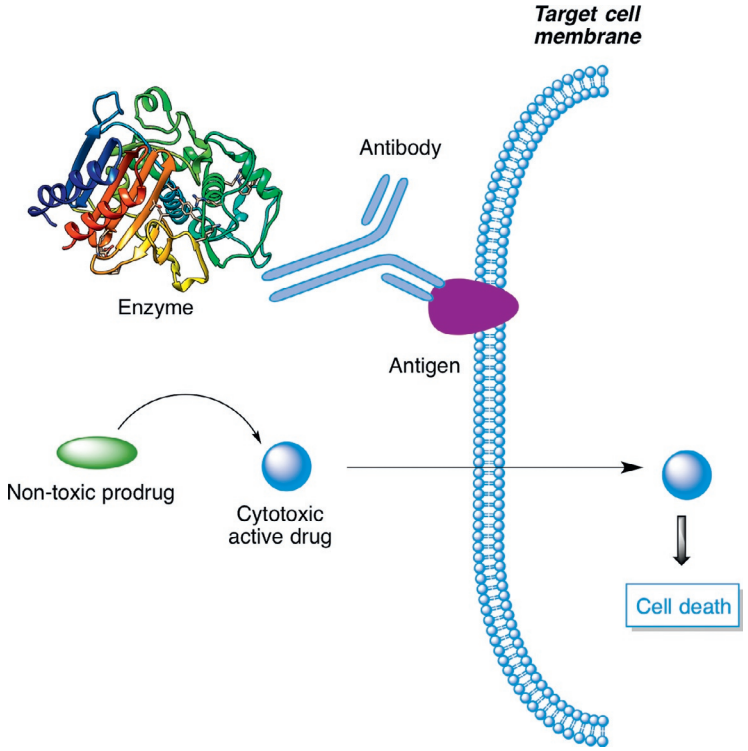


FIGURE 13.24

The basis of ADEPT therapy.

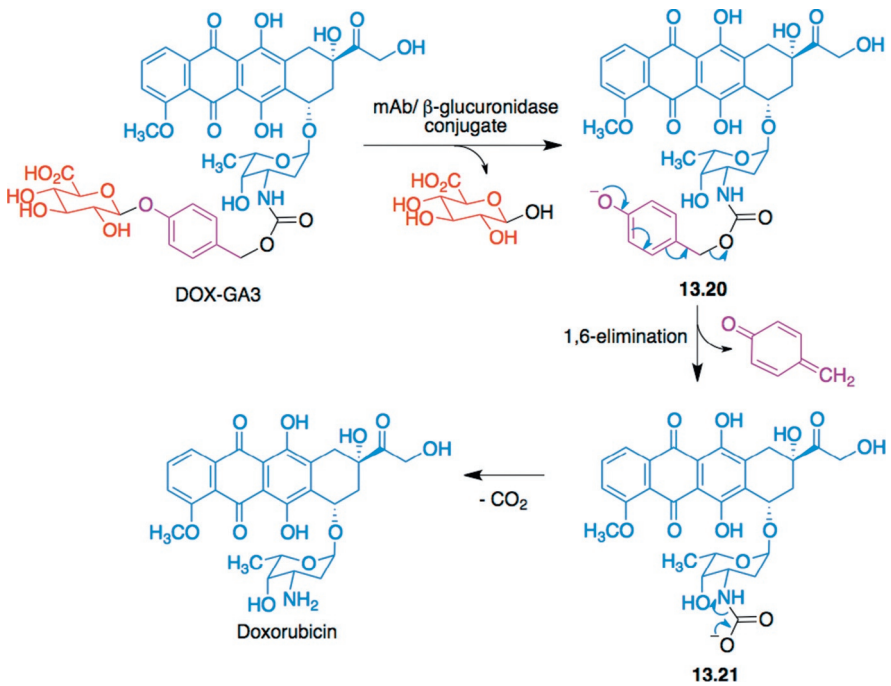


FIGURE 13.25

Bioactivation of DOX-GA3 in ADEPT therapy.

In another example of the application of the ADEPT strategy, an *E. coli* nitroreductase to activate prodrugs containing a *p*-nitrobenzylcarbamate substituent. In this way, the mitomycin C prodrug **13.22** is bioactivated by a 1,6-elimination process (Figure 13.26).[33]

Phosphatases have also been used as activating enzymes in ADEPT approaches. For instance, etoposide phosphate (Etopophos®) is activated to etoposide by a mAb –alkaline phosphatase immunoconjugate (Figure 13.27).

The first example of a locally activated prodrug designed using an ADEPT approach that reached clinical trials was the mustard prodrug CMDA in combination with the carcinoembryonic antigen antibody A5B7/bacterial carboxypeptidase G conjugate and cyclosporin to counteract the immune response to this conjugate.[34] The activity of **13.23** is probably associated with its ionized form **13.24**, where the electron density of the nitrogen atom is increased. It is also relevant to note that **13.23** showed a higher

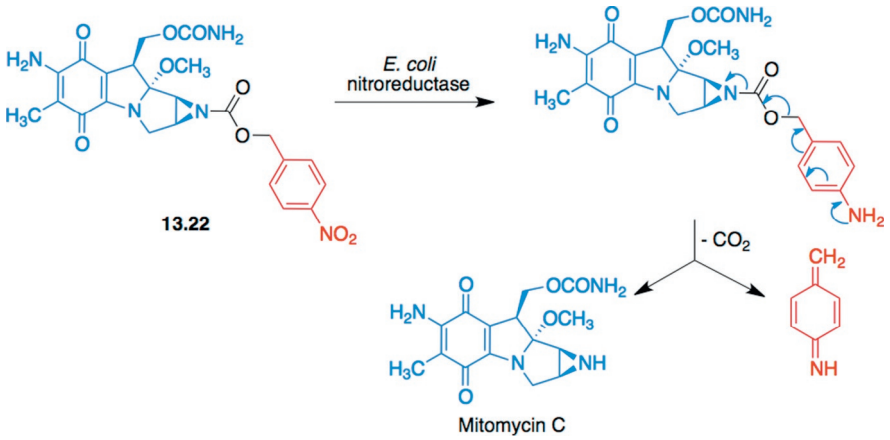


FIGURE 13.26

Bioactivation of a mitomycin C prodrug in ADEPT therapy.

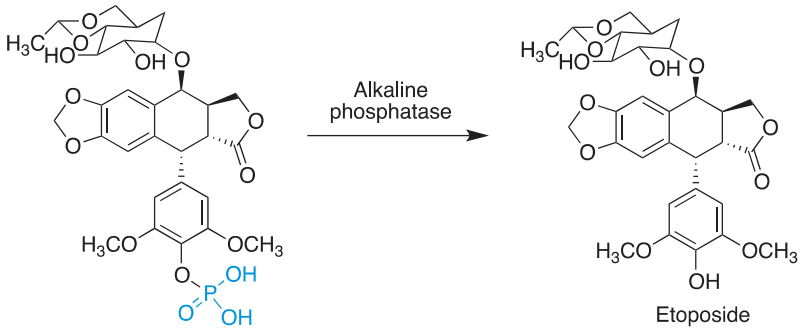


FIGURE 13.27

Bioactivation of Etopophos® in ADEPT therapy.

activity than its bis-chloroethyl analog, which can be ascribed to the higher ability of the mesylate unit to act as a leaving group. A related ADEPT approach that also underwent phase I clinical trials used the carbamate prodrug ZD-2767P, which releases the corresponding iodo nitrogen mustard (Figure 13.28).[35]

β-Lactamases have low toxicities and are stable and easy to purify. Because of these advantages, they have received much attention in ADEPT therapy and have been reported to activate a variety of prodrugs, allowing the release of many commonly used cancer drugs. For example, the bioactivation of a β-lactamase prodrug of a nitrogen mustard is shown in Figure 13.29.

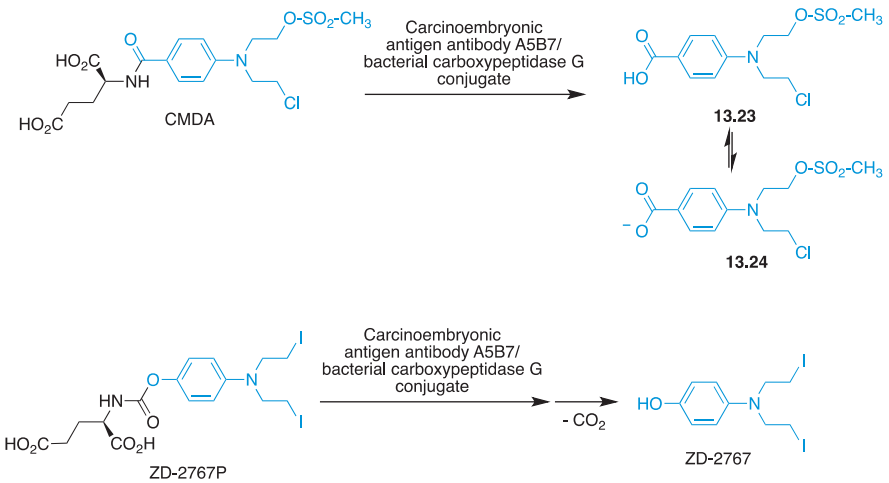


FIGURE 13.28

Some ADEPT approaches to chemotherapy with nitrogen mustards.

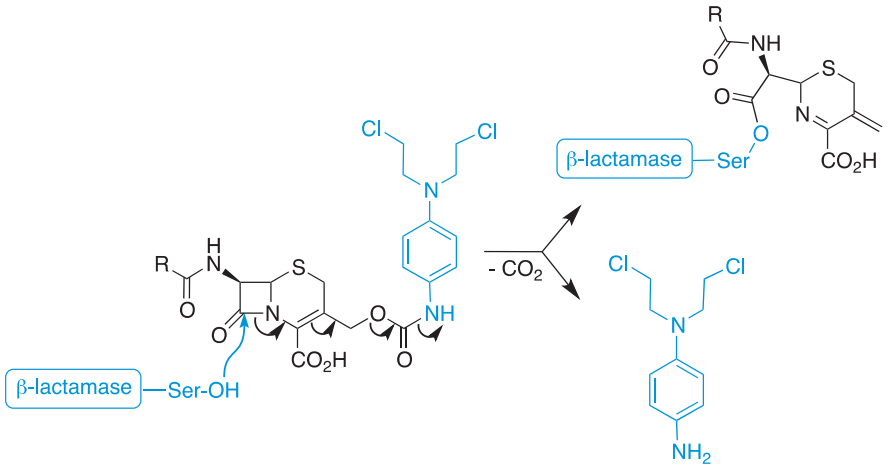


FIGURE 13.29

β-Lactamases in ADEPT therapy.

3 THERAPEUTIC NANOPARTICLES FOR DRUG DELIVERY IN CANCER: GENERAL ASPECTS

Nanotechnology, defined as the use of materials with structural features ranging from 1 to 100 nm in size, has dramatically altered the design, use, and delivery of cancer diagnostic and therapeutic agents. Nanoscale diagnostic and therapeutic agents have been in use since the development of micellar nanocarriers and polymer–drug nanoconjugates in the mid-1950s and liposomes in the mid-1960s, whereas polymeric nanoparticles were introduced by Langer and Folkman in 1976. Since then, nanoscale constructs have been developed for the systemic delivery of agents to specific disease sites, more than 20 FDA-approved diagnostic or therapeutic nanotechnologies are in clinical use, and many others are in clinical development. Nanoparticle formulations help to overcome the issue of drug solubility, which is an essential factor for drug effectiveness. Another advantage is facilitation of drug delivery across various barriers, the most important of which is the blood–brain barrier that limits access to brain tumors. Other major advantages include targeted drug delivery, reduced toxicity because of the "enhanced permeability and retention effect" (EPR; discussed below), and facilitation of a combination of diagnostics and therapeutics for cancer.[36]

Among several nanobiotechnologies based on nanoparticles that have been used to facilitate drug delivery in cancer are polymer conjugates, liposomes, and copolymer micelles. Polymer conjugates include polymer–drug conjugates (which are macromolecular small-drug carrier systems), immunoconjugated drugs, folate receptor-targeted conjugates, and polymer-directed enzyme prodrug therapies (PDEPT).

The term "polymer therapeutics" has been coined to describe water-soluble devices that use polymers as carriers and are designed for parenteral administration. They are one of the first nanodrugs, which can be defined as nanometer-scale complexes that contain at least two components, one of which is a bioactive agent.[37]

4 POLYMER CONJUGATES: MACROMOLECULAR SMALL-DRUG CARRIER SYSTEMS

In macromolecular drug carrier systems, an active drug is covalently attached to a macromolecule, an approach that has been particularly studied in the anthracyclines.[38] These conjugates may passively target solid tumor tissues by a mechanism known as the EPR effect, which is based on the increased permeability of tumor vascular endothelium due to its poor organization (Figure 13.30). This phenomenon allows that relatively large particles loaded with an antitumor drug can extravasate and accumulate inside the interstitial space, where the drug can be released as a result of normal carrier degradation.[39] More specific targeting may be achieved by using as a part of the macromolecular component an antibody directed to a tumor antigen or a peptide whose receptors are overexpressed in tumor cells.

These conjugates do not cross cell membranes, and they need to access the intracellular space by receptor-mediated endocytosis, adsorptive endocytosis, or fluid-phase endocytosis. In these processes, the cell membrane invaginates the particle, forming an intracellular vesicle (endosome) that eventually fuses with lysosomes. The macromolecular transporter is hydrolyzed and the active drug is released as a consequence of lowered pH values at both the endosomes and lysosomes, and also as a consequence of the presence of hydrolytic enzymes in lysosomes (Figure 13.31). Some extracellular drug release may

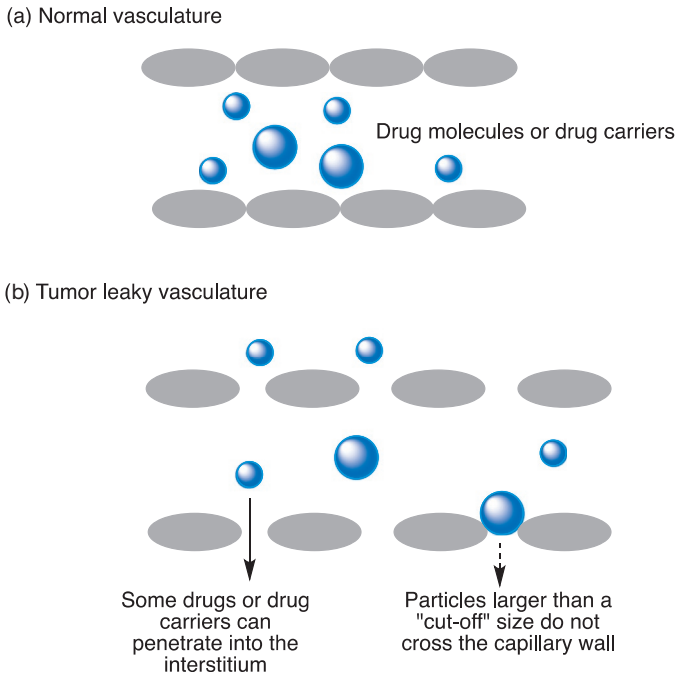


FIGURE 13.30

Enhanced permeability and retention in tumor vasculature: the EPR effect.

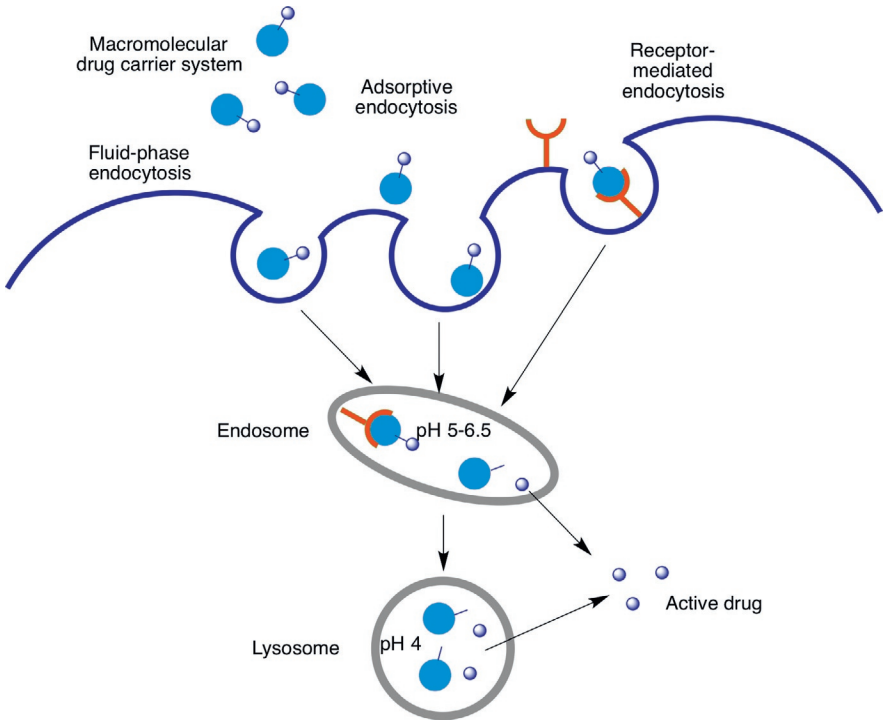


FIGURE 13.31

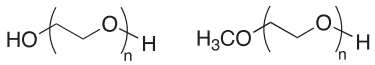
Endocytosis of macromolecular drug carrier systems and intracellular drug release.

also be produced due to the more acidic tumor environment (often 0.5–1 pH units lower than normal tissues) and to the overexpression in tumors of some extracellular proteases, such as matrix metalloproteinases and plasmin.

Polymer–drug conjugates must be water-soluble, and the polymer has to be nontoxic, nonimmunogenic, suitable for repeated administration, and chosen taking into account that its physicochemical properties govern biodistribution, elimination, and metabolism of the conjugate. The most commonly studied synthetic polymers for drug conjugation derive from polyethyleneglycol (PEG), *N*-(2-hydroxypropylmethacrylamide) (HPMA), and polyglutamic acid (PGA). PEG and HPMA are not biodegradable and have to be limited to masses less than 40 kDa to ensure eventual renal elimination, but this limitation does not affect PGA polymers.

4.1 PEGYLATED CONJUGATES

PEG is a unique polyether diol, usually manufactured by the aqueous anionic polymerization of ethylene oxide. If this polymerization is initiated with anhydrous methanol or other alcohol derivatives, it results in monoalkyl-capped polyethylene glycols, such as methoxy PEG (mPEG).



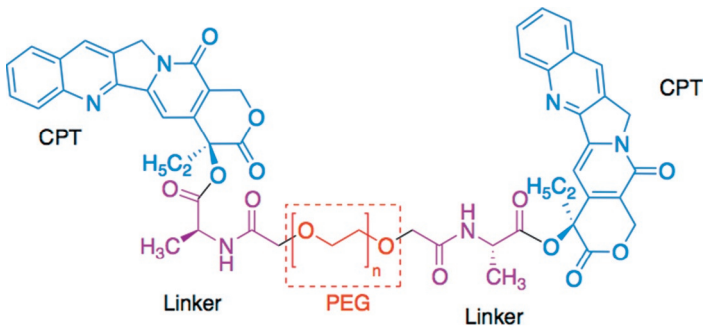
PEG mPEG

These polymers are amphiphilic, dissolve well both in organic solvents and in water, and their conjugation to proteins is accomplished mainly by reaction of an available amino group or other reactive sites such as histidine or cysteine residues. Several polymer–protein conjugates containing a therapeutic protein, mostly PEGylated enzymes or cytoquines, have entered into routine clinical use in oncology.[40] PEGylation of proteins enhances their stability, reduces their immunogenicity, and prolongs their plasma half-life; consequently, the patient requires less frequent dosing.[41] On the other hand, the PEGylation process can lead to reduction or even complete loss of the bioactivity of the therapeutic protein, associated with alteration of the overall protein charge and to reduction of its substrate or receptor binding affinity. Ten PEG drugs, not all of which are used in cancer therapy, have been approved by the FDA between 1990 and 2012: PEG liposomal doxorubicin (PLD), PEGfilgrastim (PFIL), PEGinterferon α-2a and α-2b (PA 2a and 2b), PEGademase bovine, PEGaspargase (PASP), certoluzimab PEGol (CP), PEG epoetin (PE), PEGmisovant, and PEGaptanib. For instance, peginterferon α-2b (Sylatron®), a covalent conjugate of recombinant α-2b interferon with monomethoxy PEG, was approved in 2001 for melanoma treatment. In addition, several PEG drugs, including PEGamotecan (PM) and PEGinterferon α-1a, are in clinical development.

The first antitumor PEGylated protein approved for clinical use was PEG-L-asparaginase (pegaspargase, Oncaspar®),[42] which is used to treat acute lymphoblastic leukemia, a disease that requires L-asparagine (see Chapter 2, Section 9). Pegaspargase has advantages compared to the native enzyme because of its reduced immunogenicity and its much longer plasma half-life. Other PEG–enzyme conjugates that have entered clinical trials are PEG–recombinant arginine deiminase (ADI-PEG20) and PEGylated glutaminase (PEG-PGA). The first copolymer depletes arginine from circulation, and its anticancer effect is based on the requirement of several cancer cells that are argininosuccinate synthetase deficient to obtain arginine from the circulation. Arginine is one of the 20 amino acids that are essential for protein synthesis and survival of cells. ADI-PEG20 has been studied as a single agent or in combination with 5-FU,[43] and it is currently being evaluated in a pivotal phase III trial for hepatocellular carcinoma and in the treatment of other cancers. PEG-PGA is being clinically evaluated in combination with the glutamine antimetabolite

6-diazo-5-oxo-L-norleucine (DON; see Chapter 2, Section 6.3) in patients with advanced refractory solid tumors, based on the idea that this drug should be more effective when glutamine levels are depleted.[44] Among PEG–cytokine conjugates, the granulocyte colony-stimulating factor (GCSF), a protein that stimulates the bone marrow and prevents chemotherapy-induced neutropenia, is used in its pegylated form (PEG-GCSF, pegfilgrastim, Neulasta®), which has a longer half-life and fewer allergic reactions than the free protein.[45]

Low-molecular-weight anticancer PEG–drug conjugates have also been studied, but no commercial products have been reported so far. Pegylated camptothecin (EZ-246, pegamotecan) is an ester-based prodrug of this topoisomerase poison that includes an alanine spacer.[46] Cleavage of the amide bond between PEG and the amino acid by exo-peptidases in the tumor results in an amino acid–CPT ester conjugate, which would still have its bioavailability enhanced by lactone stabilization. The ester bond would subsequently be cleaved to release CPT. Clinical studies have shown a favorable rate of hematological toxicities and diarrhea, compared with irinotecan, which suggests that pegamotecan could be combined with other active agents.[47]



Pegamotecan (EZ-246)

The main limitation of PEG as a drug carrier is the presence of only two reactive groups per polymer chain, which leads to an intrinsically low drug payload. To overcome this limitation, a conjugate of SN38 (an active metabolite of CPT) with a 40-kDa PEG dendron structure containing four arms and known as EZN-2208 is being developed (Figure 13.32).[48]

4.2 *N*-(2-HYDROXYPROPYL)METHACRYLAMIDE POLYMERS

Most HPMA conjugates incorporate the enzyme-degradable peptide linker Gly-Phe-Leu-Gly because of its stability in plasma and susceptibility to cleavage by lysosomal proteases following internalization by endocytosis. The HPMA polymer has not shown evidence of toxicity or immunogenicity in humans, which represents a great advantage over previously studied natural polymers that led to immunogenic reactions. On the other hand, HPMA has the disadvantage of not being biodegradable. Several of these polymer–drug conjugates have entered clinical trials for anticancer therapy.[49] The doxorubicin conjugate PK-1 (FCE-28068)[50] has a greatly reduced toxicity compared with free doxorubicin, and it was the first synthetic polymer-based anticancer conjugate to enter clinical trials (1994). After phase II studies in patients with breast cancer, non-small cell lung cancer (NSCLC), and colorectal cancer, PK-1 entered phase III clinical trials.

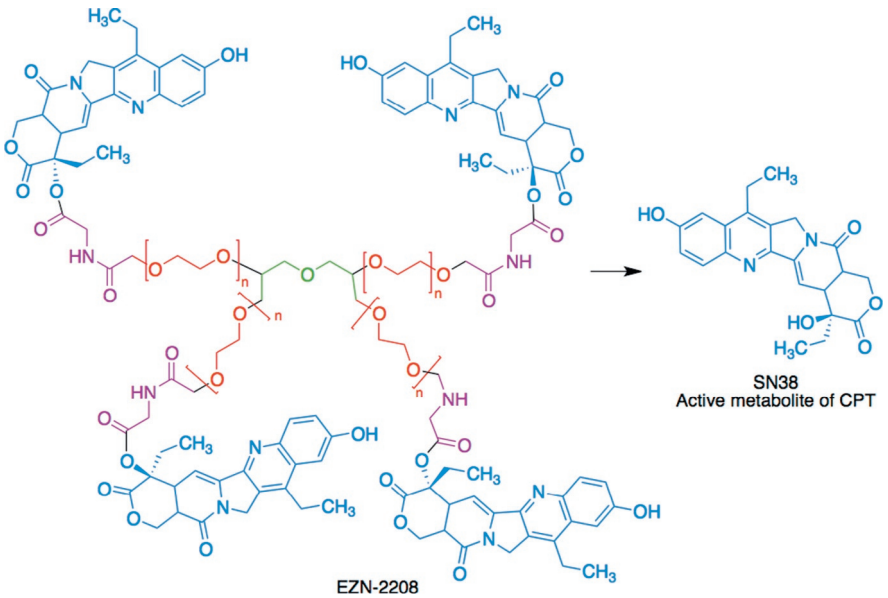
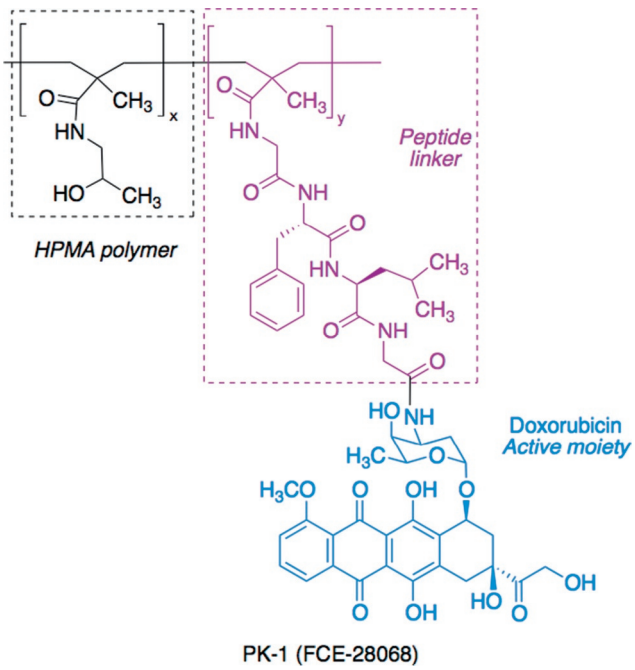
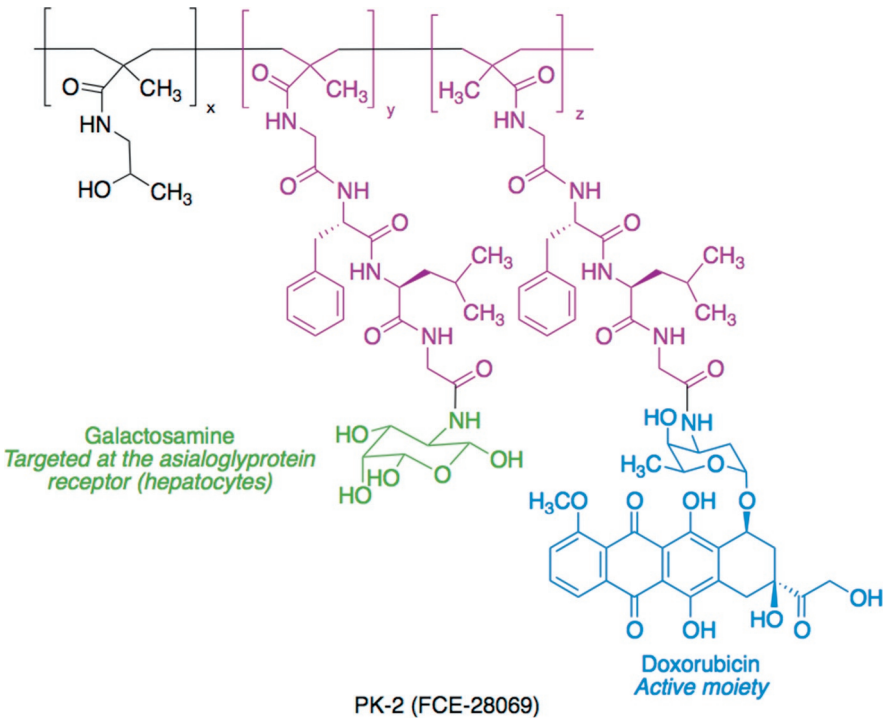


FIGURE 13.32

EZN-2208, a dendronic prodrug of SN38.

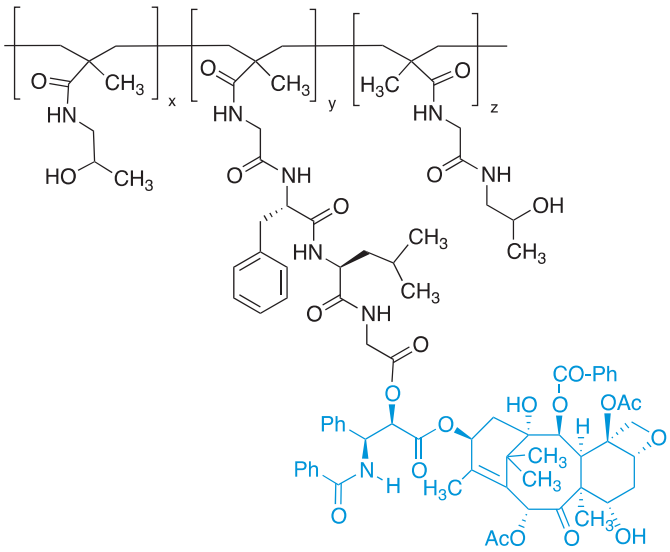


In a more sophisticated approach, a HPMA conjugate known as PK-2 (FCE-28069), which is also in clinical trials, was designed for tumor targeting using receptor-mediated endocytosis. This copolymer contains the HPMA polymer, the Gly-Phe-Leu-Gly peptide, doxorubicin, and galactosamine. This carbohydrate is targeted at the asialoglyprotein receptor, which is present in hepatocytes, and consequently this conjugate prodrug is expected to be useful in hepatocarcinomas.[51] Other ligands different from galactosamine are being explored for targeting.

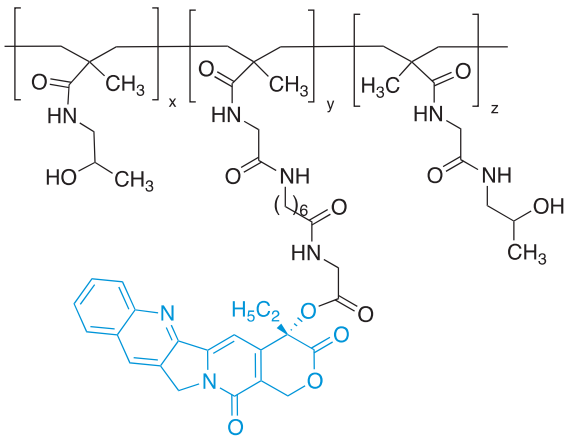


PK-2 (FCE-28069)

Other HPMA conjugates that have entered clinical studies are the paclitaxel conjugate PNU-166945[52] and the camptothecin derivative **13.25**.[53]



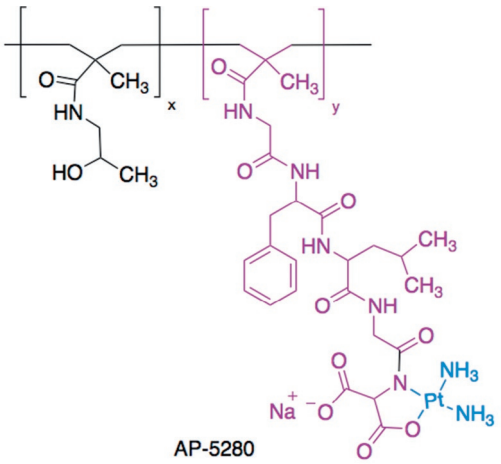
PNU-166945 (HMPA copolymer-paclitaxel)



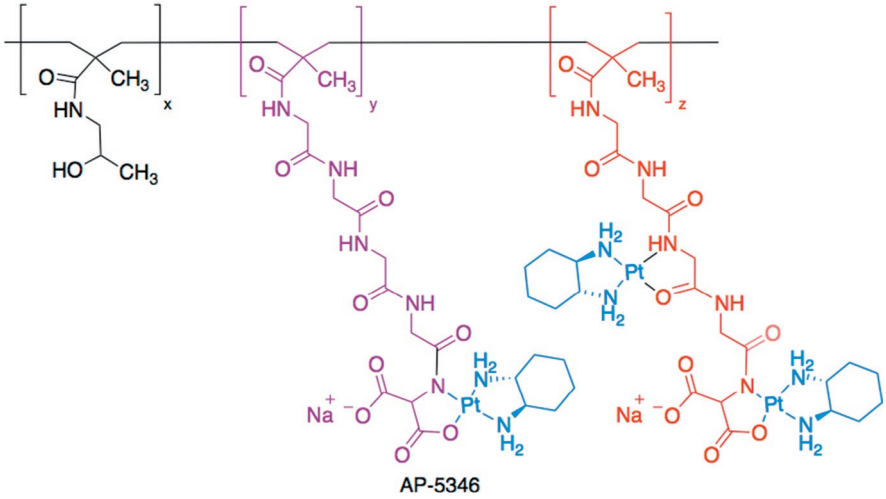
HMPA copolymer-camptothecin (**13.25**)

Cisplatin is one of the main anticancer agents currently in use, but it shows severe side effects, the most prominent ones being nephrotoxicity, neurotoxicity, nausea, and vomiting. Its intrinsic or acquired tumor resistance is also a major problem. Such limitations, coupled with a narrow therapeutic index and poor solubility, have been the driving force behind a sustained research effort into the discovery of novel

platinum agents, novel formulations, and delivery methods of existing platinum agents.[54] Two HPMA copolymer–platinate agents known as AP-5280[55,56] and AP-5346[57] are under clinical development.

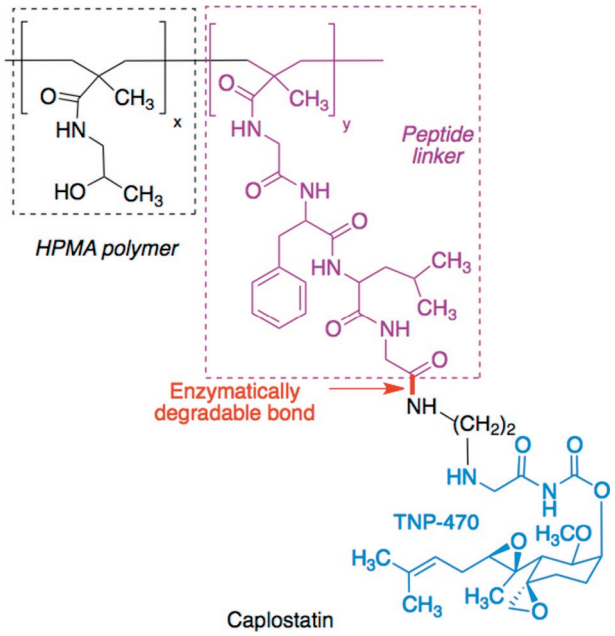


AP-5280



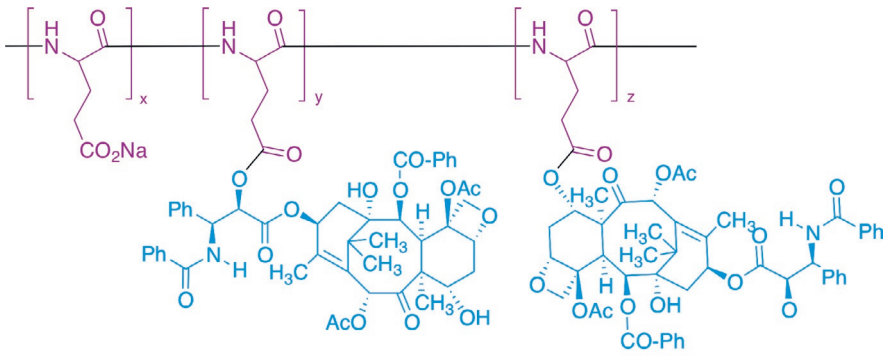
AP-5346

Caplostatin is an HPMA conjugate of TNP-470 that substantially enhances and prolongs the activity of this antiangiogenic drug. Polymer conjugation prevents TNP-470 from crossing the blood–brain barrier and decreases its accumulation in normal organs, thereby avoiding drug-related toxicities.[58] However, the drug still could not be effectively administered because it required continuous intravenous infusions resulting in costly treatments and substantial patient discomfort. An orally delivered micellar formulation of TNP-470 (see Section 7.2) has been developed.

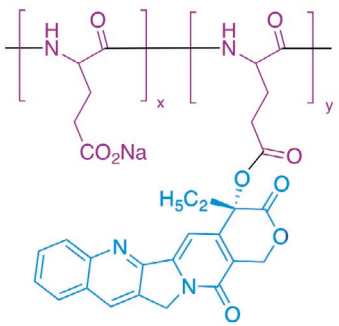


4.3 POLY-(L-GLUTAMIC) CONJUGATES

Another group of polymers designed for passive targeting through the EPR effect that are under clinical trials are the conjugates of poly-(L-glutamic) acid (PGA). They have the advantage over HPMA and PEG polymers of being biodegradable and therefore not subject to the previously mentioned 40-kDa limit in molecular mass. The main PGA conjugates under study are prodrugs of paclitaxel (CT-2103) and camptothecin (CT-2106).[59] Paclitaxel polyglumex (CT-2103, Xyotax®)[60] is the most advanced anticancer drug conjugate in clinical trials. Phase III studies have shown promising activity against NSCLC,[61] and it is also active against several cancers. In 2006, it received fast track designation from the FDA for the treatment of advanced NSCLC in patients with a poor performance status. It was proposed to minimize normal tissue exposure to free drug and evade bacterial multidrug resistance efflux pumps via pinocytotic tumoral uptake. The poor solubility of taxanes requires the inclusion of surfactant vehicles such as Cremophor EL® in their commercial formulations, but paclitaxel polyglumex does not contain this toxic vehicle due to the ability of polyglutamic acid to render highly hydrophobic molecules soluble.



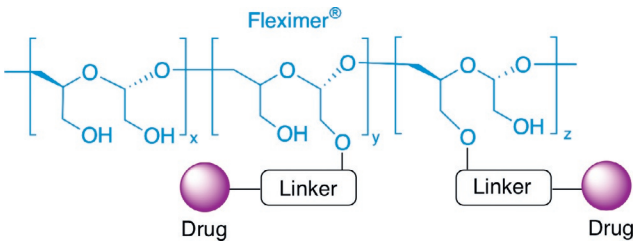
CT-2103



Paclitaxel polyglumex (CT-2106)

4.4 CONJUGATES WITH SEMISYNTHETIC HYDROPHILIC POLYALS

Polyals are hydrophilic polymers consisting of acyclic units built of nonsignaling carbohydrate substructures via either polymerization of suitable monomers or lateral cleavage of polysaccharides. They may be biocompatible and non-bioadhesive, and their intrachain acetal or ketal groups may be nonenzymatically degraded upon uptake by cells. Fleximer® [poly(1-hydroxymethylethylene hydroxymethylformaldehyde)] is a clinically validated 70-kDa biodegradable hydrophilic polyacetal that has multiple sites available for functionalization and can be loaded with different drugs through adequate linkers.[62] The use of custom-selected linkers permits control of the mechanism, rate, and localization of drug release, with subsequent improvement of pharmacokinetics, reduction of immunogenicity, and optimization of drug load of small molecules, antibody-drug conjugates, antibody fragments, siRNA, and synergistic drug combinations.



XMT-1001, which is being evaluated in clinical trials, is a water-soluble polymeric prodrug derivative of CPT[63] with an improved therapeutic window compared with CPT and irinotecan in human tumor xenograft models.[64] When administered intravenously, XMT-1001 releases camptothecin-20-(*N*-succinimido-glycinate) (CPT-SI), CPT, and camptothecin-20-(*N*-succinamidoyl-glycinate) (CPT-SA) over an extended time period (Figure 13.33).

Analogs of fumagillin (see Chapter 11, Section 6.3) have also been studied as Fleximer® conjugates. XMT-1107 is a prodrug of XMT-1191, a first-in-class antiangiogenic compound with broad

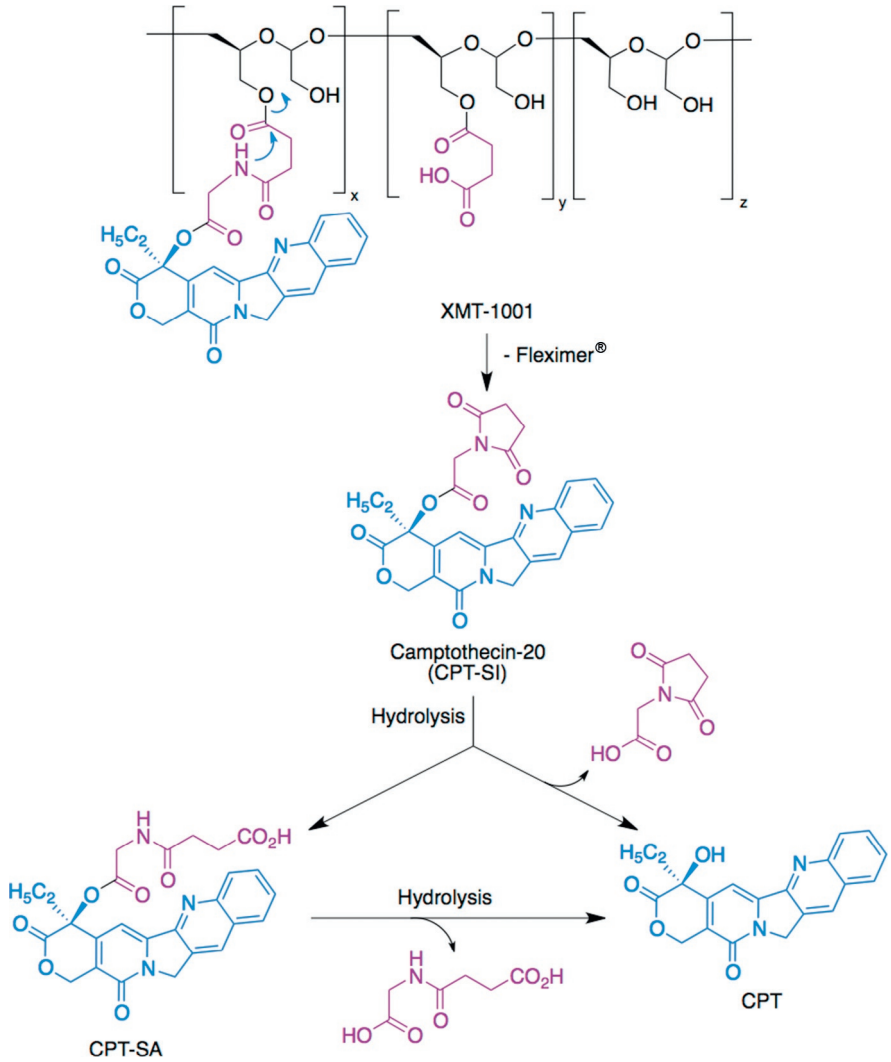


FIGURE 13.33

XMT-1001, a Fleximer®-derived camptothecin prodrug.

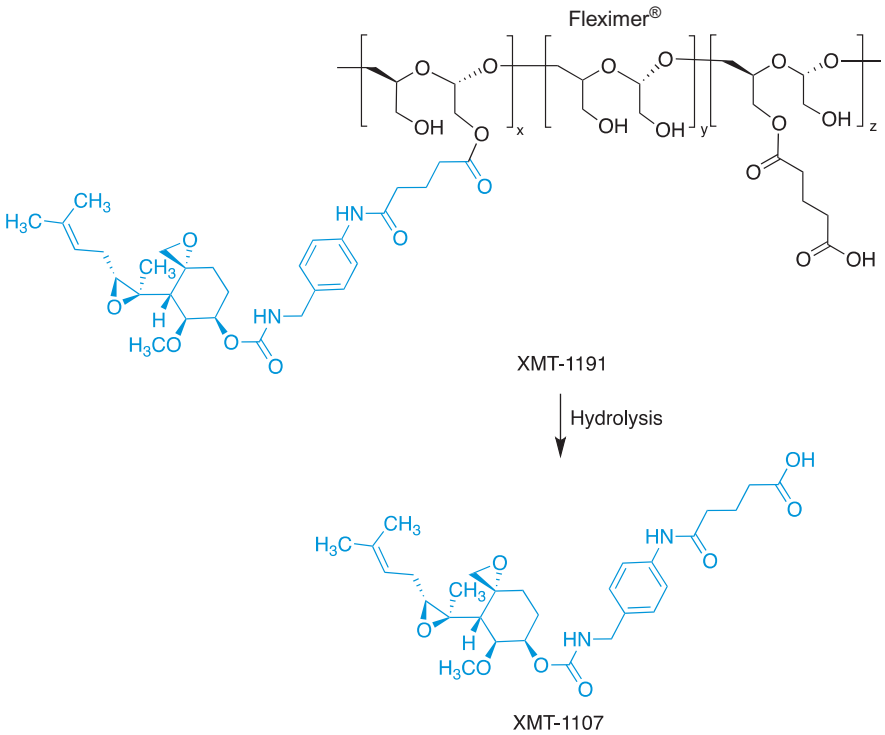


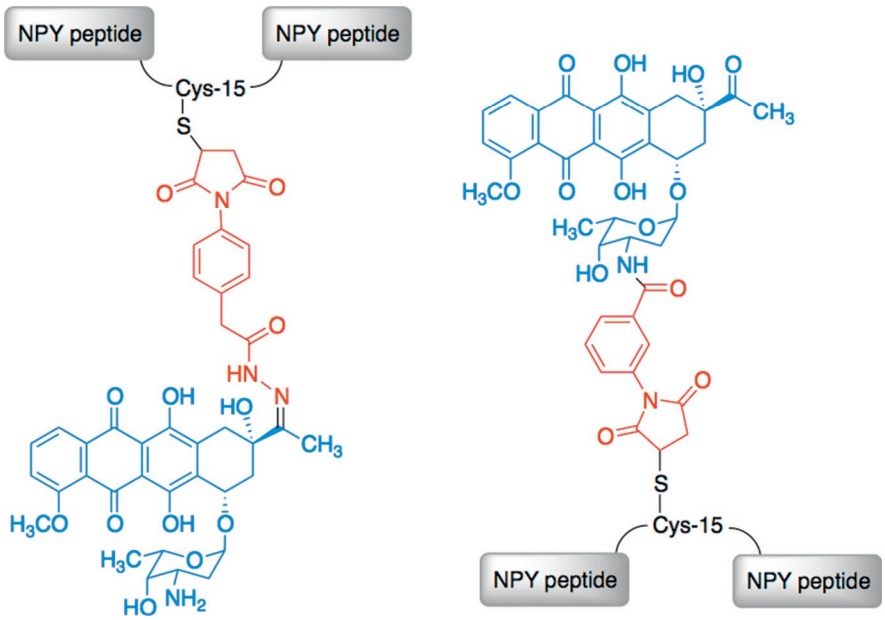
FIGURE 13.34

XMT-1107, a Fleximer®-derived prodrug of XMT-1191.

potential across multiple oncology indications that entered clinical studies in 2010 (Figure 13.34). It has demonstrated dramatically improved pharmacokinetics and no evidence of central nervous system toxicity, establishing it as a potential new antiangiogenic drug with significant therapeutic advantages.[65]

4.5 NEUROPEPTIDE Y CONJUGATES

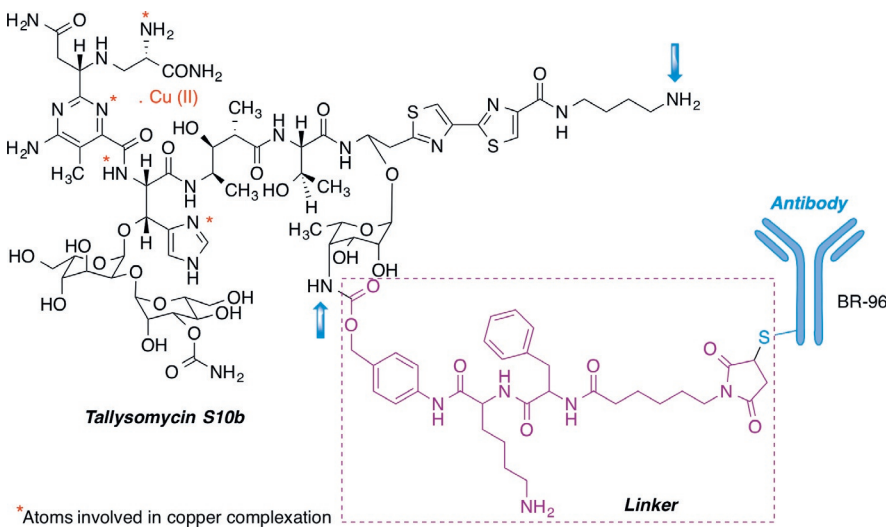
Receptors of neuropeptide Y (NPY), a 36-amino acid peptide of the pancreatic polypeptide family, are often overexpressed in neuroblastomas. For this reason, conjugates of daunorubicin or doxorubicin with this neuropeptide target and bind to these cells and, after being internalized, release the free drug. Both drugs were covalently linked to NPY via two spacers that differ in stability: an acid-sensitive hydrazone bond at the 13-keto position of daunorubicin and a stable amide bond at the 3′-amino position of daunorubicin and doxorubicin. A Cys residue at position 15 of NPY was used for attaching the maleimide end-moiety of these linkers.[66]



4.6 ANTIBODY–DRUG CONJUGATES

Selective mAbs may be linked to anticancer agents to reduce the exposure of sensitive organs and tissues to drugs while enhancing the exposure of the tumor and metastatic foci (see Chapter 12, Section 2). Although most antibodies normally remain bound to the cell surface, some of them may be endocytosed, which permits increased drug potency when the antibody is internalized after binding to the corresponding tumor antigen. Because of the tumor specificity provided by the antibody, highly potent compounds that would otherwise be too toxic can be employed as payloads. Linkers include hydrazones, disulfides, thioethers, and peptides. For the thioethers, drug release is mediated by intracellular antibody degradation.[67] Use of peptides as linkers may present advantages because the hydrolysis is enzymatic and the enzymes can be selected for preferential expression in tumors.

Antibody–drug conjugates (ADCs) of tallysomycin have been prepared using peptide sequences for intracellular drug release, such as the conjugate formed by a copper (II) complex of tallysomycin S_{10b} (a bleomycin analog) and the antibody BR-96. The only two amino groups present in the drug (marked with arrows in the following scheme) are available for acylation. A *p*-aminobenzyl carbamate was used as the spacer between the dipeptide linker and the drug to ensure that the scissile bond is not sterically encumbered, and a maleimide group was employed as a handle for conjugation to the antibody BR-96 via a cysteine residue. This conjugate enhanced the activity of the free drug by up to 825-fold.[68]



Gentuzumab ozogamicin (Mylotarg®) was the first clinically approved ADC. It contains a humanized mAb, known as hP67.6, that binds specifically to CD-33, a sialic acid-dependent adhesion protein found on the surface of normal and leukemic myeloid cells, including acute myeloid leukemia blasts, but not on normal bone marrow hematopoietic stem cells. In gentuzumab ozogamicin, hP67.6 is coupled to a prodrug form of the natural product calicheamicin, which belongs to the enedyine class. After binding, the internalized conjugate breaks down, allowing calicheamicin to interact with the minor groove of DNA. Because the antigen is not expressed on normal hematopoietic cells, this conjugate is highly selective for leukemia cells. Gemtuzumab ozogamicin was indicated for patients with $CD33^+$ acute myeloid leukemia who have relapsed after initial treatment and are considered ineligible for more aggressive cytotoxic chemotherapy.[69] Since the approval of Mylotarg® in 2000, many other ADCs have entered clinical trials. Although in 2010 its manufacturer voluntarily withdrew the drug when a clinical trial showed that the drug added no benefit over conventional therapies, trials with gemtuzumab ozogamicin have continued.

Some highly cytotoxic agents, such as the marine natural product vedotin (monomethylauristatin E; see Chapter 9, Section 2.1.2), have been developed and approved as conjugate drugs. Brentuximab vedotin (Adcetris®) is an ADC directed against protein CD30, which is expressed in classical Hodgkin's lymphoma and systemic anaplastic large cell lymphoma. This conjugate was approved by the FDA in 2011 and by the European Medicines Agency (EMA) in 2012 for treatment of $CD30^+$ lymphoproliferative disorders such as Hodgkin's lymphoma.[70] In glembatumumab vedotin (CDX-011, CR011-vcMMAE), which is in phase II clinical studies against breast cancer and unresectable melanomas[71] the drug is connected to a fully human mAb directed against CG56972 by a three-part chain that comprises an aromatic spacer, a valine–citrulline dipeptide linker, and a maleimidocaproyl linker, which becomes covalently attached to the antibody by a Michael addition from a cysteine residue to the maleimide double bond (see Figure 13.35).

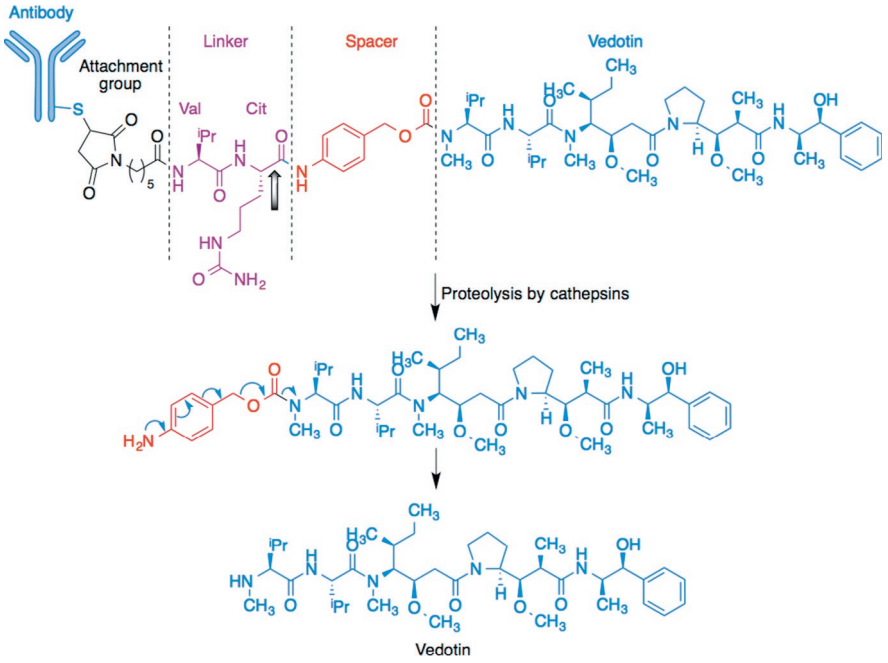
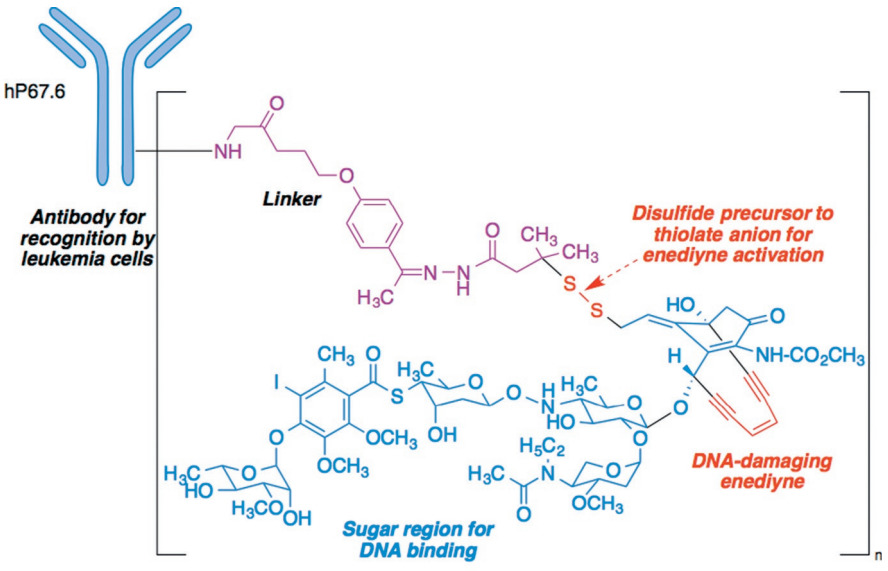


FIGURE 13.35

Bioactivation of glembatumumab vedotin.



Gemtuzumab ozogamicin (CMA-676) n = 2 - 3

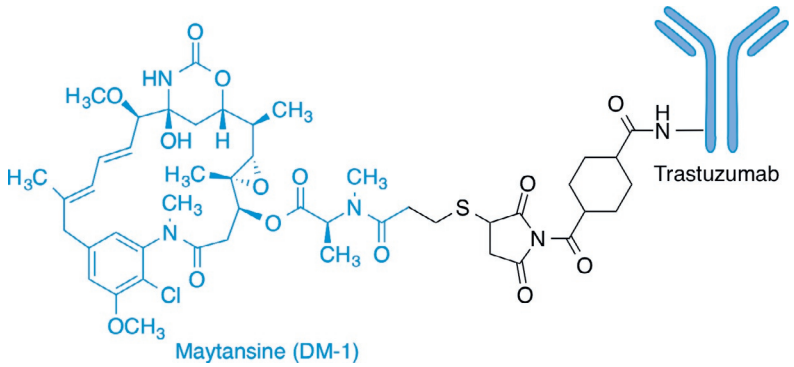
The dipeptide linker was chosen for its stability in the extracellular fluid and its sensitivity to proteases of the cathepsin family, which are highly expressed in the lysosomes. Thus, upon internalization of the conjugate inside the cell, these proteases cleave the bond shown by the arrow, spontaneous fragmentation of the *p*-aminobenzylcarbamate takes place, and vedotin is released as a chemically unmodified, active drug.[72]

Due to their very high cytotoxicity, many other antibody conjugates of the monomethyl auristatins E and F are under clinical trials. These are summarized in Table 13.1.

Table 13.1 Main Antibody Conjugates of Dolastatin 10 Analogs

Name	"Warhead"	Antibody (Target)	Clinical Status
Brentuximab vedotin (Adcetris®)	Monomethylauristatin E	Brentuximab, cAC10 (CD30)	Commercialized
Glembatumumab vedotin (CDX-01, CR011-vcMMAE)	Monomethylauristatin E	Human mAb CR011 (CG56972)	Phase II (breast cancer, melanoma)
ABT-414	Monomethylauristatin F	ABT-806 (EGFR)	Orphan drug
PSMA-ADC	Monomethylauristatin E	PSMA (prostate-specific antigen)	Phase II (prostate cancer, glioblastome)
DCDT-2980S	Monomethylauristatin E	Humanized IgG1 Ab (CD22 epitope)	Phase II (leukemia)
DCDS-4501A	Monomethylauristatin E	MAb (CD79b)	Phase II (limphomas)
Enfortumab vedotin (AGS-22MSE and AGS-22ME)	Monomethylauristatin E	Human IgG1k Ab	Phase I
Vorsetuzumab mafdotin (SGN-75)	Monomethylauristatin F	Humanized mAb 1 F6 (CD70)	Phase I
SGN-19A (SGN-CD19A)	Monomethylauristatin F	Humanized antibody (CD19)	Phase I
BAY 79-4620 (3ee9/MMAE)	Monomethylauristatin E	Ab against human carbonic anhydrase IX	Phase I
AGS-16C3F (AGS-16M8F)	Monomethylauristatin F	Human IgG2k MAb (AGS-16)	Phase I
DMUC-5754A (RG-7458)	Monomethylauristatin E	mAb (MUC16 epitope)	Phase I
DNIB-0600A (RG-7599)	Monomethylauristatin E	Humanized IgG1 mAb (NaPi2b epitope)	Phase I
A1-mcMMAF (PF-06263507)	Monomethylauristatin F	Humanized mAb (5 T4 tumor antigen)	Phase I
DMOT-4039A	Monomethylauristatin E	MMOT-0530A (antigen overexpressed in pancreatic and ovarian cancer)	Phase I
DSTP-3086S (RG-7450; thio-anti-STEAP1-MC-vc-PAB-MMAE)	Monomethylauristatin E	Ab against STEAP1 (six-transmembrane epithelial antigen of prostate 1)	Phase I
MLN-0264	Monomethylauristatin E	5 F9 human MAb (guanylyl cyclase C)	Phase I
SGN-LIV1A	Monomethylauristatin E	Human mAb (LIV-1)	Phase I
AGS-15E (AGS-15ME)	Monomethylauristatin E	AGS15 human IgG2 mAb (SLITRK6)	Phase I

Trastuzumab-DM1, also known as trastuzumab emtansine or ado-trastuzumab emtansine (Kadcyla®), is an ADC that contains the humanized anti-HER-2 MoAb trastuzumab and delivers a thioether-linked to maytansine (DM1), an antimitotic agent that binds tubulin. Because trastuzumab targets the Her-2^+ breast tumor cells, this conjugate was designed to release the drug upon complete degradation of the mAb in the lysosomes of these cells.[73] After a phase III clinical study, this ACD was approved in 2013 specifically for treatment of HER-2^+ metastatic breast cancer in patients who have been treated previously with trastuzumab and a taxane.[74]



Monoclonal antibodies are also employed in nuclear medicine to target radioactive nuclides to specific tumor cells.[75,76] For instance, ibritumomab tiuxetan (Zevalin®) consists of two parts—the murine anti-CD20 ibritumomab, which targets mature B cells, and the EDTA analog tiuxetan, which provides a chelation site for yttrium-90 or indium-111 (Figure 13.36). It was the first targeted radioconjugate agent approved for cancer treatment and is indicated for refractory B-cell non-Hodgkin's lymphoma.

The combination of the antibody tositumomab and its radioactive derivative ^{131}I-tositumomab (Bexxar®) was the basis of an anti-neoplastic radioimmunotherapeutic mAb-based regimen that is indicated for the treatment of patients with CD20 antigen-expressing non-Hodgkin's lymphoma.[77]

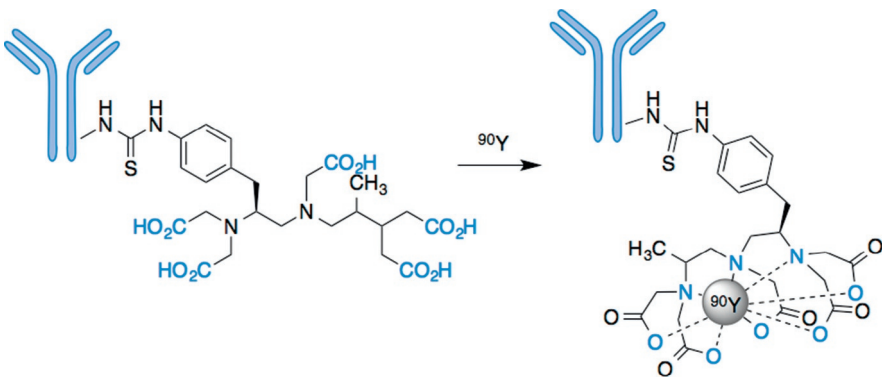


FIGURE 13.36

Structure of ^{90}Y-ibritumomab tiuxetan.

Nevertheless, it was withdrawn from the market in early 2014 due to a decline in its use and the existence of similar drugs. Tositumomab itself is a murine IgG2a mAb directed against the CD20 antigen, which is found on the surface of normal and malignant B lymphocytes.

Several mAbs labeled with different radioactive nuclides, including the α-emitter ^{211}At,[78] are currently under study in cancer models.

5 POLYMER-DIRECTED ENZYME PRODRUG THERAPY APPROACHES

PDEPT is a two-step antitumor approach that uses a combination of a polymeric prodrug and a polymer–enzyme conjugate to generate selective cytotoxicity. In a preliminary study of this approach, administration of the previously mentioned doxorubicin conjugate PK-1 was followed by administration of a conjugate of the proteolytic enzyme cathepsin B (Figure 13.37). This conjugate also used the copolymer of HPMA and the Gly-Phe-Leu-Gly tetrapeptide linker, and its antitumor activity showed advantages compared to PK-1 alone.[79]

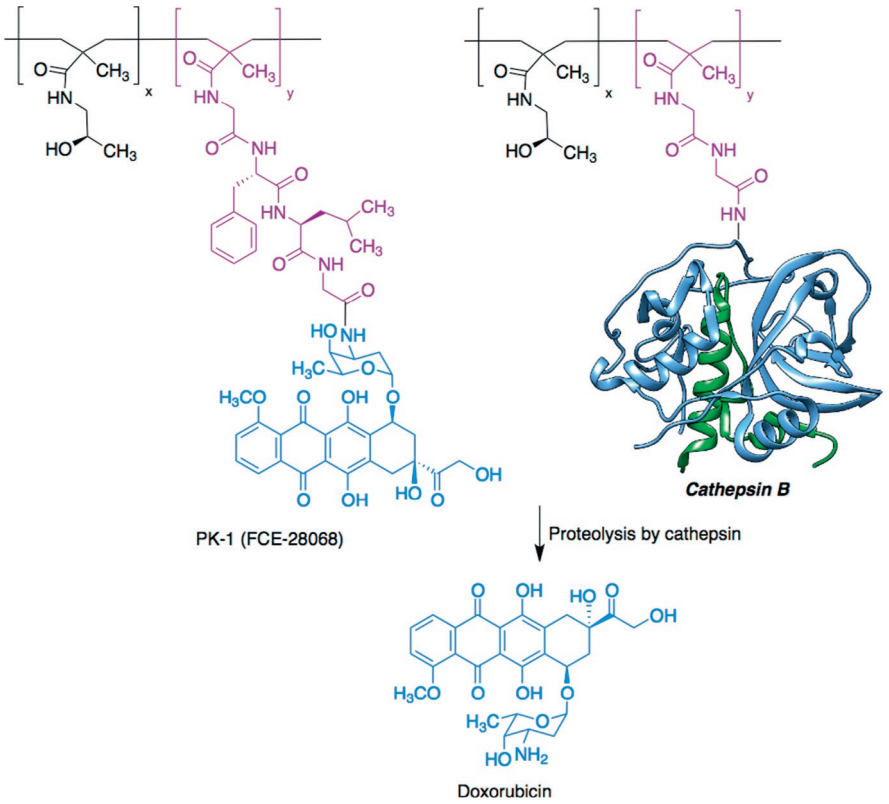


FIGURE 13.37

Targeting and liberation of doxorubicin by the PDEPT approach. The structure of cathepsin B was generated from Protein Data Bank reference 1K3B using Chimera 1.81.

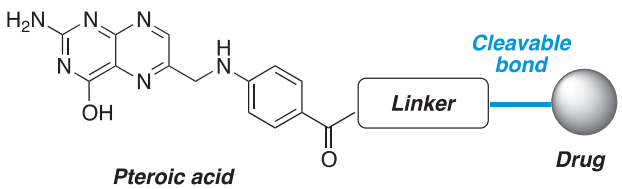
The PDEPT strategy has been claimed to have advantages over ADEPT and GDEPT because the polymer–enzyme conjugates have reduced immunogenicity and the polymeric prodrug has a relatively short residence time in plasma, which allows subsequent administration of polymer–enzyme without prodrug activation in the circulation.

6 FOLATE RECEPTOR-TARGETED CHEMOTHERAPY

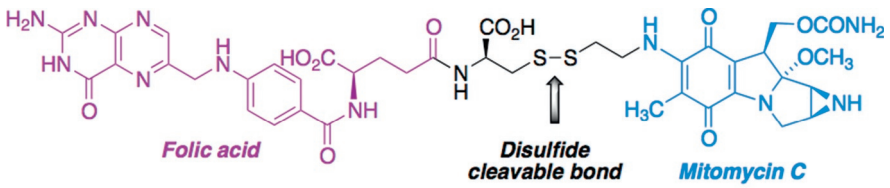
Folic acid has become a useful ligand for targeted cancer therapies because it binds to a tumor-associated antigen known as the folate receptor (FR). The human FR (FRα) is a glycophosphatidylinositol-anchored membrane glycoprotein that binds physiological folates and mediates their intracellular transport via receptor-mediated endocytosis. It is upregulated in many tumors and appears to increase as the cancer progresses. It is possible to link folic acid to drugs specifically for FR^+ tumor cells, in which these folate–drug conjugates are internalized after binding, with subsequent drug delivery.

Initial folate targeting studies were conducted with radiolabeled and fluorescent proteins covalently attached to folic acid, but subsequently this technique has been extended to conjugates of radiopharmaceutical agents, magnetic resonance imaging (MRI) contrast agents, low-molecular-weight chemotherapeutic agents, antisense oligonucleotides and ribozymes, proteins, liposomes with entrapped drugs, drug-loaded nanoparticles, and plasmids.[80]

A typical structure for a folate–drug conjugate contains pteroic acid (Pte), a linker to avoid the lower affinity of the conjugate for the FR that occurs when the drug is positioned too close to the Pte core, a cleavable bond that is very often a disulfide moiety, and, finally, the drug. The linker is usually hydrophilic and most often is a peptide that contains a glutamic acid residue attached to the pteroate portion, thus giving rise to a folic acid moiety. It may also be a polymer or a carbohydrate.



Many folate–drug conjugates have been preclinically studied. The structure of one of these drugs, the EC16–mitomycin C conjugate, is given here.



EC16-mitomycin

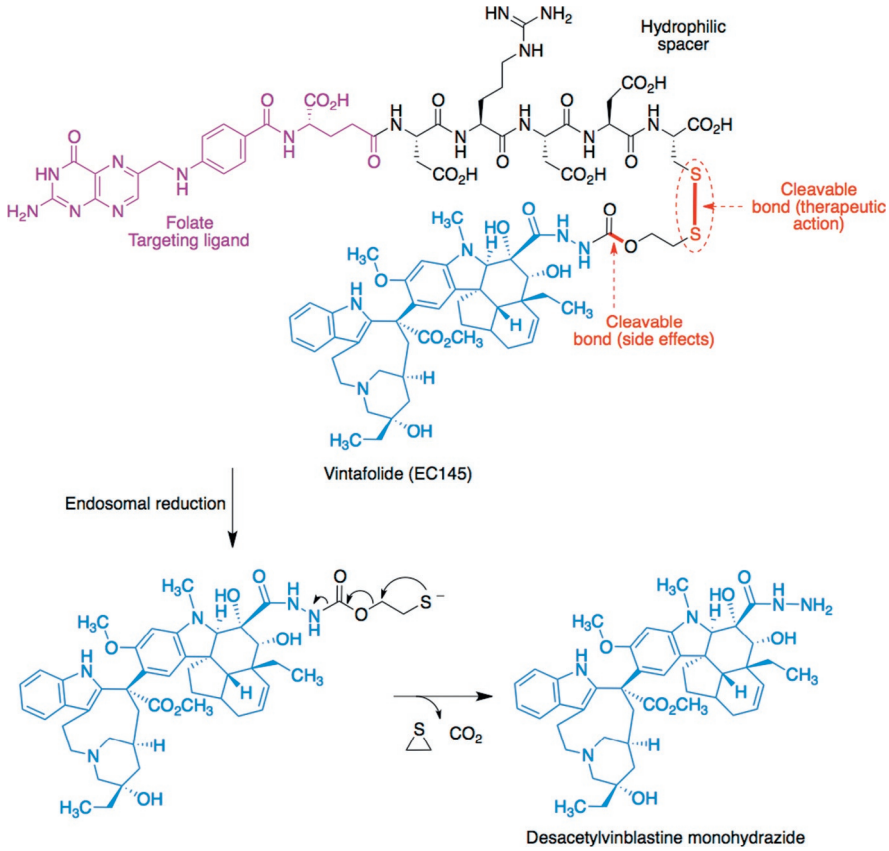
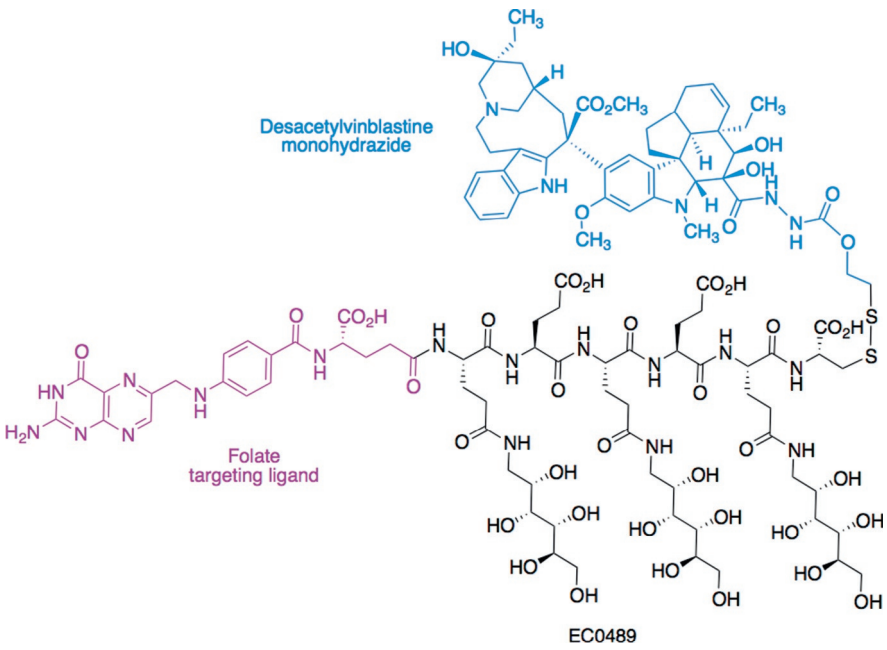


FIGURE 13.38

Structure and bioactivation of vintafolide.

Among folate–drug conjugates that have reached clinical trials vintafolide (EC-145) is a water-soluble, folate-targeted conjugate of a *Vinca* alkaloid developed as a treatment for patients with FR^+ cancers, such as platinum-resistant ovarian cancer.[81] The bioactivation of this compound is initiated by reduction of the disulfide bond between the cysteine units of the spacer and the linker, which takes place in the endosome formed in the endocytosis process through a mechanism that is not completely understood. The release of the active species occurs via a self-immolative process that transforms the linker into a molecule of thiirane (which is later hydrolyzed to 2-mercaptoathanol) and another of carbon dioxide (Figure 13.38).[82]

Constipation was identified as the dose-limiting toxicity of vintafolide during a phase I trial. The origin of this problem was the release of unconjugated vinca alkaloid to the bile following hepatic hydrolysis of the carbamate moiety that connects the alkaloid to the spacer, which led to the development of analogs with an increased hydrolytic stability (see the discussion of EC0489 below).



Vintafolide is being studied in association with etarfolatide (^{99m}Tc-EC20), a folate-targeted radiopharmaceutical imaging agent used to identify FR^+ cancers. This allows identifying tumors that overexpress this receptor without the need for a tissue biopsy and hence to select the patients who will be most likely to respond to folate-targeted therapy.[83] Etarfolatide, similarly to most ^{99m}Tc radiopharmaceuticals of diagnostic importance, needs to be prepared immediately before its administration from the chelating agent EC20 and radioactive ^{99m}Tc, together with $SnCl_2$ for the reduction of Tc^{7+} to a lower valency state, which facilitates its chelation (Figure 13.39). Etarfolatide has undergone a number of clinical trials[84] and is being developed together with vintafolide. This combination received orphan drug status in Europe in 2012.[85]

Constipation was identified as the dose-limiting toxicity of vintafolide during a phase I trial. Interestingly, the origin of this problem was identified to be the release of unconjugated vinca alkaloid to the bile following hepatic hydrolysis of the carbamate moiety that connects the alkaloid to the spacer, which led to the development of analogs with an increased hydrolytic stability such as EC0489 is another desacetyl vinblastine hydrazide-derived conjugated drug containing a folate structural fragment as a targeting unit. This compound was designed to have reduced hepatic clearance by placing carbohydrate spacers formed by 1-amino-1-deoxyglucitolyl-γ-glutamate units between the folate and the alkaloid moieties.[86] This new conjugate has entered phase I trials in patients with refractory or metastatic solid tumors who have exhausted standard therapeutic options.[87]

The folate–drug conjugate EC-0225 is unique in that it contains two active moieties (the vinca alkaloid and mitomycin C) and a single folate molecule. In preclinical studies in animals, results were similar to those of vintafolide with doses approximately threefold lower, which justified starting a phase I trial.

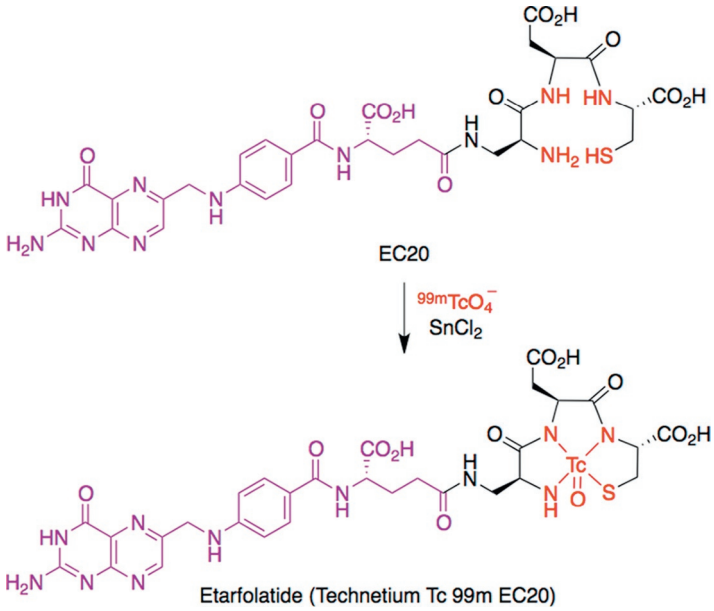
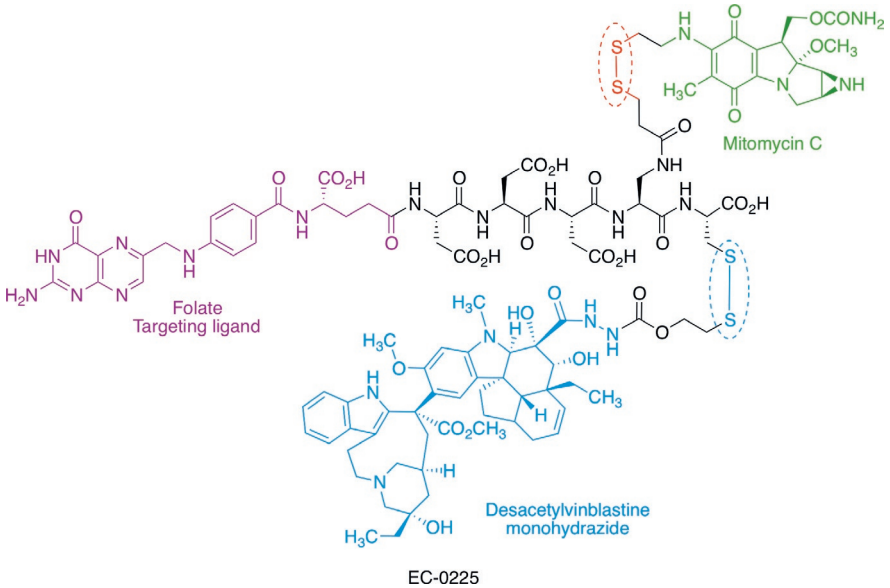
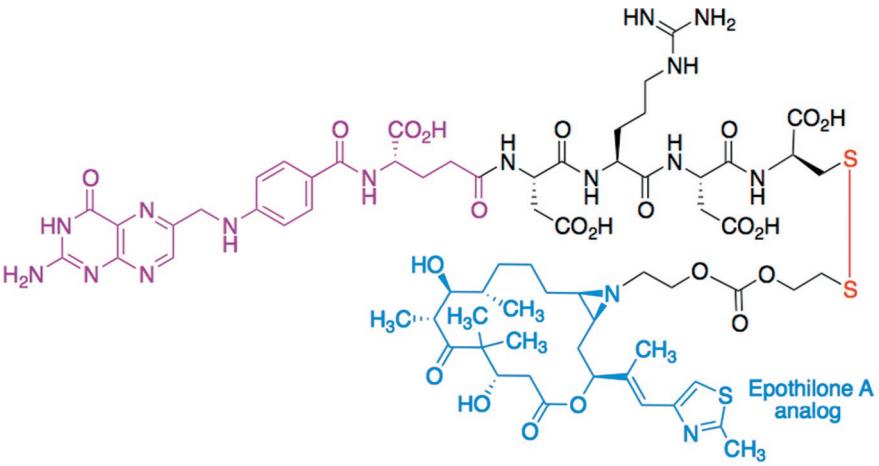


FIGURE 13.39

Structure of ^{99}Tc-etarfolatide.

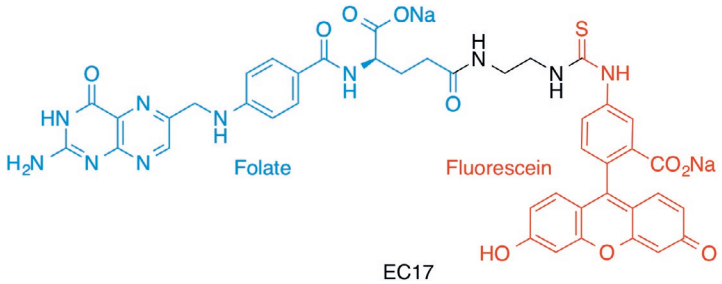


Epofolate (BMS753493) is a conjugate of a synthetic analog of epothilone A that is also in early clinical trials.[88]



Epofolate (BMS753493)

A new approach to cancer immunotherapy[89] aims to render the tumors more immunogenic. For example, the folate-targeted hapten EC-17 is a tumor-specific fluorescent agent that enables the surgeon to locate and remove a malignant tumor with FR-alpha expression and is prepared from folate and fluorescein isothiocyanate (FITC) through an ethylenediamine spacer, is under study as a potential treatment for metastatic renal and ovarian cancer.



EC17

The general strategy of the multistep process involved in this new approach can be summarized as follows (Figure 13.40):

1. The surface of FR^+ tumor cells is saturated with a folate–hapten conjugate (in this case, EC-17) against which the cancer-bearing host has a preexisting or induced immunity. Folate receptors are overexpressed on the surfaces of many cancer cells, including kidney and ovarian cancer cells.
2. Once bound to the cancer cell through the folate moiety of the conjugate, circulating anti-fluorescein antibodies may recognize and bind to the FITC moiety. When these tumor cells are saturated with millions of folate receptor-targeted haptens, they attract anti-hapten antibodies to the tumor cell surface.

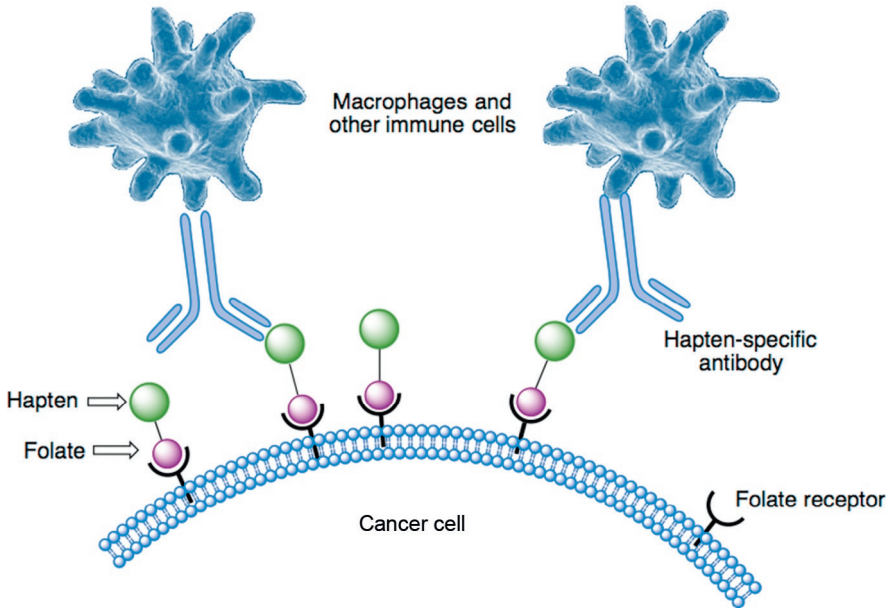


FIGURE 13.40

Cancer immunotherapy with a folate-targeted hapten.

3. The antibody-coated tumor cells are recognized by immune cells, such as natural killer cells, macrophages, neutrophils, and mast cells having Fc receptors on their surface. When these receptors are stimulated they induce an antitumor response against the anti-hapten antibody opsonized tumor cells, which leads to their destruction.

7 LIPOSOMES AND OTHER NANOPARTICLES IN ANTICANCER DRUG TARGETING

A critical advantage in treating cancer with non-solution-based therapies is the previously mentioned leaky vasculature inherently present in cancerous tissues due to their rapid vascularization. However, the injected nanoparticles are usually taken up by the liver, spleen, and other parts of the reticuloendothelial system (RES), depending on their surface characteristics and size. To obtain particles with longer circulation times, and hence greater ability to target to a given tumor, they should be 100 nm or less in diameter and have a hydrophylic surface in order to reduce clearance by macrophages. In addition, coating of hydrophilic polymers can originate a cloud of chains at their surface that will repel plasma proteins.[90]

Cancer-related nanotechnological devices of this type include liposomes for the therapy of different cancers,[91] nanosized MRI contrast agents for neuro-oncological interventions,[92] copolymer micelles, and novel nanoparticle-based methods for the high-specificity detection of DNA and proteins.[93]

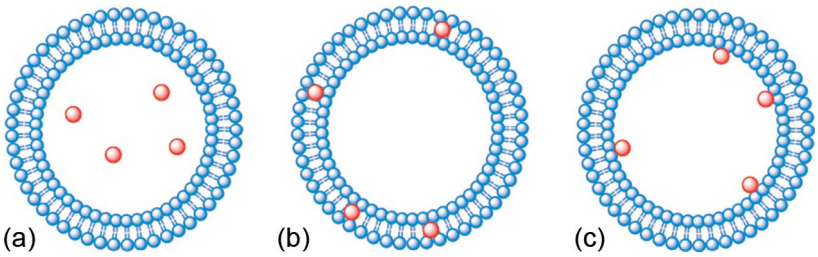


FIGURE 13.41

Possible locations of drugs in liposomes.

7.1 LIPOSOMES

Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules, usually phospholipids. Since the observation that phospholipids form closed bilayered structures in aqueous systems, liposomes have become quite common pharmaceutical carriers.[94] Hydrophilic drugs such as doxorubicin can be loaded into the internal aqueous compartment, whereas lipid-soluble drugs are usually carried into the lipid bilayer or in the interface between the lipid bilayer and the aqueous compartment (Figure 13.41). Cationic liposomes are made of positively charged lipids and are increasingly being researched for use in gene therapy due to their favorable interactions with negatively charged DNA and cell membranes.

The drug-loaded liposomes enter the tumor tissues through the previously mentioned EPR effect.[95] They can then be adsorbed or fused with the cell membrane and release their contents into the cytoplasm or use the endocytosis process. The antitumor effect of liposomal-encapsulated anticancer agents depends on the ability of the liposome to carry the anticancer agent to the tumor, its releasing into the extracellular fluid, and its penetration into the cell. Liposomal formulations modify the toxicity profile of a drug due to the alterations in tissue distribution. Thus, liposomal doxorubicin reduces the incidence and severity of cumulative dose-related cardiomyopathy while preserving antitumor activity of the conventional drug.[96] However, administration of a drug in a liposome may also result in new toxicities.

Standard liposomes may be attacked by the RES marker molecules soon after their injection, with premature release of the drug. To avoid the interaction with opsonizing proteins, liposomes may be coated with a protective polymer giving the so-called "stealth®" liposomes. The most common polymer used for this purpose is PEG, which provides a water-soluble coating. Current research on this topic is focused on attaching PEG to the liposomes in such a way that it can be removed in order to facilitate cell uptake under local pathologic conditions, especially the decreased pH commonly found in tumors. This can be achieved by linking the polymer to the phosphatidylethanolamine molecules that make up the liposome through an *N*-glutaryl spacer (Figure 13.42). The four ester bonds present in a phospholipid may all be subject to hydrolysis in water, but the carboxyl esters are hydrolyzed faster than the phosphate esters producing fatty acids and lysophospholipids.[97]

Non-PEGylated formulations include the following: DaunoXome® (liposomal daunorubicin),[98] approved in the United States and Europe to treat AIDS-related Kaposi's sarcoma; Myocet® and Lipo-Dox® (liposomal doxorubicin), used in combinational therapy for several cancers; DepoCyt® (liposomal cytarabine), used by intrathecal administration for treatment of lymphomatous and

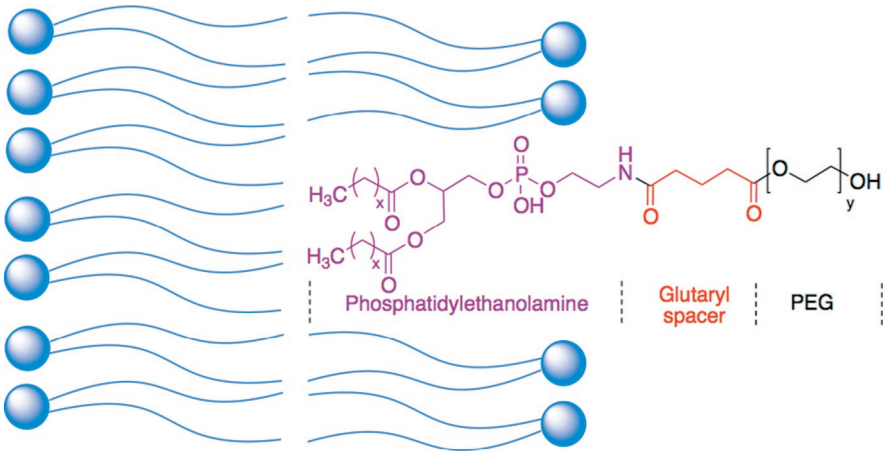


FIGURE 13.42

An example of a PEGylated bilayered phospholipid.

neoplastic meningitis; Marqibo® (liposomal vincristine), approved by the FDA in 2012 for the treatment of metastatic malignant uveal melanoma; EEP-ETU (liposomal paclitaxel), which is completing phase II clinical trials; and CPX-1 (liposomal formulation containing irinotecan and floxuridine), which is completing phase III clinical trials for ovarian, breast, and lung cancer.

Among the PEGylated formulations, Doxil® (Caelyx®) is a, PEG–liposomal doxorubicin employed for the treatment of Kaposi's sarcoma for more than 16 years and more recently approved for metastatic breast cancer and recurrent ovarian cancer. Doxil® produces mild myelosuppression, minimal hair loss, and a low risk of cardiotoxicity (Figure 13.43).[99] Thermodox® is another liposomal doxorubicin that releases its content upon heat. It is in phase III for treatment of hepatocellular carcinoma and recurrent chest

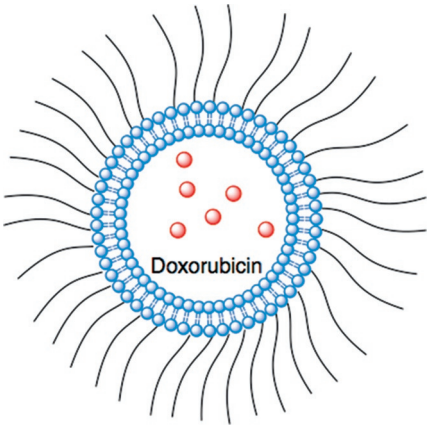
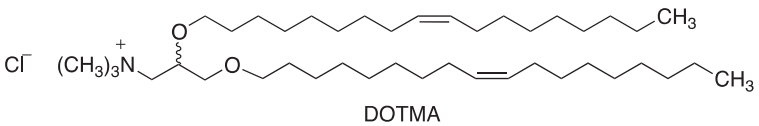


FIGURE 13.43

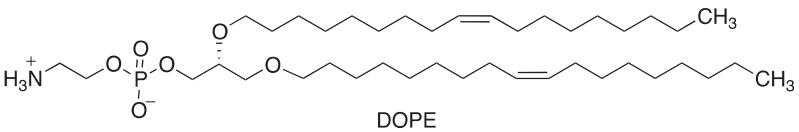
A doxorubicin-carrying PEGylated liposome (Doxil®).

wall breast cancers. Similarly, Lipoplatin® (liposomal cisplatin) is a PEGylated formulation used for treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder, and testicular cancer.

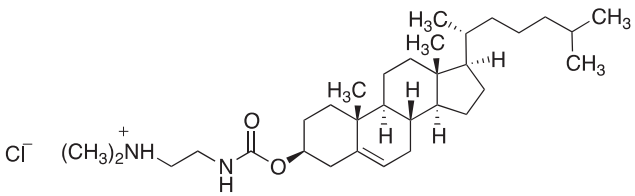
Cationic liposomes have been studied as non-viral vectors in gene therapy and are formed by positively charged amphiphilic molecules whose positive charges interact electrostatically with negative charges in DNA phosphate groups, forming complexes that are capable of entering the cells. Some of the positively charged amphiphilic molecules (cationic lipids) used to this purpose are DOTMA, DOPE and DC-Chol.



DOTMA



DOPE



DC-Chol

The inherent target selectivity of "stealth" liposomes, based on the preferential accumulation and leaking into the tumor vascular bed, can be dramatically enhanced by their chemical coupling to tumor-specific Abs, antibody fragments, or other targeting moieties—essentially any molecule that selectively recognizes and binds to target antigens or receptors overexpressed or selectively expressed on cancer cells. Vascular-targeted liposomes are based on the fact that endothelial cells in the angiogenic vessels within solid tumors express several proteins that are absent or barely detectable in established blood vessels. Preclinical studies have been conducted on numerous liposome-based agents actively targeted to tumor neovasculature. The targets for these formulations have included membrane type 1 matrix metalloproteinase, endoglin (CD105), vascular cell adhesion molecule-1 (CD106), epithelial cell adhesion molecule (CD326), $\alpha v\beta 3$ integrin (CD51/CD61), and aminopeptidase N (CD13). CD13 has become widely recognized as a rational target for therapeutic development,[100] and several NGR-conjugated agents are now in preclinical and clinical development. One example of this approach is the NGR–peptide-targeted liposomal doxorubicin (TVT-DOX), in which the linear peptide containing the asparagine–glycine–arginine motif (NGR) specifically binds to CD13-expressing cells (Figure 13.44).[101]

Antibody-targeted immunoliposomes bear the corresponding antibody covalently coupled either to the reactive phospholipids in the membrane or to the PEG hydroxy groups (Figure 13.45). Alternatively, they may be hydrophobically anchored into the liposomal membrane after being modified

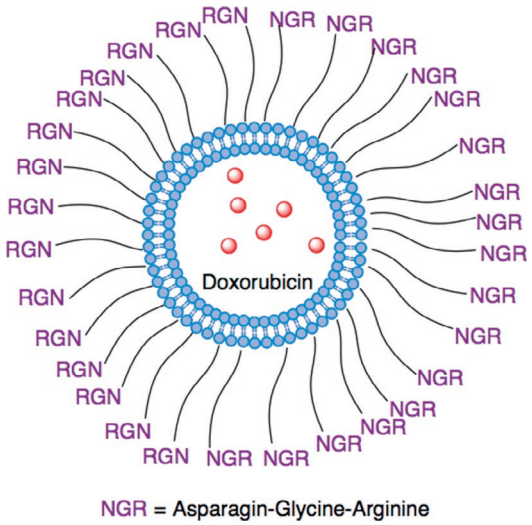


FIGURE 13.44

Structure of the NGR-peptide-targeted liposomal doxorubicin.

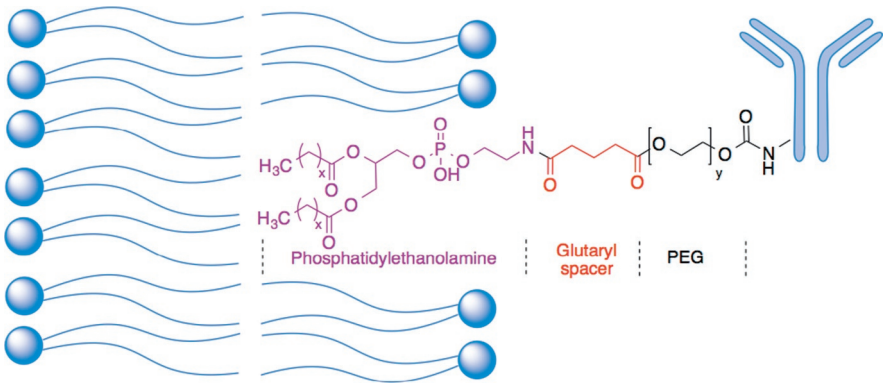


FIGURE 13.45

Antibody-containing immunoliposomes.

by attachment of a lipophilic moiety. The enhanced antitumor efficacy of these formulations has been clinically demonstrated, as in the case of the anti-EGFR ILs-dox, designed for patients with various solid tumors overexpressing the epidermal growth factor receptor. ILs-dox contains fragments of the mAb C225 (cetuximab) covalently linked to PEGylated liposomes loaded with doxorubicin.[102] MCC-465 is other PEGylated immunoliposome-encapsulated doxorubicin that has entered clinical trials. It is tagged with the $F(ab')_2$ fragment of the human mAb GAH, which interacts with more than 90% of cancerous stomach tissues but not with all normal tissues. Although this product does not appear to have progressed through development after a phase I clinical trial, it demonstrated superior cytotoxic activity against several human stomach cancer cells.[103]

Polymersomes (polymer-based liposomes) are attracting much attention as nanoparticle drug carriers because their vesicle membranes are formed by amphiphilic synthetic diblock copolymers and may be tailored to respond to a desired stimulus.[104] For instance, polymersomes that encapsulate doxorubicin and superparamagnetic iron oxide (γ-Fe_2O_3) deliver this anticancer drug upon local application of a high-frequency magnetic field, probably through induction of a hyperthermia effect at the level of the polymersome membrane.[105]

7.2 COPOLYMER MICELLES

Copolymer micelles are amphiphilic macromolecules with distinct hydrophobic and hydrophilic block domains. Within each copolymer system, aqueous exposure induces the hydrophobic and hydrophilic segments to phase separate, forming nanoscopic supramolecular core/shell structures. L-Lactide and poly(ethylene glycol) are frequently used to generate the hydrophobic core and the hydrophilic shell segments, respectively (Figure 13.46). The drug may be covalently linked to the amphiphilic segments or physically entrapped into the micelle.

Among representative studies on polymeric drug carriers responding to physical or biological signals for effective cytosolic drug delivery is the acidic pH-activating system folate–PEG-poly(aspartate hydrazone doxorubicin) [folate-PEG-(PAsp-Hyd-DOX)] (Figure 13.47), in which a multifunctionalized block copolymer contains a folate ligand and a hydrazone bond between the drug and the polymer. This bond is intracellularly cleaved at low pH, resulting in drug release.[106]

Copolymer micelles are rapidly becoming powerful nanomedicine platforms for cancer therapeutic applications due to their small size (10–100 nm), *in vivo* stability, ability to solubilize water-insoluble

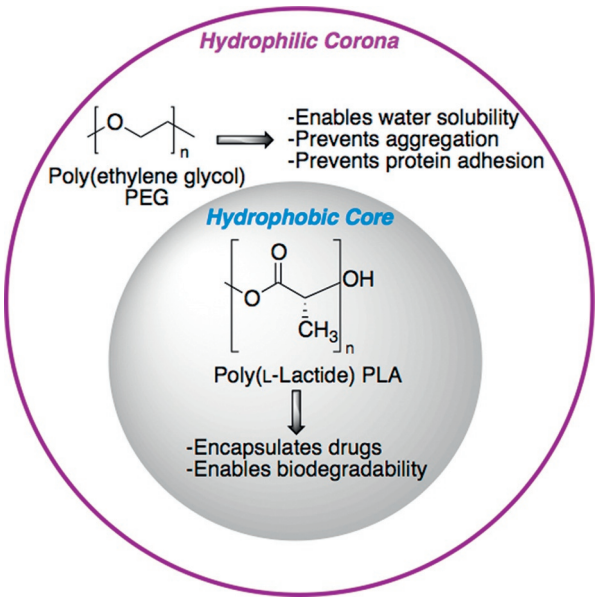


FIGURE 13.46

Structure of a L-lactide-poly(ethylene glycol) copolymer micelle.

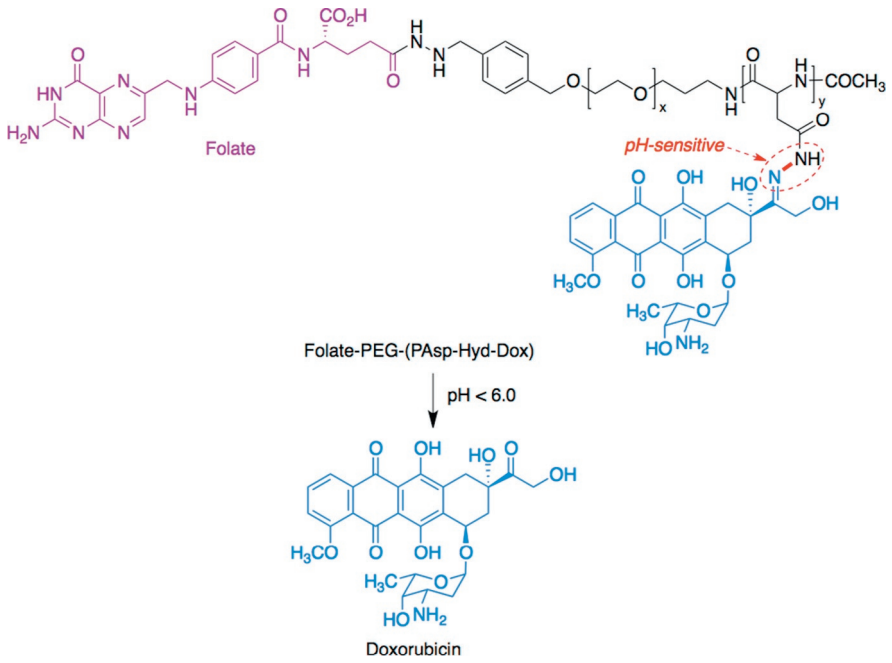


FIGURE 13.47

Structure and activation of the folate–PEG–poly(aspartate hydrazone doxorubicin) copolymer.

anticancer drugs, and prolonged blood circulation times. The drug is protected from enzymatic degradation by the micelle shell, and its biodistribution is mainly determined by the micelles surface properties, size, and stability. Furthermore, along with passive targeting, the delivery of micellar drugs to tumors can potentially be enhanced by the modification of the surface of the polymer micelles with targeting molecules.

Some micellar formulations for anticancer therapy are under clinical evaluation, among which Genexol-PM® has been approved for use in patients with breast cancer.[107] Genexol-PM® is a biodegradable paclitaxel-encapsulated poly(ethylene glycol)-poly(D,L-lactide) copolymer micellar formulation that is in phase III clinical studies for NSCLC. The copolymer increases the water solubility of paclitaxel and allows delivery of higher doses than those achievable with paclitaxel alone.

NK105 is another paclitaxel-incorporating micellar nanoparticle, which is in phase II study for second-line treatment of advanced or recurrent gastric cancer. PEG is the hydrophilic segment, and modified polyaspartate is the hydrophobic segment. Carboxylic groups of the polyaspartate block were modified by esterification with 4-phenyl-1-butanol, and paclitaxel was incorporated into the inner lipophilic core of the micelle system by physical entrapment through hydrophobic interactions (Figure 13.48).

Nanoplatin® (NC-6004) is a cisplatin-incorporated micelle that is in phase II clinical trials in patients with advanced solid tumors. In this case, the drug is incorporated into the micelle by coordination to carboxylic groups in the polyglutamic acid core (Figure 13.49). This formulation has a lower toxicity and a higher antitumor activity than cisplatin.[108]

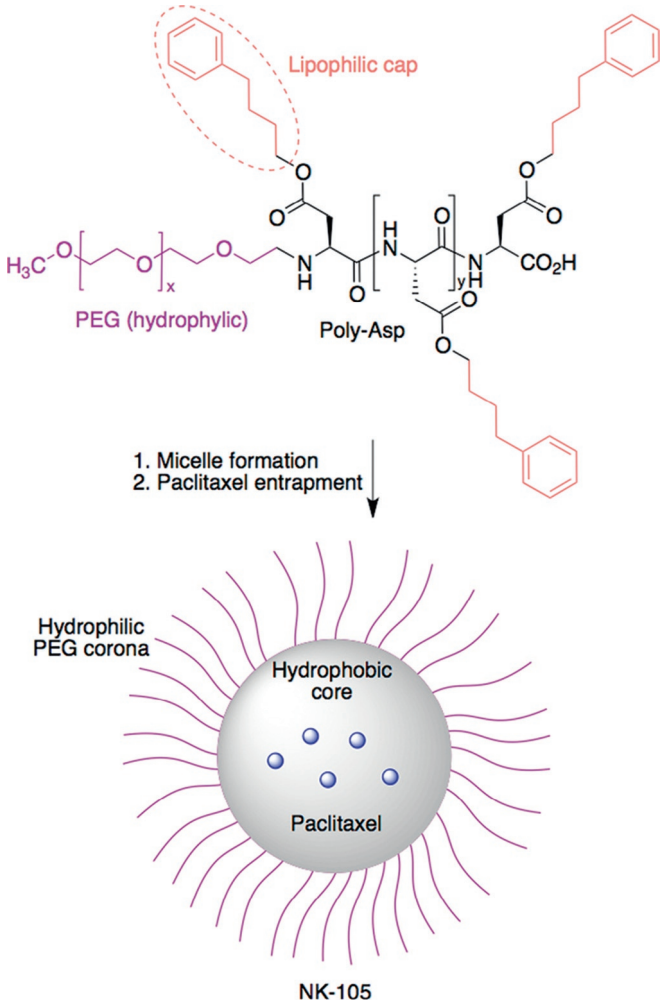


FIGURE 13.48

Structure of NK105, a paclitaxel-incorporating micellar nanoparticle.

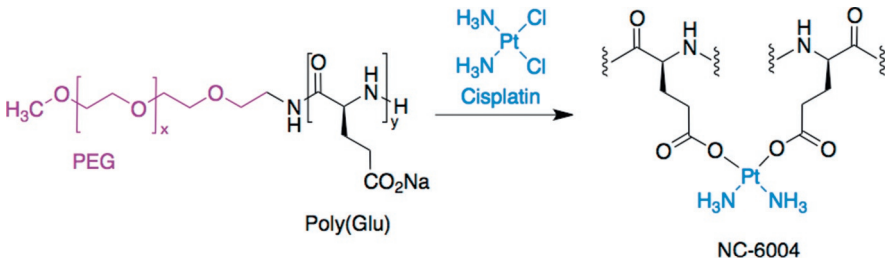


FIGURE 13.49

Structure of nanoplatin (NC-6004), a cisplatin-transporting micelle.

TNP-470 is an antiangiogenic synthetic analog of fumagillin whose development was suspended in the 1990s, despite its efficacy for a wide range of cancers, mainly because of neurologic side effects and also because the relative insolubility of TNP-470 precluded its oral administration. To prevent TNP-470 from crossing the blood–brain barrier, it was reformulated as the conjugate caplostatin (see Section 4.2), but the drug still could not be effectively administered and required continuous intravenous infusions. These problems were solved with the development of the orally active, nontoxic micellar formulation lodamin, which can be chronically administered for cancer therapy or metastasis prevention.[109] It is prepared by conjugating TNP-470 to a copolymer formed by monomethoxy polyethylene glycol/polylactic acid. Because this copolymeric form is amphiphilic, in an aqueous medium it self-assembles into micelles where TNP-470 is protected within the core. Orally administered lodamin enters rapidly into circulation and maintains higher levels in serum relative to free TNP-470.

7.3 GOLD NANOPARTICLES

Due to their unique optical and electronic properties, gold nanoparticles have found biomedical applications in specific aspects of diagnosis and treatment of cancer,[110] acting by several mechanisms.

Gold nanoparticles accumulate at tumor sites due to the EPR effect, and the high density of atoms on their surfaces greatly enhances their receptor binding affinity. One of these nanomedicines that has entered clinical trials is CYT-6091, a PEGylated colloidal gold–rhTNF used as a platform for the delivery of tumor necrosis factor R (TNFR) to solid tumors.[111]

Gold nanoparticles are also being developed for the treatment of hormone-dependent malignancies such as breast and prostate cancers in which estrogen and androgen receptors can serve as targets for tissue-selective drug delivery. For instance, tamoxifen is selectively delivered from PEGylated gold nanoparticles to breast cancer cells at concentrations more than 10,000-fold higher than the drug alone (Figure 13.50).[112]

Active targeting significantly improved the cellular accumulation in the target cells of gold nanoparticles.[113] Those conjugated with cetuximab were quickly internalized by pancreatic adenocarcinoma and colorectal adenocarcinoma cancer cells overexpressing EGFR.[114] Cetuximab was conjugated via a covalent hydrazide–thiol heterobifunctional linker by oxidizing with $NaIO_4$ the

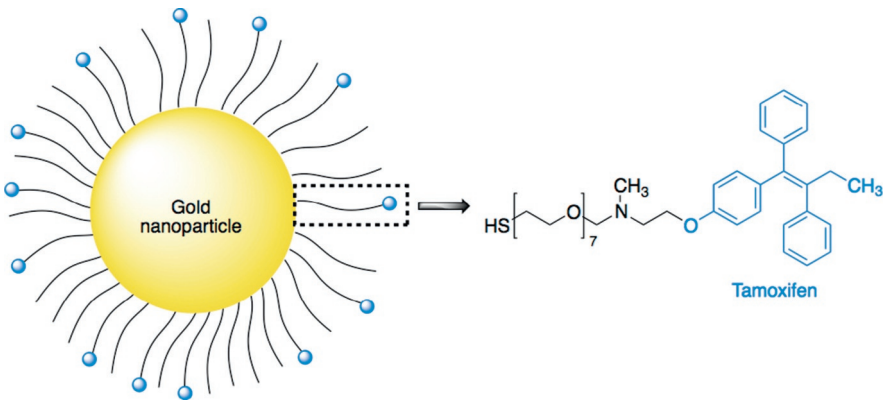


FIGURE 13.50

A gold nanoparticle for the delivery of tamoxifen.

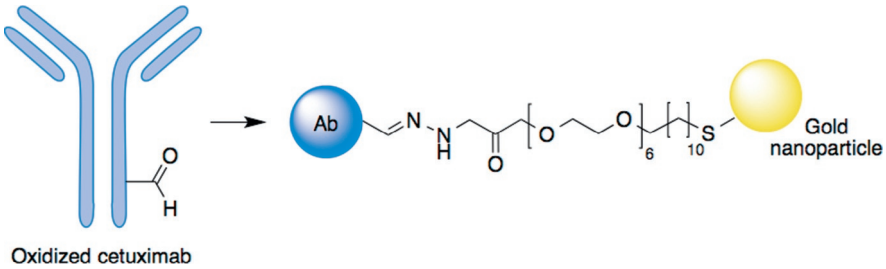


FIGURE 13.51

An antibody-targeted gold nanoparticle.

carbohydrate moieties to aldehyde groups on the Fc region of the antibody. It was then allowed to covalently bind to the linker by formation of an hydrazine. Finally, this Ab-linker species was attached to the gold nanoparticles via a thiol–gold binding reaction (Figure 13.51).

Mild hyperthermic cancer treatments have been in clinical use since the early 1980s because normal tissues tolerate hyperthermia at higher temperatures and for longer periods of time than do malignant tissues. In 2008, it was shown that gold nanorods (single-walled carbon nanotubes) could be used in near-infrared (NIR) laser photothermal therapy to achieve selective tumor cell ablation and resorption/remission *in vivo*. NIR-absorbing PEGylated gold nanorods were systemically or intratumorally administered in mice bearing head and neck tumor models (squamous cell carcinoma), and the subsequent exposure to near-infrared lasers of the nanoparticle-loaded tumors for just 10 minutes resulted in temperature increases of more than 20°C, with minimal damage to surrounding tissues.

7.4 DENDRIMERS AS CARRIERS FOR THE DELIVERY OF CHEMOTHERAPEUTIC AGENTS

Dendrimers are a family of nanosized, three-dimensional polymers characterized by a unique treelike branching architecture and compact spherical geometry in solution. Their development started in the 1970s, but the first family of hyperbranched polymers was developed in 1984. They are sought for a variety of applications in chemistry, biology, and medicine, being particularly interesting for the delivery of anticancer drugs and imaging agents. However, their translation into cancer therapies is lagging due to the difficulty of synthesizing the proposed systems in large quantities at clinical-grade purity and to the regulatory hurdles that demand detailed characterization of the polymeric carriers, the linkages, and the incorporated drug.[115]

7.5 NANOPARTICLE ALBUMIN-BOUND TECHNOLOGY

In addition to angiogenesis, tumors have adapted other mechanisms to meet their increased need for nutrients. One of them is the gp60 pathway, by which nutrients are preferentially transported across the endothelial barrier when attached to albumin. They also secrete into the tumor's interstitium the glycosylated 43-kDa specialized protein called SPARC (secreted protein, acidic and rich in cysteine), also known as osteonectin and as BM-40, that modulates the interaction between cell and extracellular

matrix, being a key regulator of critical cellular functions such as proliferation, survival, and cell migration (see Chapter 11, Section 8.2 and Section 7 of Chapter 14).[116] SPARC, a highly charged receptor, specifically attracts and binds albumin and albumin-bound nutrients to concentrate them within the tumor's interstitium, thereby avoiding their diffusion outside the tumor cell.[117] These mechanisms have served as the basis for the development of a nanoparticle albumin-bound technology to transport and deliver drugs into tumors instead of nutrients. Albumin-bound paclitaxel (ABI-007, Abraxane®) represents one of the strategies adopted to overcome the solvent-related problems of taxanes (other surfactant-free formulations include taxane analogs, prodrugs, polyglutamates, and liposomes). The nanoparticle colloidal suspension of Abraxane® is prepared by high-pressure homogenization of paclitaxel in the presence of human serum albumin at a concentration of 3.4%, similar to that of albumin in the blood. This formulation was approved by the FDA in 2005 and by the EMA in 2008 for the treatment of metastatic breast cancer,[118,119] having also been evaluated for NSCLC, ovarian cancer, melanoma, and cervical cancer.

REFERENCES

1 For reviews, see. (a) Rooseboom M, Commandeur JNM, Vermeulen NPE. *Pharmacol Rev* 2004;**56**:53; (b) Kratz F, Müller IA, Ryppa C, Warnecke A. *ChemMedChem* 2008;**3**:20.
2 Tew KD. *Expert Opin Invest Drugs* 2005;**14**:1047.
3 Denmeade SR, Mhaka AM, Rosen DM, Brennen WN, Dalrymple S, Dach I, et al. *Sci Transl Med* 2012;**4**:140ra86.
4 Brown JM, Wilson WR. *Nature Rev Cancer* 2004;**4**:437.
5 For reviews, see; (a) Naylor MA, Thomson P. *Mini Rev Med Chem* 2001;**1**:17; (b) Ahn G-O, Brown M. *Front Biosci* 2007;**12**:3483; (c) Wouters A, Pauwels B, Lardon F, Vermorken JB. *Oncologist* 2007;**12**:690; (d) Tanabe K, Zhang Z, Ito T, Hattaa H, Nishimoto S-I. *Org Biomol Chem* 2007;**5**:3745.
6 Patterson LH. *Cancer Metastasis Rev* 1993;**12**:119.
7 http://www.clinicaltrials.gov/ct/show/NCT00394628.
8 Patterson LH. *Drug Metab Rev* 2002;**34**:581.
9 Patterson LH, Murray GI. *Curr Pharm Des* 2002;**8**:1335.
10 Antonini I, Lin TS, Cosby LA, Dai YR, Sartorelli AC. *J Med Chem* 1982;**25**:730.
11 Hatzigrigoriou E, Papadopoulou MV, Shields D, Bloomer WD. *Oncol Res* 1993;**5**:29.
12 De Oliveira RB, Alves RJ. *Quim Nova* 2002;**25**:976.
13 Palmer BD, van Zijl P, Denny WA, Wilson WR. *J Med Chem* 1995;**38**:1229.
14 Patterson AV, Ferry DM, Edmunds SJ, Gu Y, Singleton RS, Patel K, et al. *Clin Cancer Res* 2007;**13**:3922.
15 McKeage MJ, Gu Y, Wilson WR, Hill A, Amies K, Melink TJ, et al. *BMC Cancer* 2011;**11**:432.
16 Ware DC, Palmer BD, Wilson WR, Denny WA. *J Med Chem* 1993;**36**:1839.
17 Kriste AG, Tercel M, Anderson RF, Ferry DM, Wilson WR. *Radiat Res* 2002;**158**:753.
18 Shibamoto Y, Ling Z, Hatta H, Mori M, Nishimoto S-I. *Jpn J Cancer Res* 2000;**91**:433.
19 For a review of radiation-induced activation of 5-fluorouracil prodrugs, see Ito T, Tanabe K, Yamada H, Hatta H, Nishimoto S. *Molecules* 2008;**13**:2370.
20 Denny WA, Wilson WR, Tercel M, Van Zijl P, Pullen SM. *Int J Radiat Oncol Biol Phys* 1994;**29**:317.
21 Shibamoto Y, Zhou L, Hatta H, Mori M, Nishimoto S. *Int J Radiat Oncol Biol Phys* 2001;**49**:407.
22 Aghi M, Hochberg F, Breakfield XO. *J Gene Med* 2000;**2**:148.
23 Russell SJ, Peng K-W. *Trends Pharmacol Sci* 2007;**28**:326.
24 Chung-Faye G, Palmer D, Anderson D, Clark J, Downes M, Baddeley J, et al. *Clin Cancer Res* 2001;**7**:2662.
25 Knox RJ, Friedlos F, Jaman M, Roberts JJ. *Biochem Pharmacol* 1988;**37**:4661.

26 For a review, see Kerr SH, Kerr DJ. *Cancer Lett* 2009;**286**:114.
27 McCarthy HO, Yakkundi A, McErlane V, Hughes CM, Keilty G, Murray M, et al. *Cancer Gene Ther* 2003;**10**:40.
28 Cowen RL, Williams KJ, Chinje EC, Jaffar M, Sheppard FCD, Telfer BA, et al. *J Cancer Res* 2004;**64**:1396.
29 (a) Carrió M, Visa J, Cascante A, Estivill X, Fillat C. *J Gene Med* 2002;**4**:141; (b) Huch M, Abate-Daga D, Roig JM, González JR, Fabregat J, Sosnowski B, et al. *Hum Gene Ther* 2006;**17**:1187.
30 Tyminski E, Leroy S, Terada K, Finkelstein DM, Hyatt JL, Danks MK, et al. *Cancer Res* 2005;**65**:6850.
31 Houba PHJ, Boven E, van der Meulen-Muileman IH, Leenders LGG, Scheeren JW, Pinedo HM, et al. *Int J Cancer* 2001;**91**:550.
32 Springer CJ, Nicolescu-Duvaz I. *J Clin Invest* 2000;**105**:1161.
33 Mauger AB, Burke PJ, Somani HF, Friedlos F, Knox RJ. *J Med Chem* 1994;**37**:3452.
34 Basgshawe KD, Begent RH. *J Adv Drug Deliv Rev* 1996;**22**:365.
35 Francis RJ, Sharma SK, Springer C, Green AJ, Hope-Stone LD, Sena L, et al. *Br J Cancer* 2002;**87**:600.
36 Vasir JK, Reddy MK, Labhasetwar VD. *Curr Nanosci* 2005;**1**:47.
37 Jaracz S, Chen J, Kuznetsova LV, Ojima I. *Bioorg Med Chem* 2005;**13**:5043.
38 Kratz F, Warnecke A, Schmid B, Chung D-E, Gitzel M. *Curr Med Chem* 2006;**13**:477.
39 Jain RK. *Cancer Metastasis Rev* 1987;**6**:559.
40 Vicent MJ, Duncan R. *Trends Biotechnol* 2006;**24**:39.
41 (a) Delgado C, Francis GE, Fischer D. *Crit Rev Ther Drug Carrier Syst* 1992;**9**:249; (b) For a review, see Kimchi-Sarfaty C, Schiller T, Hamasaki-Katagiri N, Khan MA, Yanover C, Sauna ZE. *Trends Pharmacol Sci* 2013;**13**:534.
42 Graham ML. *Adv Drug Deliv Rev* 2003;**55**:1293.
43 Delman KA, Brown TD, Thomas M, Ensor CM, Holtsberg FW, Bomalaski JS, et al. *Proc Am Soc Clin Oncol* 2005, abstr 4139.
44 (a) Unger C, Müller C, Jäger E, Bausch M, Roberts J, Al-Batran S, et al. *Proc Am Soc Clin Oncol* 2005, abstr 3130; (b) Mueller C, Al-Batran S, Jaeger E, Schmidt B, Bausch M, Unger C, et al. *J Clin Oncol* 2008;**26**:15S,2533.
45 Campos LT, Folbe M, Meza L, Charu V, Dansey R, Xie F. *Proc Am Soc Clin Oncol* 2005, abstr 8115.
46 Greenwald RB, Conover CD, Choe YH. *Crit Rev Ther Drug Carrier Syst* 2003;**55**:217.
47 Scott LC, Yao JC, Benson AB, Thomas AL, Falk S, Mena RR, et al. *Cancer Chemother Pharmacol* 2009;**63**:363.
48 (a) Kurzrock R, Goel S, Wheler J, Hong D, Fu S, Rezai K, et al. *Cancer* 2012;**118**:6144; (b) For a review of PEG–prodrug conjugates, see Banerjee SS, Aher N, Patil R, Khandare JJ. *Drug Deliv* 2012;103973.
49 Vicent MJ, Duncan R. *Trends Biotechnol* 2006;**24**:39.
50 Vasey PA, Twelves C, Kaye SB, Wilson P, Morrison R, Duncan R, et al. *Clin Cancer Res* 1999;**5**:83.
51 Seymour LW, Ferry DR, Anderson D, Hesslewood S, Julyan PJ, Poyner R, et al. *J Clin Oncol* 2002;**20**:1668.
52 (a) Meerum Terwogt JM, Bokkel Huinink WW, Schellens JHM, Schot M, Mandjes IAM, Zurlo MG, et al. *Anticancer Drugs* 2001;**12**:315; (b) For a review of paclitaxel nanoparticle formulations in clinical trials, see Ma P, Mumper RJ. *J Nanomed Nanotechnol* 2013;**4**:1000164.
53 Caiolfa VR, Zamal M, Fiorini A, Frigerio E, D'Argy R, Ghigleri A, et al. *J Controlled Release* 2000;**65**:105.
54 For a review of targeting and delivery of platinum-based anticancer drugs, seeWang X, Guo Z. *Chem Soc Rev* 2013;**42**:202.
55 Gianasi E, Wasil M, Evagorou EG, Keddle A, Wislon G, Duncan R. *Eur J Cancer* 1999;**3**:994.
56 Rademaker-Lakhai JM, Terret C, Howell SB, Baud CM, de Boer RF, Pluim D, et al. *Clin Cancer Res* 2004;**10**:3386.
57 Sood P, Thurmond KB, Jacob JE, Waller LK, Silva GO, Stewart DR, et al. *Bioconjugate Chem* 2006;**17**:1270.
58 (a) Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, et al. *Nature Med* 2004;**10**:255; (b) Satchi-Fainaro R, Mamluk R, Wang L, Short SM, Nagy JA, Feng D, et al. *Cancer Cell* 2005;**7**:251.

59 Homsi J, Simon GR, Garrett CR, Springett G, De Conti R, Chiappori AA, et al. *Clin Cancer Res* 2007;**13**:5855.
60 Shaffer SA, Baker Lee C, Kumar A, Singer JW. *Eur J Cancer* 2002;**38**:S129.
61 Singer JW, Shaffer S, Baker B, Bernareggi A, Stromatt S, Nienstedt D, et al. *Anticancer Drugs* 2005;**16**:243.
62 Papisov MI, Hiller A, Yurkovetskiy A, Yin M, Barzana M, Hillier S, et al. *Biomacromolecules* 2005;**6**:2659.
63 Yurkovetskiy AV, Fram RJ. *Adv Drug Deliv Rev* 2009;**61**:1193.
64 Walsh MD, Hanna SK, Sen J, Rawal S, Cabral CB, Yurkovetskiy AV, et al. *Clin Cancer Res* 2012;**18**:2591.
65 (a) Akullian LC, Stevenson CA, Yurkovetskiy AV, Benson JD, Fram RJ, Lowinger TB. *Proc Am Assoc Cancer Res* 2009, abstr 670; (b) Bendell JC, Shapiro G, Sausville EA, Fields Jones S, Hilton JF, Shkolny D, et al. *J Clin Oncol* 2014, 32:abstr 2526.
66 Langer M, Kratz F, Rothen-Rutishauser B, Wunderli-Allenspach H, Beck-Sickinger AG. *J Med Chem* 2001;**44**:1341.
67 For a review, see Senter PD. *Curr Opin Chem Biol* 2009;**13**:1.
68 Walker MA, King HD, Dalterio RA, Trail PA, Firestone RA, Dubowchik GM. *Bioorg Med Chem Lett* 2004;**14**:4323.
69 Larson RA, Sievers EL, Stadtmauer EA, Löwenberg B, Estey EH, Dombret H, et al. *Cancer* 2005;**104**:1442.
70 For reviews of brentuximab vedotin, see. (a) Copeland A. *Drugs Future* 2010;**35**:797; (b) Ansell SM. *Expert Opin Invest Drugs* 2011;**20**:99; (c) Haddley K. *Drugs Today* 2012;**48**:259; (d) Newland AM, Li JX, Wasco LE, Aziz MT, Lowe DK. *Pharmacotherapy* 2013;**33**:93
71 Zhou LT, Liu FY, Li Y, Peng YM, Liu YH, Li J. *Neoplasma* 2012;**59**:1.
72 Doronina SO, Toki BE, Torgov MY, Mendelsohn BA, Cerveny CG, Chace DF, et al. *Nature Biotechnol* 2003;**21**:778.
73 Beeram M, Krop I, Modi S, Tolcher A, Rabbee N, Girish S, et al. *J Clin Oncol* 2007;**25**:18S(abstr 1042).
74 Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga S, et al. *N Engl J Med* 2012;**367**:1783.
75 Milenic DE, Brady ED, Brechwiel MW. *Nature Rev Drug Discov* 2004;**3**:488.
76 For a general review of the design of targeting ligands in medicinal chemistry, see Storr T, Thompson KH, Orvig C. *Chem Soc Rev* 2006;**35**:534.
77 Hedge S, Carter J. *Annu Rep Med Chem* 2004;**39**:337.
78 Gustafsson AME, Bäck T, Elgqvist J, Jacobsson L, Hultborn R, Albertsson P, et al. *Nucl Med Biol* 2012;**39**:15.
79 Satchi R, Duncan R. *Br J Cancer* 2001;**85**:1070.
80 Leamon CP, Reddy JA. *Adv Drug Deliv Rev* 2004;**56**:1127.
81 Luyckx M, Votino R, Squifflet J-L, Baurain J-F. *Int J Womens Health* 2014;**6**:351.
82 Yang J, Chen H, Vlahov IR, Cheng J-X, Low PS. *Proc Natl Acad Sci U S A* 2006;**103**:13872.
83 Reddy JA, Xu L-C, Parker N, Vetzel M, Leamon ChP. *J Nucl Med* 2004;**45**:857.
84 http://clinicaltrialsfeeds.org/clinical-trials/results/term=Technetium+Tc+99m+EC20.
85 For a review of the use of vintafolide for the treatment of folate receptor-α-positive platinum-resistant ovarian cancer, see Ambrosio AJ, Suzin D, Palmer EL, Penson RT. *Expert Rev Clin Pharmacol* 2014;**7**:443.
86 Leamon CP, Reddy JA, Klein PJ, Vlahov IR, Dorton R, Bloomfield A, et al. *J Pharmacol Exp Ther* 2011;**336**:336.
87 Harb WA, Conley BA, LoRusso P, Sausville EA, Heath EI, Chandana SR, et al. *J Clin Oncol* 2010;**28**(Suppl. 3088), meeting abstracts.
88 Peethambaram PP, Hartmann LC, Goss GD, Jonker DJ. Plummer R. *J Clin Oncol* 2010;**28**(15 Suppl.):e13005.
89 Lu Y, Sega E, Leamon CP, Low PS. *Adv Drug Deliv Rev* 2004;**56**:1161.
90 Brannon-Peppas L, Blanchette JO. *Adv Drug Deliv Rev* 2004;**56**:1649.
91 Park JW. *Breast Cancer Res* 2002;**4**:95.
92 Kircher MF, Mahmood U, King RS, Weissleder R, Josephson L. *Cancer Res* 2003;**63**:8122.

93 Ferrari M. *Nature Rev Cancer* 2005;**5**:161.
94 Torchilin VP. *Nature Rev Drug Discov* 2005;**4**:145.
95 Greish K. *J Drug Target* 2007;**15**:457.
96 Huwyler J, Drewe J, Krahenbuhl S. *Int J Nanomed* 2008;**3**:21.
97 Zamboni WC. *Clin Cancer Res* 2005;**11**:8230.
98 Forssen EA. *Adv Drug Deliv Rev* 1997;**24**:133.
99 O'Shaughnessy JA. *Clin Breast Cancer* 2003;**4**:318.
100 Chen Y, Wu JJ, Huang L. *Mol Ther* 2010;**18**:828.
101 Garde SV, Forte AJ, Ge M, Lepekhin EA, Panchal CJ, Rabbani SA, et al. *Anticancer Drugs* 2007;**18**:1189.
102 Elbayoumi TA, Torchilin VP. *Clin Cancer Res* 2009;**15**:1973.
103 Matsumura Y, Gotoh M, Muro K, Yamada Y, Shirao K, Shimada Y, et al. *Ann Oncol* 2004;**15**:517.
104 For a review, see Venkatraman SS, Ma LL, Natarajan JV, Chattopadhyay S. *Front Biosci (Schol Ed)* 2010;**2**:801.
105 Oliveira H, Pérez-Andrés E, Thevenot J, Sandre O, Berra E, Leccommandoux S. *J Control Release* 2013;**169**:165.
106 Shim WS, Kim JH, Kim K, Kim YS, Park RW, Kim IS, et al. *Int J Pharm* 2007;**331**:11.
107 Yang D, Yu L, Van S. *Cancers* 2011;**3**:17.
108 Plummer R, Wilson RH, Calvert H, Boddy AV, Griffin M, Sludden J, et al. *Br J Cancer* 2011;**104**:593.
109 Benny O, Fainaru O, Adini A, Cassiola F, Bazinet L, Adini I, et al. *Nature Biotechnol* 2008;**26**:799.
110 Dreaden EC, Alkilany AM, Huang X, Murphy CJ, El-Sayed MA. *Chem Soc Rev* 2012;**41**:2740.
111 Libutti SK, Paciotti GF, Byrnes AA, Alexander HR, Gannon WE, Walker M, et al. *Clin Cancer Res* 2010;**16**:6139.
112 Dreaden EC, El-Sayed MA. *Acc Chem Res* 2012;**45**:1854.
113 Dickerson EB, Dreaden EC, Huang XH, El-Sayed IH, Chu HH, Pushpanketh S, et al. *Cancer Lett* 2008;**269**:57.
114 (a) Curley SA, Cherukuri P, Briggs K, Patra CR, Upton M, Dolson E, et al. *J Exp Ther Oncol* 2008;**7**:313; (b) Glazer ES, Zhu C, Massey KL, Thompson CS, Kaluarachchi WD, Hamir AN, et al. *Clin Cancer Res* 2010;**16**:5712.
115 For a review, see Medina SH, El-Sayed MEH. *Chem Rev* 2009;**109**:3141.
116 Podhajcer OL, Benedetti L, Girotti MR, Prada F, Salvatierra E, Llera AS. *Cancer Metastasis Rev* 2008;**27**:523.
117 Ackland SP, Bull JM, Boyle FM. *Asia Pac J Clin Oncol* 2009;**5**:147.
118 Brigger I, Dubernet C, Couvreur P. *Adv Drug Deliv Rev* 2002;**54**:631.
119 Moghimi SM, Hunter AC, Murray JC. *FASEB J* 2005;**19**:311.

CHAPTER

DRUGS THAT MODULATE RESISTANCE TO ANTITUMOR AGENTS

14

CONTENTS

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00014-4

1 INTRODUCTION

Tumor development principally occurs following the accumulation of genetic and epigenetic alterations in tumor cells, but these changes also lead to the transformation of chemosensitive cells to chemoresistant ones. Similarly to the resistance observed in bacteria after their exposure to antimicrobial agents, the selective pressure created in tumor cells when an essential target is therapeutically inactivated makes them evolve mechanisms of multidrug resistance (MDR) that include the production of a drug-resistant variant of the targeted protein or substitution of its cellular function by upregulating alternate pathways.[1] Because cell growth and proliferation may involve multiple molecular pathways, when preexisting DNA mutations are blocked, new ones allow the development of new pathways.

Treatment failure due to resistance to chemotherapy is linked with metastasis.[2] The sequence of events leading to metastasis requires the coordinated expression of multiple genes, signaling events, and favorable environmental conditions, distinct from those promoting growth of a primary tumor. A group of genes called metastasis suppressor genes (MSGs) influence aggressiveness and metastatic potential of cancers. It has been shown that resistance to anticancer drugs in a breast-resistant cell cancer line decreases more than 50–60% after inducing MSG levels.[3]

Cancer resistance to drugs or ionizing radiation may be intrinsic (treatment not effective for a particular tumor) or acquired (developed after initial effectiveness). Among cancers that are susceptible to chemotherapy, more than 50% rapidly develop drug resistance because cancer cells elude chemotherapy effects through a myriad of ways. To obtain anticancer treatments that are effective with drug-resistant variants as well as with wild-type cells, combination therapies are frequently used; alternatively, promiscuous drugs that interact with different targets can be used. However, drug resistance is still a major problem in oncology, affecting old therapies, new "targeted" drugs, and personalized cancer treatments.[4] For instance, patients with HER2-positive metastatic breast cancer treated with trastuzumab (Herceptin®) may develop a resistance mechanism that implies the expression by these cancer cells of the protein p95HER2, an NH_2-terminally truncated form of HER2 that lacks the extracellular domain for trastuzumab binding. This fact explains why not all the selected patients show response to this antibody and why many of them eventually develop resistance within 1 year.[5] The efficacy of the monoclonal antibody for epidermal growth factor receptor (EGFR)-expressing metastatic colorectal carcinoma cetuximab (Erbitux®) is hampered by mutation of the *K-Ras* gene, which occurs in 35–45% colorectal cancers. A large percentage of melanoma patients who do respond to therapy with the b-Raf inhibitor vemurafenib (Zelboraf®) develop resistance within 2–18 months after initial treatment through mechanisms not fully understood. In patients with lung cancers bearing gefitinib (Iressa®)- or erlotinib (Tarceva®)-sensitive EGFR mutations, resistant subclones containing an additional EGFR mutation emerge in the presence of these drugs. Consequently, detection of these changes should be a routine assay to select which patients maintain the efficacy of a given treatment. However, given the almost intrinsic genetic instability of many late-stage cancers, many experts are doubtful that widespread metastatic cancer can be cured.

Tumor cells usually enhance the expression and function of deactivating enzymes, survival factors, and signaling pathways, as well as transporters involved in drug efflux. Resistance to cancer chemotherapy is also associated with the DNA damage repair machinery, a network of upstream factors induced by tumor cells in response to damaged DNA that transmit pro- and antiapoptotic signals. For instance, reduction of the efficiency of the base excision repair (BER) mechanism with drugs targeting apurinic–apyrimidinic endonuclease 1 (APE1), DNA polymerase β (Polβ), or poly(ADP-ribose)

polymerase (PARP) makes tumor cells more sensitive to base lesions and single-stranded breaks produced by ionizing radiation or alkylating drugs; inhibitors of components of double-strand DNA breaks (DSBs) repair mechanisms, such as homologous recombination (HR) or nonhomologous end-joining (NHEJ), are radiosensitizers; inhibitors of O^6-methylguanine DNA methyltransferase (MGMT) sensitize cancer cells to alkylating agents; and inactivators of components of the nucleotide excision repair (NER) make cells more sensitive to cisplatin and similar chemotherapeutic drugs.

The vast knowledge that has been obtained about resistance mechanisms has not yet resulted in the expected clinic advances to improve diagnostic assays, predict individual therapy responses, or develop effective chemo- or radiosensitizers to enhance the efficiency of the therapy.[6] In this chapter, the main intratumor and extratumor processes that are involved in the development of radio- and chemoresistance are discussed, omitting the previously mentioned role of cancer stem cells (see Chapter 11, Section 7).

2 ABC EFFLUX PUMPS IN ANTICANCER DRUG RESISTANCE

Multidrug transporters are present in almost all cells to protect them from xenobiotics through an active excretion mechanism. The multidrug resistance (MDR) phenotype is mostly associated with the overexpression of P-glycoprotein (Pgp) and of multidrug resistance-associated protein-1 (MRP1). Both proteins are members of the superfamily of membrane transport carriers known as ATP-binding cassette (ABC) proteins, which hydrolyze ATP as an energy source to drive the outwardly directed transport of substrates against a concentration gradient and therefore reduce their intracellular concentration.[7] To date, most studied compounds that reverse this event, which are known as MDR modulators, resistance modifiers, or chemosensitizers, are Pgp inhibitors. Three generations of these inhibitors have enhanced the understanding of the mechanisms involved in chemotherapy resistance, but their clinical success has been limited and none of them have reached the market.

2.1 GENERAL FEATURES OF ABC EFFLUX PUMPS

ABC transporters are membrane "pump" proteins that eliminate toxic chemicals present in organs related to digestion and excretion, such as the biological membranes of the intestinal wall. The genes responsible for their synthesis are activated by environmental stress (e.g., by foreign chemicals or heat). These transporters have great importance in drug absorption and, through the removal of drugs from the cells, are the major cause for failure of anticancer chemotherapy (Figure 14.1a). Consequently, much effort has been placed in the development of blockers of these transport proteins in order to restore the sensitivity of the cell to the anticancer drug (Figure 14.1b).[8]

ABC proteins are organized similarly and contain transmembrane domains, which contain 5–10 membrane-spanning R-helices where the substrate binding sites are located and nucleotide-binding domains (NBDs) with ATPase activity that provides energy to allow the transport process. The structures of three representative types of ABC transporters involved in resistance to anticancer agents are shown in Figure 14.2.

Pgp is a membrane-associated 170-kDa glycoprotein that effluxes approximately 50% of all anticancer agents used clinically today without chemically modifying them. It is overexpressed in many

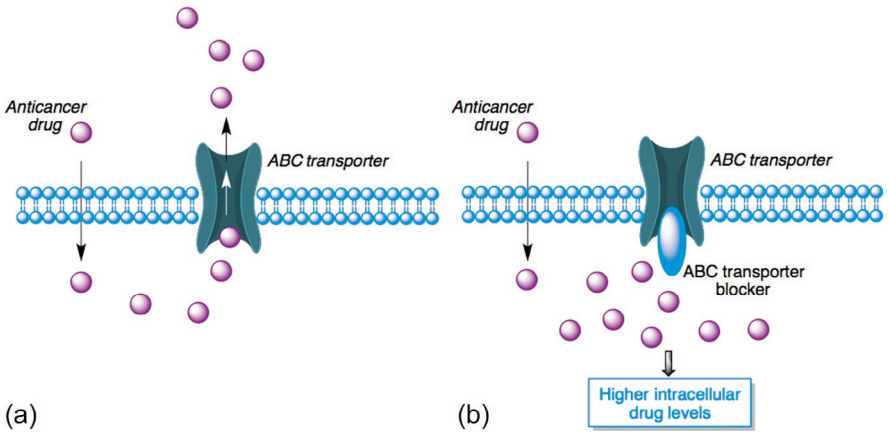


FIGURE 14.1

Effects of ABC efflux pumps (a) and their blockade (b) on the intracellular concentrations of anticancer drugs.

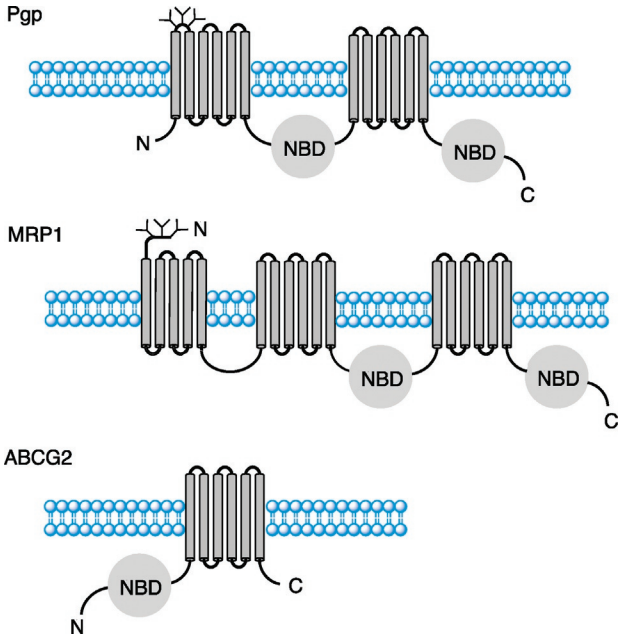


FIGURE 14.2

Schematic structures of the Pgp, MRP1, and ABCG2 transporters.

intrinsically resistant tumors and in others that acquire resistance during chemotherapy treatment. In fact, when the *mdr* gene that encodes Pgp is transfected into drug-sensitive cells, they became resistant.[9] Generalizations about the structural features required for a compound to be a Pgp inhibitor are hampered by the very heterogeneous chemical structure of compounds that have shown this property. The extensive list of traditional Pgp substrates includes the anthracyclines (doxorubicin and daunorubicin), vinca alkaloids (vinblastine and vincristine), colchicine, epipodophyllotoxins (etoposide and teniposide), and paclitaxel. In addition to these compounds, some of the modern antitumor drugs, such as the antileukemia drug imatinib, the marine natural product trabectedin, and the calicheamicin conjugate gemtuzumab ozogamicin, are excreted by this mechanism.

The functionally related protein MRP1 confers resistance to the vinca alkaloids, anthracyclines and epidophyllotoxins, as well as glucuronide, glutathione, and sulfate conjugates of drugs. Other members of the ABC family, such as MRP2 and MRP3, transport the same drugs, whereas MRP4 and MRP5 transport nucleotide and nucleoside analogs. The breast cancer resistance protein (BCRP) is a member of the ABCG subfamily that partially overlaps the substrate specificity of Pgp and MRPs, conferring resistance to mitoxantrone, methotrexate, topotecan, and SN-38.[10]

The ABC transporters are asymmetrically distributed when they are present in the same cell. For instance, in intestinal epithelium cells, Pgp, MRP2, and BCRP are located at the brush borders, whereas MRP1 and MRP3 are located at the basolateral surface. They are also differently expressed in normal conditions. Thus, Pgp is expressed in several organs, such as intestine, lung, kidney, liver, adrenal gland, certain hematological cells, blood–brain barrier, and placenta, which suggests that it is important in limiting the oral absorption of xenobiotics and contributes to limit access to the central nervous system through the blood–brain barrier. Several of these roles have been confirmed in knockout mice. Most MDR modulators act by binding to Pgp, inhibiting its drug-effluxing activity, and some act by indirect mechanisms, including inhibition of the expression of the *mdr1* gene.[11]

Because Pgp and MRPs are membrane-bound proteins, their study by nuclear magnetic resonance or X-ray diffraction techniques is difficult. To date, there is no detailed information on their structures and their substrate or inhibitor binding sites, which prevents proper *de novo* design approaches, but crystallization of prokaryotic ABC membrane proteins has helped in the design of Pgp inhibitors. The crystal structure of mouse Pgp, which has 87% sequence identity to human Pgp, was described in 2009,[12] and that of *Caenorhabditis elegans* was disclosed in 2012.[13]

2.2 INHIBITION OF P-GLYCOPROTEIN

Human Pgp is formed by 1280 amino acids and has two homologous halves, each containing a transmembrane domain with six α-helices (TM1–6 on TMD1 and TM7–12 on TMD2) and a hydrophilic nucleotide-binding domain (NBD1 and NBD2) that is located at the cytoplasmatic face of the membrane. Pgp is glycosylated at the first extracellular loop and phosphorylated by protein kinase C, which respectively affect its integration in the membrane and its transport function. Pgp may be modulated by competitive inhibitors that directly interact with TMDs or NBDs[14] or by noncompetitive inhibitors that interact with an allosteric residue relevant for its activity.

ABC proteins, and Pgp in particular, recognize a wide spectrum of compounds, but they all have in common a relatively high lipophillicity—a feature that has led to the proposal of two models to explain how this protein works. In the *hydrophobic vacuum cleaner* model, drugs partitioning into the membrane spontaneously translocate to the cytoplasmic leaflet and gain access to the Pgp substrate-binding

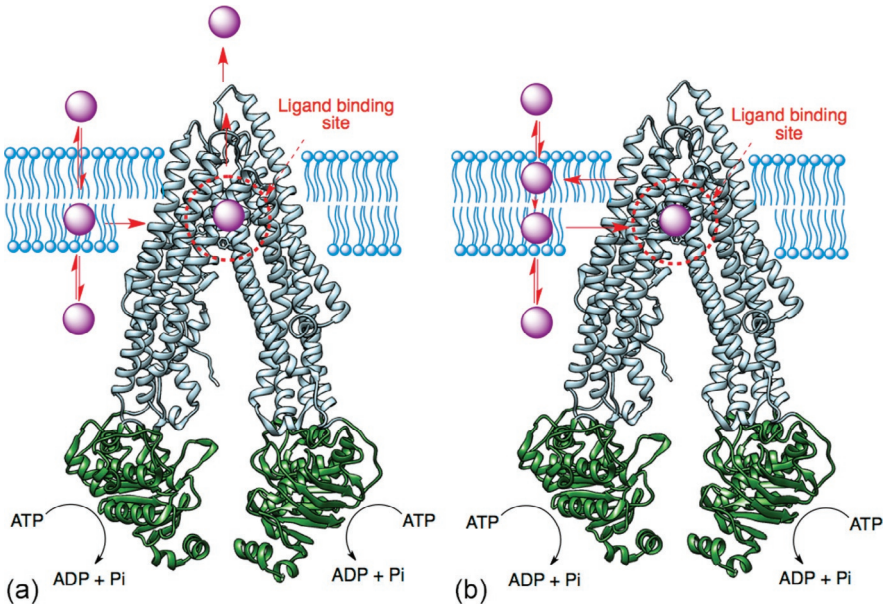


FIGURE 14.3

The hydrophobic vacuum cleaner (a) and flippase (b) models for the action of Pgp and other ABC proteins. The three-dimensional structure of Pgp was generated from Protein Data Bank reference 3G61 and displayed with Chimera 1.81.

pocket, from where they are finally effluxed into the extracellular aqueous phase (Figure 14.3a). In the flippase model, drugs partitioning into the membrane spontaneously translocate to the inner leaflet, interact with the Pgp substrate-binding pocket, and are then flip back to the outer membrane leaflet, thus starting a futile cycle that lowers the intracellular drug concentration (Figure 14.3b).[15]

The main mechanisms of action of Pgp modulators are depicted in Figure 14.4 and are summarized as follows:

1. According to the flippase model, substrates bind to the protein within the cytoplasmic leaflet of the bilayer and are transported to the extracellular leaflet faster than their intrinsic flip-flop rate, generating a drug concentration gradient. A modulator such as verapamil rapidly translocates spontaneously to the inner leaflet, where it is recognized as a substrate and transported back to the outer leaflet, thus locking Pgp in a futile cycle of modulator transport and ATP hydrolysis that enhances the intracellular concentration of the cytotoxic drug.
2. Some Pgp modulators, such as zosuquidar, are not transported but have a prolonged very high affinity to the drug-binding pocket, which blocks transport of drug substrates, whereas others, such as tariquidar (XR9576), bind at sites distinct from the drug-binding pocket.
3. Noncompetitive inhibitors may interact with key residues in other regions of the protein.

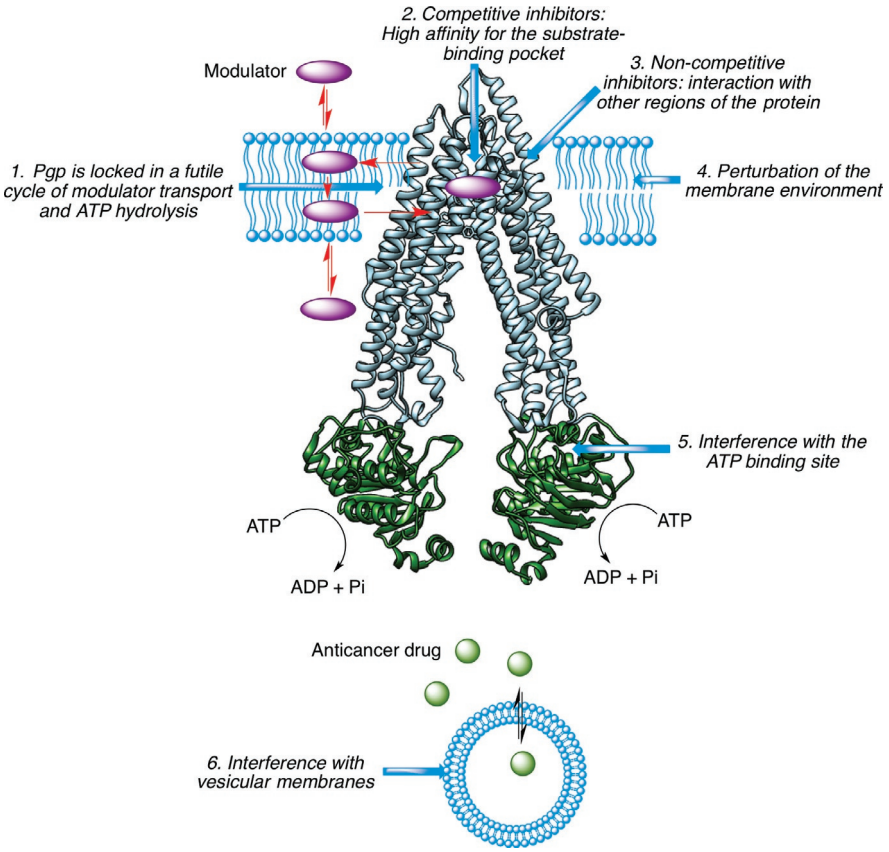


FIGURE 14.4

Summary of the mechanisms of action of Pgp modulators.

4. Perturbation of the membrane environment by fluidizers, anesthetics, and surfactant vehicles can also reverse Pgp-mediated MDR in a nonspecific way,[16] increasing the intrinsic flip-flop rate of drug substrates and restoring their cytotoxicity.[17]
5. Interference with the ATP-binding region of Pgp may provide an alternative mechanism for its modulation. For example, Pgp is inhibited by vanadate-induced trapping of ADP at the ATPase site.[18]
6. Other mechanisms involve the interference with drug sequestration by cellular organelles.

Some modulators, such as verapamil, valspodar (PSC-833), and zosuquidar (LY335979), induce apoptosis in a process that may be related to a failure of cytokinesis or to the production of reactive oxygen species (ROS) as a result of Pgp-mediated ATP turnover.[19]

To overcome the efflux of cytotoxic chemotherapeutic drugs and sensitize MDR cancers, Pgp modulators and anticancer drugs have to be simultaneously administered. Consequently, because inhibition of this drug pump also affects normal tissues, the cytotoxicity is also enhanced in normal cells.[20]

Another important problem is that because many modulators are also substrates for the cytochrome P450 enzyme (CYP450 3A), the drug clearance is reduced, leading to an increased cytotoxicity. To circumvent these problems, clinical studies with MDR modulators have been conducted with reduced doses of chemotherapy compared to those routinely used in clinical practice, but theses doses may be too low for some patients or still too high for others. One promising compound that seems to overcome the latter problem is zosuquidar, which has a Pgp/CYP3A4 affinity ratio of approximately 60.[21]

The first evidence of Pgp inhibition by natural products came from interactions of flavonoids[22] present in grapefruit juice with numerous drugs.[23] They enhance the bioavailability of paclitaxel,[24] tamoxifen,[25] and vincristine,[26] among others, although their mechanisms are largely undetermined.[27] The observed effects may be due to (1) nonspecific interaction with the cell membrane, resulting in increased passive membrane permeability; (2) an induced decrease in Pgp expression; or (3) direct binding either to the Pgp substrate binding site or to the NBD.[28] Some flavonoids, such as quercetin, which is the most predominant flavonoid in the human diet, interact with different targets involved in MDR. This compound competitively inhibits the Pgp, MRP1, and BCRP members of the MDR family, as well as the metabolizing enzymes CYP3A4 and glutathione *S*-transferase π (GSTπ; see Section 3.2). However, although hydrolysis of food β-glycosides allows the presence of a sufficiently high concentration of flavonoids in the intestine to inhibit intestinal Pgp, the flavonoid metabolites produced after intestinal absorption induce an insignificant systemic inhibition because they are organic anions that do not interact with Pgp.

The pharmacological approach to circumvent MDR began with the report by Tsuruo that the calcium channel blocker verapamil and the phenothiazine derivative trifluperazine potentiate the activity of vincristine.[29] Given the difficulties in establishing structure–activity relationships because of the existence in Pgp of several ligand binding sites[30] and the use of IC_{50} values without considering if modulators act by the same mechanism and binding mode, the identification of lead compounds in this area was mainly based on serendipity. The first attempts to identify a pharmacophore for Pgp inhibition[31] used a software package to analyze a set of structurally diverse ligands, which led to the identification of a number of pharmacophoric substructures (Figure 14.5).[32] However, because most of

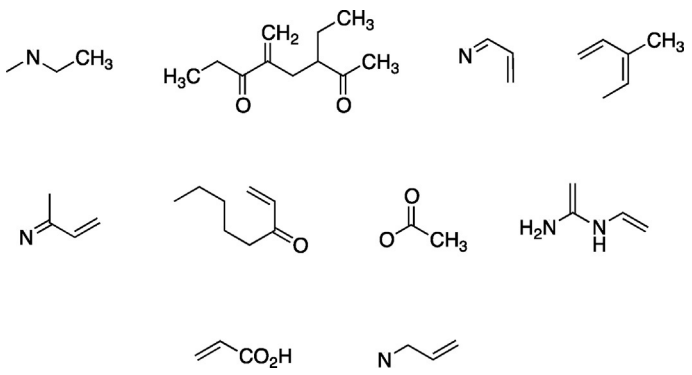


FIGURE 14.5

Proposed pharmacophoric fragments of Pgp inhibitors.

these fragments can be found in a large number of bioactive molecules, these results were not very helpful in the design of new ligands.

A subsequent study of more than 100 Pgp substrates led to the proposal of a set of structural elements for Pgp recognition that is mainly based on the number and strength of hydrogen bonds, which are related to the affinity to the protein.[33] Further rationale for this theory derives from the fact that the transmembrane sequences of Pgp involved in substrate interaction contain a high number of amino acids with hydrogen bond donor sites, such as the OH groups of Ser, Thr, and Tyr; the NH_2 group of Gln; the indolic NH group of Trp; and the SH group of Cys.

Two different types of recognition elements were identified:

1. Type I units, which contain two hydrogen bond acceptor (electron donors) groups with a separation of 2.5 ± 0.5 Å
2. Type II units, which contain either three hydrogen bond acceptor (electron donors) groups with a separation of 4.6 ± 0.6 Å between the outer units or two electron donor groups with the same spatial separation[34,35]

All molecules containing at least one of these two types of units are predicted to be Pgp substrates (Figure 14.6).

Another computer-aided substructural search coupled to a quantitative structure–activity relationship (SAR) study of 609 compounds showed several pharmacophores, the most important of which was

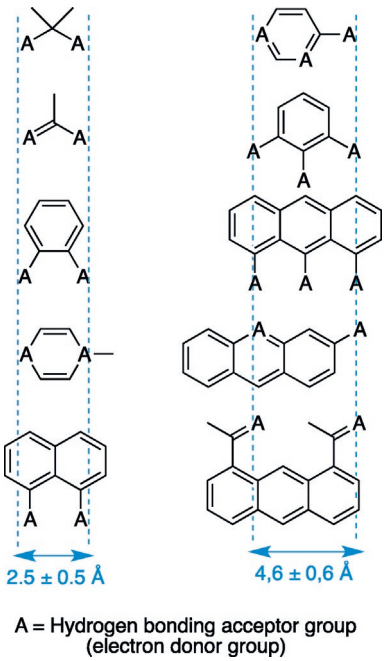


FIGURE 14.6

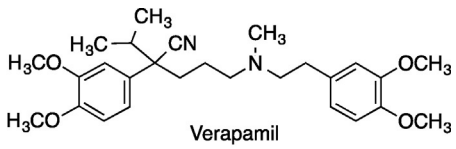
Proposed structural elements for Pgp recognition.

the C-C-X-C-C fragment, where X is N (preferentially tertiary) or O. The most significant physicochemical property was found to be lipophillicity as measured by the $(\log P)^2$ parameter, whereas hydrophylic fragments such as carboxylic acids, phenols, anilines, and quaternary ammonium compounds are deactivating.[36] These findings correlate well with an independent study that concluded that an effective Pgp modulator candidate should have a log P higher than 2.92, a molecular axis with at least 18 atoms, a high energy value for the highest occupied molecular orbital (HOMO), and at least one tertiary, basic nitrogen atom.[37]

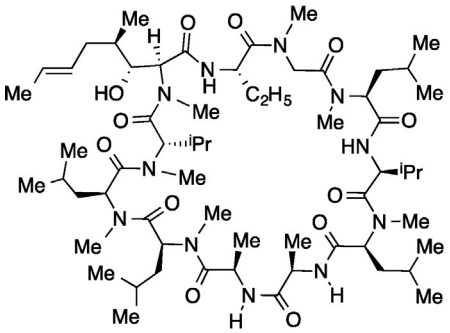
The role of lipids in MDR cells and the changes induced in the properties of membrane lipids by MDR modulators has been reviewed.[38] In this context, it has been proposed that MDR reversors should be designed to be lipophilic ($\log P \approx 4$), monobasic drugs with a near neutral pK_a (7–8).[39] In addition, electrostatic interactions between the modulator and the membrane phospholipids also play an important role.

A novel approach for predicting Pgp inhibition using molecular interaction fields has generated *in silico* models based on the synergistic combination of specific (pharmacophore) and nonspecific (general physicochemical) descriptors.[40] Potent inhibitors with a dibenzoxazepine scaffold have emerged from a pharmacophore model based on known Pgp inhibitors,[41] and dual activity as antitumor agents and Pgp inhibitors was found in 1-aminated thioxanthone derivatives designed by using homology modeling and docking.[42] Historical perspectives of Pgp inhibitors have been reviewed.[43]

The first generation of Pgp inhibitors includes many different compounds that were developed for other therapeutic purposes but showed their resistance-modulation effect when administered in combination with some anticancer treatments.[44] Consequently, when studied as possible Pgp modulators, they showed undesirable side effects at concentrations necessary to inhibit Pgp clinically. Among them are the previously mentioned calcium channel blocker verapamil and the immunosuppressive agent cyclosporin A (CsA). Verapamil, vinblastine, and digoxin appear to have superimposable binding sites to Pgp through different binding modes.[45]

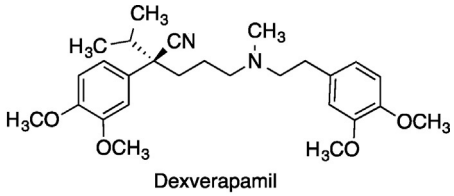


Verapamil

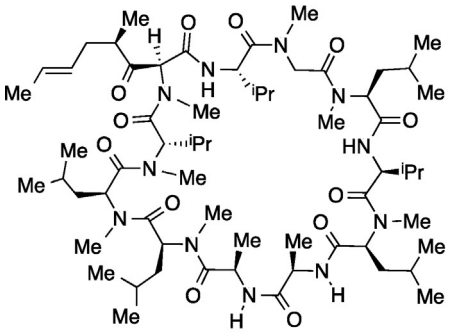


Cyclosporin A

The second-generation compounds were analogs of the previously mentioned drugs, which were designed to reduce their toxicity by suppressing their additional pharmacological effects. Among them, the (*R*)-isomer of verapamil, named dexverapamil, lacks its cardiac effects while retaining the ability to inhibit Pgp, and valspodar (PSC 833) is a structural analog of CsA that lacks its immunosuppressive effects. Unfortunately, both compounds are nonselective as Pgp inhibitors. For instance, valspodar inhibits Pgp, MRP2, and other proteins of the ABC superfamily. In addition, its clinical value is uncertain because its specificity for Pgp is similar to that for cytochrome P450, which explains why valspodar is involved in pharmacokinetic interactions with most anticancer drugs, requiring dose adjustment and leading to a high interpatient variability.[46] On the other hand, valspodar and cyclosporin were thought to have an anticancer effect independently of their action on MDR,[47] but recent clinical results with valspodar in patients with acute myeloid leukemia (AML) have been disappointing.[48]



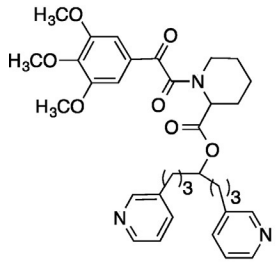
Dexverapamil



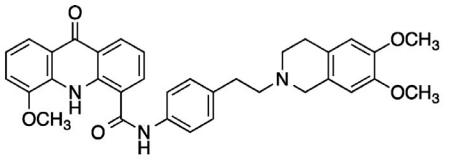
Valspodar (PSC 833)

For third-generation chemosensitizers, the development of compounds more specific and more potent as Pgp inhibitors than the earlier studied agents was attempted; however, most of these compounds showed unexpected toxic effects. Among those that have entered clinical trials, biricodar (Incel®, VX-710) and dofequidar (MS-209) inhibit Pgp and MRP1, elacridar (GF120918) inhibits Pgp and BCRP, tariquidar (XR9576) interacts with other ABC transporters rather than Pgp, and zosuquidar (LY335979) and ONT-093 (OC144-093) are selective inhibitors of Pgp. Biricodar is a simplified analog of tacrolimus, an immunosuppressive macrolactone that has an activity similar to that of cyclosporin. It has shown an optimal pharmacological profile in combination with paclitaxel,[49] and a phase II study has demonstrated its good safety and tolerability.[50] Dofequidar, in

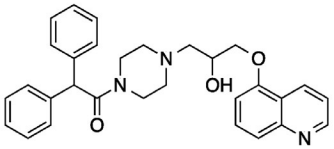
combination with etoposide or adriamycin, resulted in marked inhibition of metastases in refractory small cell lung cancer patients,[51] and another clinical study showed that it increases the antitumor efficiency of docetaxel.[52] The related compound MS-073 has been used to study the brain distribution of several neurokinin-1 antagonists, proving that some of them are effectively transported by Pgp across the blood–brain barrier.[53] Tariquidar (XR9576) underwent a phase II study in chemotherapy-resistant advanced breast cancer.[54] Zosuquidar (LY-335979), one of the most potent Pgp inhibitors described to date,[55] has fewer pharmacokinetic interactions than other MDR modulators because of its low affinity for P450 cytochromes, and advanced clinical studies on AML patients showed that it restores drug sensitivity.[56] Pharmacokinetic studies with ONT-093 (OC-144-093) showed that this compound does not interact significantly with the metabolism of paclitaxel because it is not a CYP3A substrate[57] and that it is selective toward Pgp, being a good candidate for a clinically useful MDR modulator.[58]



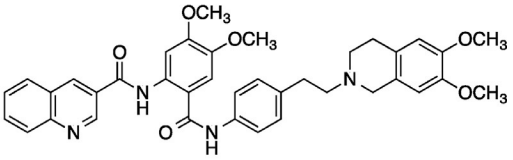
Biricodar (VX-710)



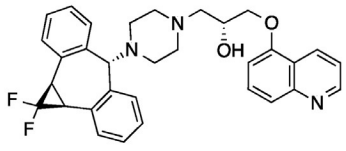
Elacridar (GF-120918)



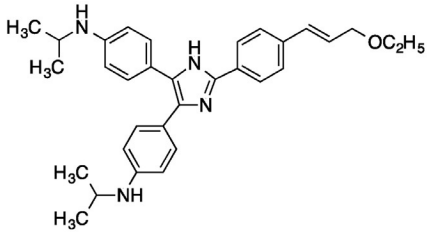
Dofequidar (MS-209)



Tariquidar (XR 9576)

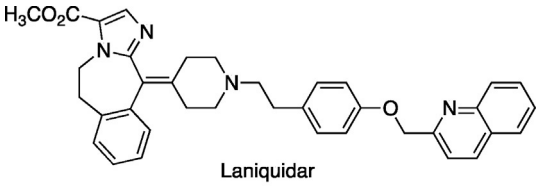


Zosuquidar (LY-335979)

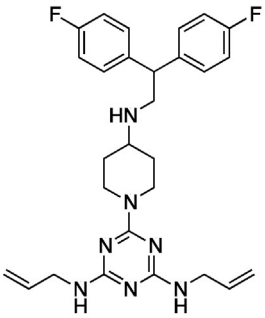


ONT-093
OC-144-093

Other members of the third-generation chemosensitizers are laniquidar, a potent orally active MDR inhibitor that entered phase II clinical trials in metastatic breast cancer in combination with taxols,[59] and the triazineaminopiperidine derivative S-9788, which inhibits Pgp specifically but showed cardiac toxicity in phase I clinical trials—a drawback that could be circumvented by combining it with verapamil or valspodar.[60]



Laniquidar



S-9788

The fourth generation of Pgp inhibitors derives from diverse strategies and includes many natural products and their derivatives (peptides, surfactants, and lipids).[61] In opposition to the classical paradigm that stated selectivity as an ideal property for a Pgp inhibitor, dual-activity agents are being designed to inhibit more than one transporter from the ABC family (in fact, two of the most potent Pgp inhibitors, tariquidar and elacridar, also interact with other ABC transporters). These dual inhibitors are examples of multitarget drugs as a modern general strategy to treat complex disorders besides cancer.[62] The design of dual ligands that inhibit Pgp and stimulate nitric oxide synthase was based on the fact that the MDR of HT20-Dox-resistant cells may be reversed by their incubation with inducers of nitric oxide (NO) synthesis. It was assumed that NO reduces the number of functionally active Pgp, perhaps by altering its conformation.[63] An immunotherapy approach, based on the induction of polyclonal autoantibodies specific for Pgp, has been developed using the antigenic capacity of conjugates comprising peptides derived from at least one of the extracellular loops of this protein.[64]

Administration of efflux pump substrates and modulators may upregulate the expression of these transport proteins making the tumor cells more drug-resistant, which is another reason for the uncertainty as to whether MDR modulators will increase patient survival.[65]

In summary, treatment of cancer patients with MDR modulators is a complex process, which explains why the results of clinical trials have so far been rather disappointing.[66] However, it is expected that MDR modulation could lead to a significant improvement in cancer chemotherapy, especially in hematological malignancies.[67]

An alternative to the use of Pgp inhibitors is to introduce structural modifications in anticancer drugs aimed at circumventing Pgp by increasing their passive diffusion because when passive diffusion is sufficiently fast, the efflux pump cannot maintain a gradient and its pumping efficiency is poor. This can be achieved by eliminating groups that solvate in water, decreasing their hydrogen bonding capacity by promoting intramolecular hydrogen bonds, and introducing lipophilic substituents such as

halogen atoms, but this aim would require that neither of the other ABC transporters be involved in chemoresistance. Only a few examples of this approach have been published.[68]

2.3 INDIRECT INHIBITORS OF MDR

The Pgp expression and function is influenced by several enzymes like cyclooxygenase 2[69] or glucosylceramide synthase,[70] which can be indirect targets in MDR inhibition. Certain compounds, such as the anticancer drug ecteinascidin-743, can prevent *mdr1* gene expression,[71] which can also be achieved by RNA interference through small RNA constructs (siRNA).[72] However, inhibition of the biosynthesis of the transport proteins by use of antisense oligonucleotides related to MRP or Pgp mRNAs seems to be of low clinical relevance despite previous good *in vitro* results.[73] Inhibitors of the Pgp ATPase activity with compounds such as the vanadate ion have also been proposed as adjuvants in the chemotherapy of solid tumors.[74] Other alternative approaches that can be used to kill cells expressing the MDR phenotype[75] are based on optimization of drug delivery by use of nanoparticles or liposomes,[76] which may be combined with hyperthermia.[77]

2.4 IMPORTANCE OF THE PGP INHIBITION DATA IN NEW DRUG APPLICATIONS

All efforts to achieve clinically relevant efflux pump inhibitors have revealed that a new molecular entity should be examined as a substrate or inhibitor of these transporters in drug development and regulatory reviews. Recommendations on methodologies and strategies for studying key transporters including Pgp[78] advise that the timing of transporter investigations should be driven by efficacy, safety, and clinical trial enrollment questions.[79]

3 GLUTATHIONE AND GLUTATHIONE *S*-TRANSFERASE IN ANTICANCER DRUG RESISTANCE

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is an antioxidant intracellular tripeptide that plays an important role in the maintenance of cellular redox potential, although it also functions in many other biological processes. GSH is a radical scavenger through its transformation to the disulfide derivative, but it is also a nucleophile that reacts with electrophiles to form deactivated conjugates readily excreted by a glutathione synthase (GS)-conjugated export pump, in a reaction that may occur spontaneously or with the help of the enzyme glutathione *S*-transferase (GST).[80] In addition, GSH may directly or indirectly participate in DNA repair because it modulates the expression of transcription factors such as *c-fos* and *c-jun* that potentially affect DNA repair and apoptosis.[81] Also, through the preservation of protein mercapto groups in a reduced state due to its antioxidant function, it protects tumor cells against apoptotic cell death.[82]

Due to its reactivity and high intracellular concentrations, glutathione has been implicated in resistance of several chemotherapeutic agents, such as platinum-containing agents,[83] alkylating agents,[84] and anthracyclines.[85] For instance, Bcl-2-mediated cisplatin resistance in MCF-7 breast cancer cells is dependent on upregulation of glutathione production, which contributes to cell survival by mechanisms independent of cisplatin inactivation or inhibition of DNA adduct formation.[86]

3.1 INHIBITORS OF GLUTATHIONE BIOSYNTHESIS

Glutathione is synthesized in two steps from amino acid precursors. The first step, in which glutamic acid and cysteine are joined, is catalyzed by γ-glutamylcysteine synthetase (γ-GCS), whereas the second step, in which addition of glycine takes place, is catalyzed by GS (Figure 14.7).

Because γ-GCS is the enzyme involved in the rate-limiting step of the GSH synthesis, depletion of intracellular GSH levels has been pursued by using inhibitors of this enzyme, among which the best studied is L-buthionine-(*S,R*)-sulfoximine (L-BSO). It has proven its efficacy as an enhancer of the antitumor activity of the alkylating agent melphalan in phase I and II clinical trials.[87]

It is known that L-glutamate is phosphorylated by MgATP to form the enzyme-bound intermediate γ-glutamylphosphate, which subsequently reacts with the amino group of cysteine. Similarly, L-BSO is phosphorylated on the sulfoximine nitrogen. This phosphorylated product is tightly (although not covalently) bound to the active site of the enzyme γ-GCS, which remains inhibited until this product and MgADP are dissociated. The geometry of the sulfoximine group resembles the tetrahedral adduct formed in the attack of the cysteine amino group to the mixed anhydride γ-glutamylphosphate, and it is considered as a transition state analog (Figure 14.8). The L-buthionine-(*S*)-sulfoximine causes essentially irreversible inhibition, whereas the L-buthionine-(*R*)-sulfoximine is a reversible competitive inhibitor of L-glutamate.[88]

The extent to which GSH depletion enhances tumor cell sensitivity without augmenting toxicity to normal cells remains to be determined.[89] Furthermore, although the administration of L-BSO depletes intracellular GSH levels in circulating white blood cells up to 60–80%, an adaptive response to this GSH depletion leads to the fast upregulation of the nuclear-related factor 2 (Nrf2), which in turn upregulates glutathione synthesis.[90] The Nrf2/Keap1 pathway has a central role in the adaptation of cells to increasing oxidation because the Keap1 protein acts as a redox sensor. Thus, under basal

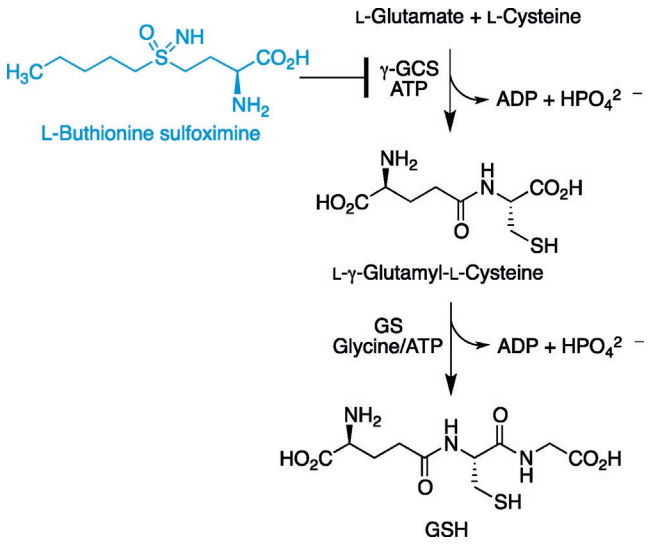


FIGURE 14.7

Biosynthesis of glutathione and its inhibition by L-buthionine-(*S,R*)-sulfoximine (L-BSO).

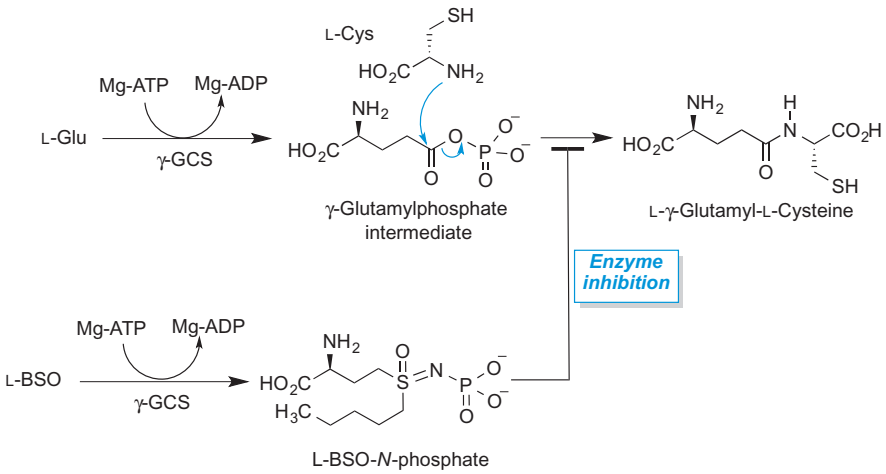


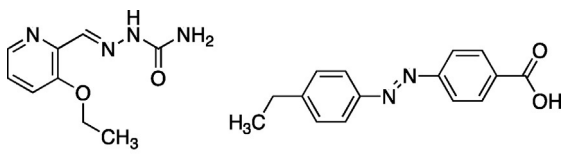
FIGURE 14.8

Inhibition of γ-glutamylcysteine synthetase by L-BSO.

conditions, Keap1, functioning as an ubiquitin ligase, targets Nrf2 for ubiquitination and proteasomal degradation and therefore leads to very low levels of Nrf2. A number of stress situations, including oxidative stress or inflammation, inhibit the activity of the Keap1-related E3 ubiquitin ligase, reducing the proteolytic degradation of Nrf2 and enabling its increased transduction to the nucleus. Nrf2 subsequently binds to antioxidant responsive elements in target genes encoding detoxifying enzymes associated with phase II metabolism, antioxidant proteins and glutathione biosynthesis enzymes (Figure 14.9).

L-BSO has been studied in both preclinical and early clinical trials, and it has proven its efficacy as an enhancer of the antitumor activity of the alkylating agent melphalan in phase I and II clinical trials.

Some inhibitors of human γ-GCSH with novel chemical structures were identified by using a three-dimensional molecular model through a virtual screening of the NCI chemical database. This strategy was validated by biologically testing of these compounds, which led to four compounds containing two novel classes of chemical skeletons that deplete GSH levels in A549 tumor cells and to two compounds with related chemical skeletons that sensitized tumor cells to melphalan treatment in clinical trials.[91] These promising chemical classes, represented by NSC79068 and NSC104960, need to be further optimized. Another strategy that has not been tested clinically involves the use of a ribozyme to cleave the γ-GCS mRNA to specifically downregulate the enzyme levels.[92]



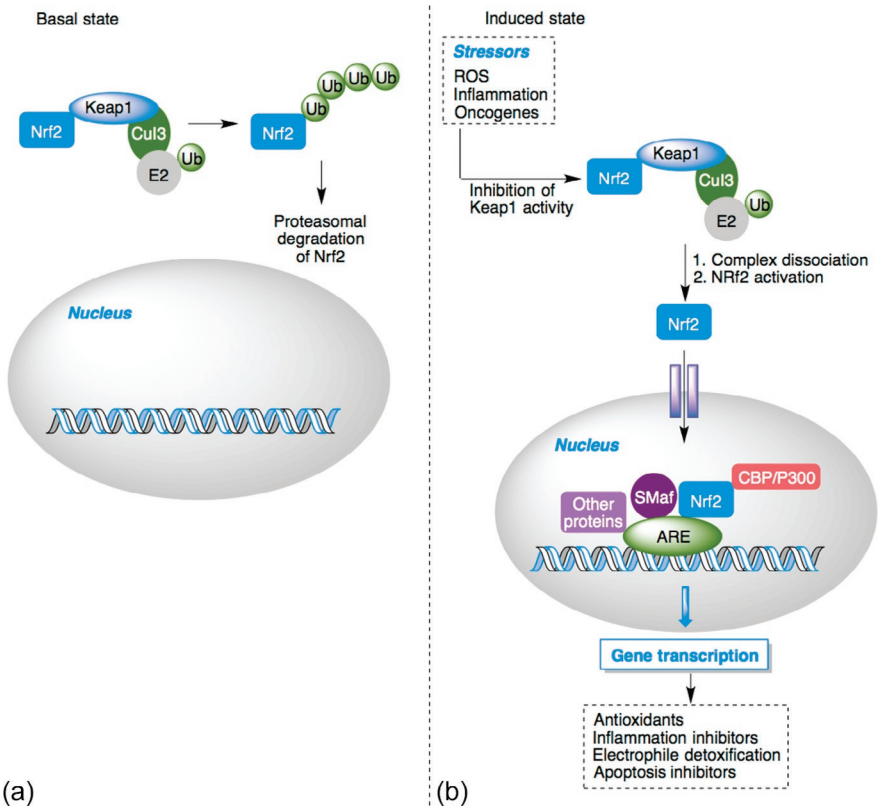


FIGURE 14.9

The Nrf2/Keap1 pathway.

3.2 INHIBITORS OF GLUTATHIONE *S*-TRANSFERASE

3.2.1 Role of Glutathione S-Transferase in Anticancer Drug Resistance

GSTs[93] are a family of phase II metabolic enzymes that have many different functions, the most important of which is the detoxification of endogenous and exogenous electrophilic compounds. In addition to their function in direct detoxification, the π and μ classes of cytosolic GSTs have a regulatory role in cancer as inhibitors of the MAP kinase pathway via protein–protein interactions with JNK/cJun (see Chapter 10, Section 6.5.4). GSTπ is a negative regulator of c-Jun N-terminal kinase 1 (JNK1), which is involved in stress response, apoptosis and cellular proliferation, especially stimulating the growth and differentiation of normal blood cells. Overexpression of GSTπ has been particularly associated with prostate,[94] colon,[95] and ovarian cancers, being regarded as a potential tumor marker.[96]

GSTs contain a site that accommodates GSH, where the proton of the thiol group is abstracted, promoting the nucleophilic conjugation of the thiolate with electrophilic substrates to give adducts that are more water-soluble and are then eliminated. In this way, they are implicated in the resistance

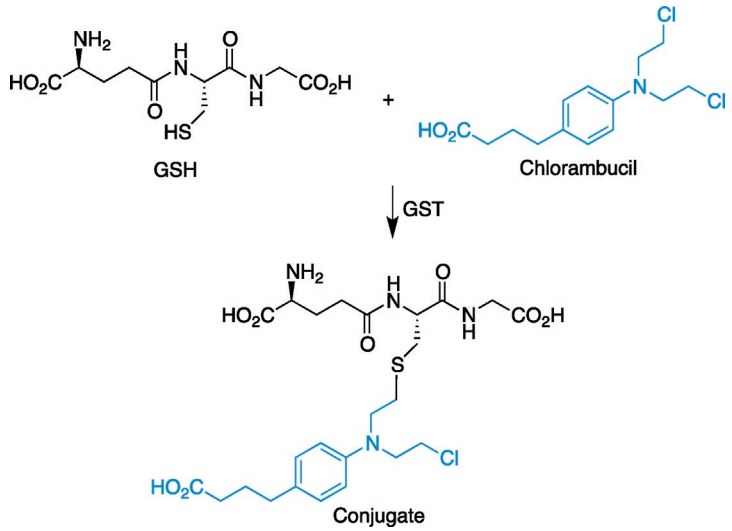


FIGURE 14.10

Mechanism of GST-mediated resistance to chlorambucil.

toward electrophilic antitumor agents, especially alkylating agents such as some nitrogen mustards and nitrosoureas (Figure 14.10).

The GSTπ enzyme is particularly important in anticancer drug resistance, and many studies have focused on the development of specific inhibitors of this isoform. Its glutathione binding site (G site) is located in a cleft between the N-terminal and C-terminal ends, but most residues that interact with glutathione belong to the N-terminal domain. The binding site for hydrophobic electrophiles (H site) is adjacent to the G site.

3.2.2 Irreversible Inhibitors of Glutathione S-Transferase

GSTπ suicide or irreversible inhibitors include agents that bind covalently to glutathione, forming thioether adducts that are stabilized at the active site of the enzyme. The diuretic drug ethacrynic acid is one of these compounds that forms a conjugate with glutathione via Michael addition to its α,β-unsaturated ketone moiety, both spontaneously and by GST catalysis, leading to glutathione depletion (Figure 14.11). The Michael adduct is an inhibitor of human GSTπ more potent than ethacrynic acid due to the potent interaction of its glutathione fragment with the enzyme (Figure 14.12). Ethacrynic acid was clinically evaluated as a resistance modulator, but it lacked specificity for the GST isoforms, and its usefulness was limited by its diuretic activity.[97]

3.2.3 Competitive Inhibitors of Glutathione S-Transferase

Competitive inhibitors of GSTπ may belong to two categories, namely non-glutathione- or glutathione-based compounds. The former group covers a broad range of chemical structures, such as tricyclic-based dibenzazepines, polyphenolic natural products, alkaloids, pyrimethamine, and dyes. Among them, the natural polyphenol quercetin entered phase I clinical trials against several types of cancer.[98]

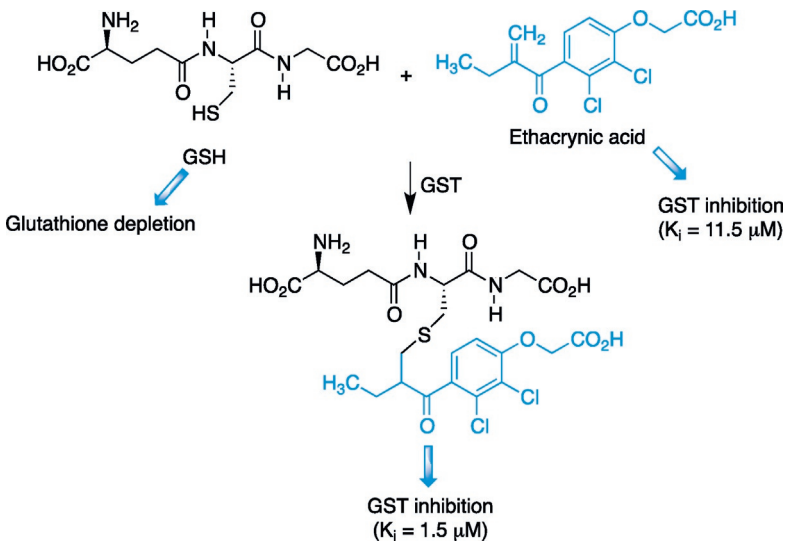


FIGURE 14.11

Mechanisms of GST inhibition by ethacrynic acid.

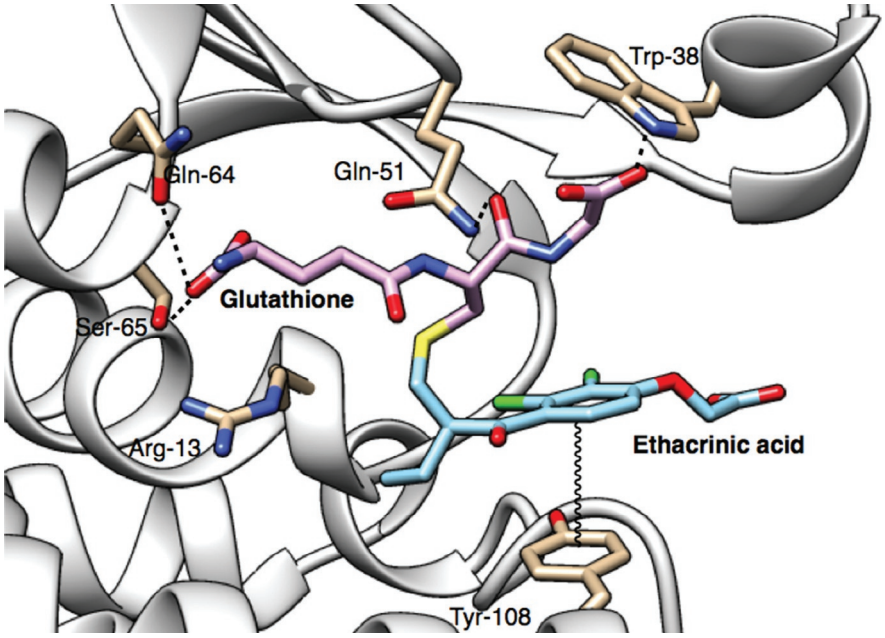
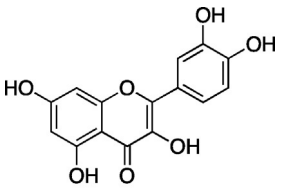


FIGURE 14.12

Structure of the complex formed between GST and the glutathione (GSH)–ethacrynic acid Michael adduct. Generated from Protein Data Bank reference 11GS and displayed with Chimera 1.8.1.

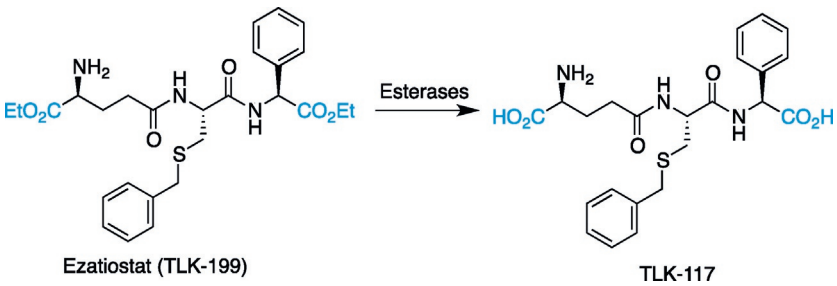


Quercetin

Another strategy for the development of GSTπ glutathione-based inhibitors is based on modification of the GSH backbone to enhance its inherent affinity for the enzyme. The inhibitory activity against GSTπ increased in glutathione peptidomimetics with linear alkyl chains on the cysteine sulfur and bulky hydrophobic substituents on the glycine residue, as in the case of TLK-177. This compound is administered in the form of the prodrug ezatiostat (Telintra®, TLK-199), which is activated by double ester hydrolysis (Figure 14.13). Although the effects of ezatiostat as a chemotherapy-potentiating agent were not sufficiently relevant to warrant continuation of the initial clinical trials for this application,[99] it was shown to act as a myeloproliferative agent by modulation of proliferation kinase pathways. In 2013, Telintra® was granted orphan drug designation by the U.S. Food and Drug Administration FDA for the treatment of myelodysplastic syndromes.

Ezatiostat has been studied in clinical trials for patients with myelodysplastic syndrome (MDS) by using a liposomal formulation for intravenous administration[100] and an oral formulation.[101] MDS is a form of pre-leukemia that may ultimately progress to leukemia, in which the bone marrow produces insufficient levels of one or more of the three major blood elements: white blood cells, red blood cells, and platelets. Although some MDS patients initially respond to treatment with growth factors, all of them require multiple transfusions and long-term therapy. Ezatiostat improves all three types of blood cells and may be a therapeutic alternative for these patients. Its combination with lenalidomide has shown good activity and tolerability.[102]

Ezatiostat increases the production of blood cells through the mechanism shown in Figure 14.14, which is related to the control of the growth and maturation of hematopoietic progenitor stem cells by the JUN pathway, because GSTπ is a negative regulator of JNK. The active tripeptide diacid TLK-117 binds to GSTπ, causing dissociation of the enzyme from the JNK/JUN complex and leading to JNK activation by phosphorylation. The diphosphorylated c-Jun formed at a later stage translocates to



Ezatiostat (TLK-199) TLK-117

FIGURE 14.13

Bioactivation of the GST inhibitor prodrug TLK-199.

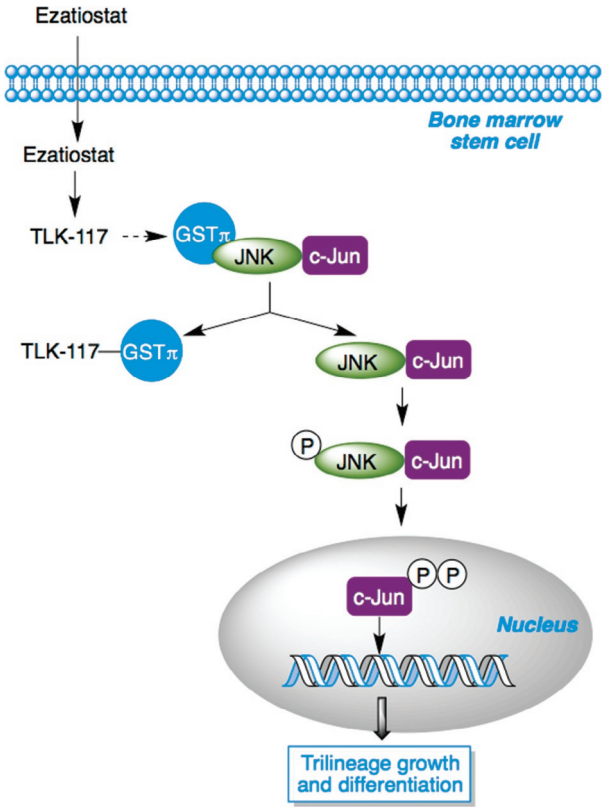


FIGURE 14.14

Mechanism of action of ezatiostat.

the nucleus, where it participates in the transcription of growth and differentiation genes that ultimately results in the stimulation of trilineage (red cells, white cells, and platelets) growth and differentiation.[103]

4 CHEMOSENSITIZERS TARGETING DNA REPAIR SYSTEMS

Ideally, a dividing tumor cell would progress into mitosis after sustaining DNA damage inflicted by chemotherapy and would subsequently undergo mitotic catastrophe and apoptosis. However, cells have the ability to halt the cell cycle in G_1, G_2, or S phases and pause to allow for DNA repair through both p53 and Chk1 pathway activation (see Chapter 10, Section 5.3). Whereas normal proliferating cells have an intact p53 pathway, this mechanism is compromised in many tumors in which the Chk1-mediated G_2/S arrest becomes a dominant defense mechanism from DNA-damaging chemotherapy. Inhibition of the chekpoint kinase Chk1 in such p53-deficient tumor cells with damaged DNA would abrogate the cell cycle arrest and force the progression into mitosis resulting in cell death, thus selectively sensitizing these tumors

to chemotherapy. Accordingly, combination therapy comprising a DNA-damaging agent with a Chk1 inhibitor can potentially have a significantly higher therapeutic index than chemotherapy alone.

The most common DNA lesions that challenge the inherent stability of the cell genome[104] are single-strand breaks (SSBs), which can occur at a frequency of tens of thousands per cell per day. Certain modulators of these pathways, rather than repair of potentially carcinogenic DNA damage, have the potential to act as anticarcinogenic compounds by promoting cell death.[105] The discovery and understanding of changes in the DNA repair pathways produced in various chemoresistant and radioresistant phenotypes have resulted in the identification of the disruption of this process with chemosensitizers as a suitable strategy to overcome intrinsic and/or acquired resistance, especially to ionizing radiation and DNA-damaging agents.

The development of new drugs targeting the DNA damage response (DDR) is a very active field of research. Many potential therapeutic DDR targets are known, and some of their inhibitors have entered clinical trials.[106–108] Unfortunately, the enhanced therapeutic efficacy of antitumor drugs when they are administered with these chemosensitizers may be linked to undesirable effects derived from inhibition of DNA repair in normal tissues. Furthermore, these agents may improve the risk of secondary malignancies because of the potential mutagenesis and carcinogenesis that may take place after inhibition of DDR. However, for a targeted treatment such as radiation, an enhanced therapeutic window may be more successfully achieved because inhibition of the DNA damage response will preferentially sensitize cells in the field of radiation.

The pathways involved in DDR may be grouped into four functions: DNA repair, DNA repair of accessory functions, DNA damage signaling, and cell survival.[109] There are four damage-type-specific DNA repair pathways that have led to new targets in adjuvant cancer therapeutics: direct repair (DR), base excision repair (BER), homologous recombination (HR), and non-homologous end joining (NGEJ) (Figure 14.15).

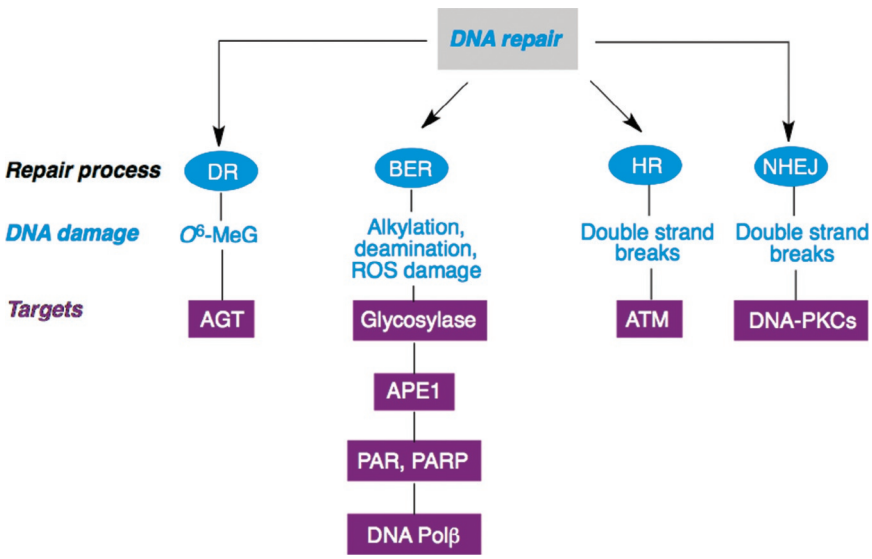


FIGURE 14.15

Anticancer targets in DNA repair processes.

The DR pathway restricts the therapeutic response of tumors to chloroethylating or methylating agents through the repair factor O^6-alkylguanine DNA alkyltransferase (AGT).[110] The BER pathway reduces tumor sensitivity to alkylating or oxidative agents by repairing oxidized-reduced, alkylated, or deaminated bases through multiple enzymes that include DNA glycosylases, which remove the damaged base generating an apurinic–apyrimidic (AP) site; apurinic–apyrimidinic endonuclease 1 (APE-1), which cleaves the phosphodiester bonds at the 5′ end of the AP site; and Polβ, which is recruited to fill this gap with assistance from PAR and PARP1. The HR and NHEJ pathways repair DSBs produced after the use of ionizing radiation or the administration of alkylating agents, topoisomerase inhibitors, or drugs that generate ROS. The targets of these two DNA repair pathways are ATM (ataxia–telangiectasia mutated) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), respectively.

4.1 INHIBITORS OF O^6-ALKYLGUANINE DNA ALKYLTRANSFERASE (MGMT OR AGT)

As discussed in Chapter 5, DNA damage by several types of alkylating agents, such as nitrosoureas and temozolomide, is initiated by alkylation of the guanine O^6 atom. This damage is repaired by O^6-alkylguanine DNA alkyltransferase (AGT), also known as O^6-methylguanine DNA methyltransferase (MGMT), which covalently transfers the guanine O^6-alkyl group of alkylated DNA to the enzyme cysteine residue Cys-145 in its active site before mispairing of bases or covalent cross-links can occur (Figure 14.16). This reaction is stoichiometric in terms of MGMT, which is deactivated. Therefore, MGMT may be considered as a sacrificial or suicide enzyme and an interesting anticancer target for combination therapy, because its inhibition potentiates the antitumor effect of those alkylating agents for which O^6-alkylation is the determinant of cancer cell death,[111] especially carmustune and temozolamide.[112] However, it must be remembered that alkylating agents also react at other DNA positions, especially guanine N-7 and adenine N-3, a damage that is repaired by the base excision repair system.

The main type of MGMT inhibitors are O^6-alkylguanine derivatives[113] that act as competitive inhibitors by analogy with the natural substrate and inactivate the enzyme by transferring their O^6-alkyl

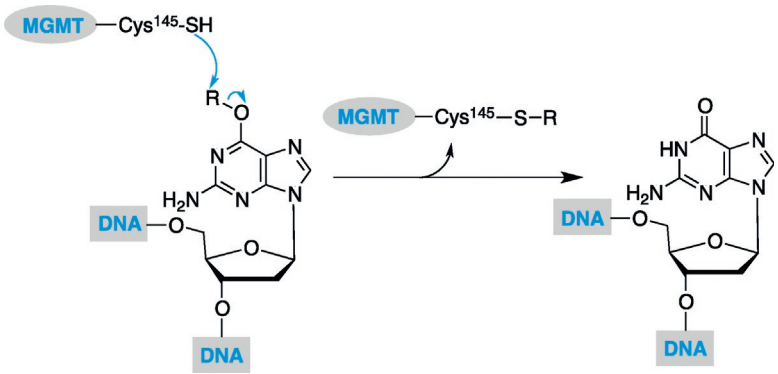


FIGURE 14.16

Repair of O^6-alkylguanine residues by alkylguanine transferase (MGMT).

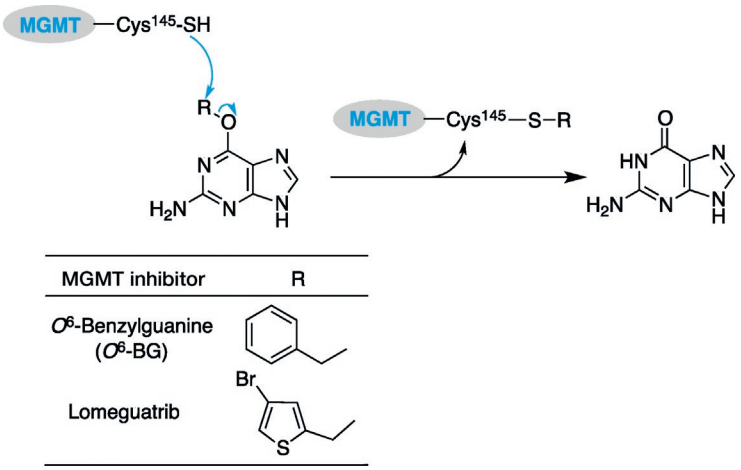


FIGURE 14.17

MGMT inactivation by O^6-benzylguanine and lomeguatrib.

group to its Cys-145 residue (Figure 14.17). Among these compounds, O^6-benzylguanine and lomeguatrib are being clinically evaluated in combination with temozolomide,[114,115] carmustine,[116] or BCNU. Triple combinations including a topoisomerase I inhibitor, such as irinotecan, are also under clinical evaluation.[117]

The acidic (pK_a 4.8) Cys-145 residue of MGMT is susceptible to nitrosylation. It has been proven that the potent nitrosylating agent nitroaspirin (NCX-4016), which may be useful to overcome tumor immunosuppression, is also a clinically relevant inhibitor of human MGMT that increases the efficacy of alkylating agents (Figure 14.18).[118]

Platinum anticancer drugs, which are not influenced by the MGMT proteins because their primary mode of action involves interaction with the guanine N-7 rather than with O-6, can reduce their

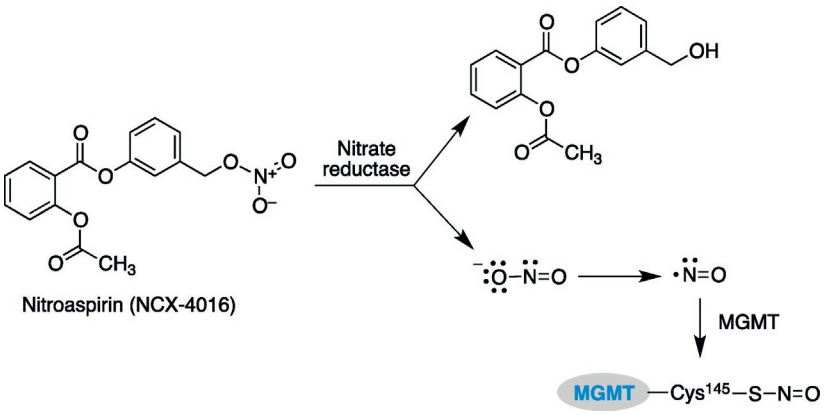


FIGURE 14.18

Nitrosylation of MGMT by nitroaspirin.

expression through downregulation of the corresponding mRNA. For this reason, clinical assays have been carried out to study the combination of cisplatinum with nitrosoureas (e.g., nimustine) and temozolomide.[119]

4.2 ANTITUMOR ADJUVANTS TARGETING THE BER PROCESS

This is a primary DNA repair process that corrects base lesions arising after damage due to oxidative, alkylation, deamination, and depurination/depyrimidination processes, and it plays an essential role in mediating cytotoxicity in an acidic tumor microenvironment.[120] BER acts through two general pathways: short-patch and long-patch. The short-patch BER pathway leads to the repair of a single nucleotide, whereas the long-patch BER pathway produces the repair of at least two nucleotides. The BER pathway is initiated by one of the many DNA glycosylases that recognizes and catalyzes the removal of damaged bases generating an apurinic or apyrimidinic (AP) site. The process is completed by the coordinated action of additional enzymes that carry out the cleavage of the AP site by an AP endonuclease-3′-phosphodiesterase (APE-1), leading to a single strand break, the subsequent gap filling with replacement of the damaged base, and the final ligation (Figure 14.19).[121] Most compounds targeting enzymes in this pathway are under preclinical studies.

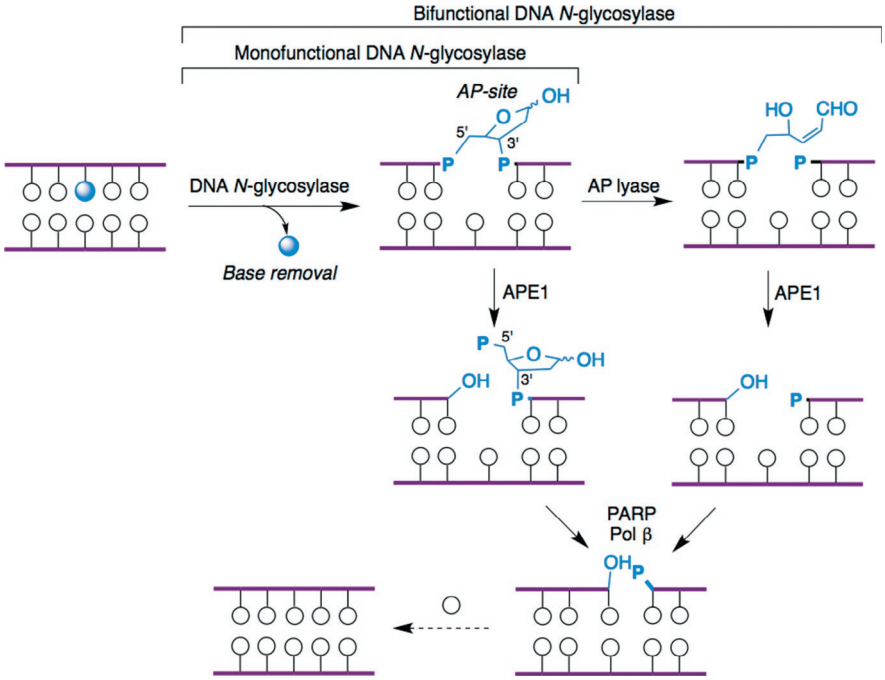


FIGURE 14.19

The base-excision repair (BER) process.

4.2.1 Inhibitors of DNA Glycosylases

DNA glycosylases monitor the presence of aberrant bases in order to remove them. They flip the damaged nucleotide out of the double helix and place it into their active site, where wrong bases are bound through π-stacking interactions. Glycosylases are grouped into four superfamilies, namely the UDG and AAG families, which are small, compact glycosylases, and the MutM/Fpg and HhH-GPD families, which comprise larger enzymes with multiple domains.

Monofunctional glycosylases, which normally are involved in the repair of deaminated or alkylated bases, hydrolyze the *N*-glycosidic bond that links these bases to the DNA backbone. The hydrolysis is carried out by a hydroxide anion generated by deprotonation of a molecule of water by an Asn or Asp enzyme residue (Figure 14.20).[122]

Bifunctional glycosylases normally remove bases that have sustained oxidative damage, and their catalytic cycle involves an initial S_N1-like attack at the C-1′ position by Lys or Pro residues that remove the aberrant base. In a second step, because of their purinic–apyrimidinic (AP) lyase activity, they catalyze a subsequent β-elimination reaction of the 3′-phosphodiester bond on the protonated Schiff base intermediate and subsequent hydrolysis, which results in strand scission (Figure 14.21).

Inhibition of the activity of DNA glycosylases can in principle be used to potentiate the activity of base-damaging anticancer drugs or radiation therapy, although the field is in its early stages of development and there are still no useful drugs based on this concept.[123] Some analogs of oligonucleotides, more chemically stable and obtained using solid phase DNA synthetic methodology,[124] are DNA glycosylase inhibitors because these enzymes are end product inhibited.[125,126] These compounds are reduced abasic site analogs (e.g., compound **14.1**), oligonucleotides containing pyrrolidine moieties that mimic the positive charge at the transition state (e.g., **14.2**), and nucleotides with stabilized glycosidic bonds that cannot be processed by DNA glycosylases (e.g., **14.3**).

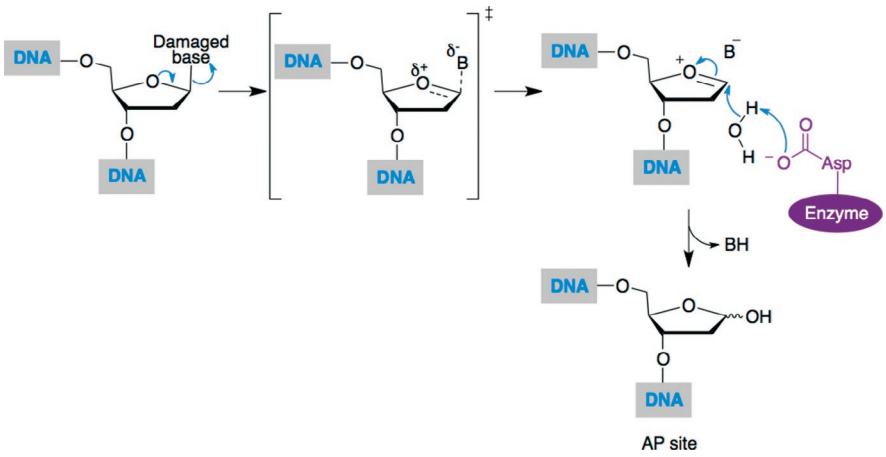


FIGURE 14.20

Mechanism of the reaction catalyzed by monofunctional DNA glycosylases.

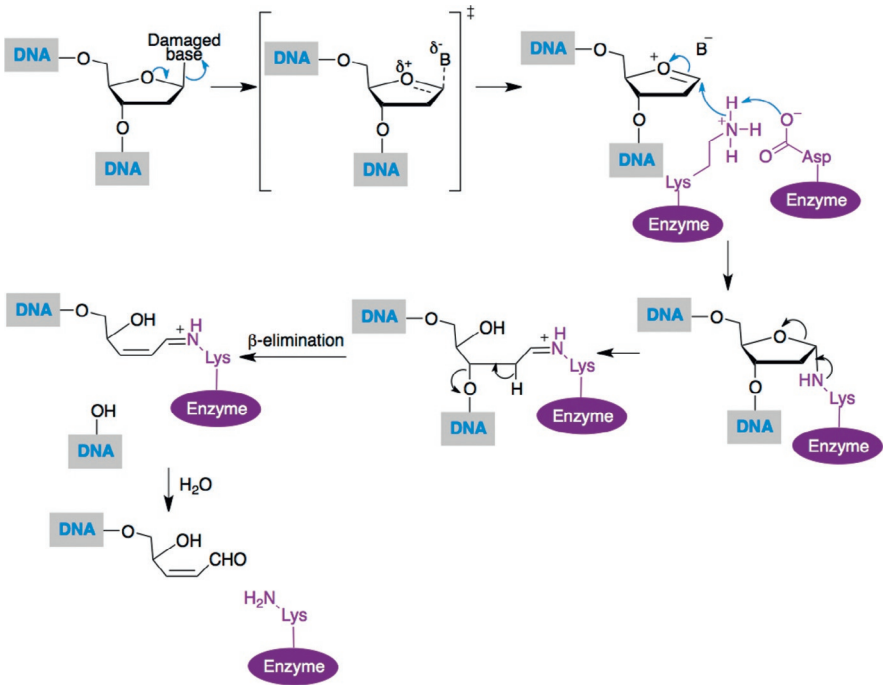
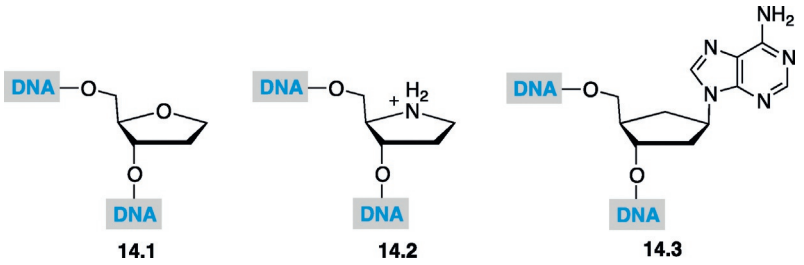


FIGURE 14.21

DNA strand scission by bifunctional glycosylases.



4.2.2 Inhibitors of APE-1 and Compounds Targeting the AP Site at DNA

APE-1 is a multifunctional protein of approximately 35.5 kDa that possesses several nuclease activities, including the cleaving of the phosphodiester bonds at the 5′ end of the AP sites. In one of the proposed mechanisms for the APE-1 catalyzed reaction,[127] His-309 acts as a base and abstracts a proton from a water molecule to generate the active site nucleophile, while Asp-283 helps to stabilize the generated positive charge by forming a hydrogen bond with the His–NH group. The metal ion bound by

Glu-96 interacts with the negatively charged phosphate group aiding the hydroxyl nucleophilic attack, while Asp-210 protonates the 3′-leaving group (Figure 14.22).

The increased expression of APE-1 is correlated with resistance to chemotherapy and radiotherapy, making it an attractive anticancer target. One example of a selective APE-1 inhibitor, identified through a high-throughput screening assay of a chemical library of 5000 compounds, was 7-nitroindole-2-carboxylic acid (CRT-0044876), which was shown to bind to the APE-1 active site[128] by computational methods. This compound potentiates the cytotoxicity of several DNA base-targeting drugs, although the reproducibility of its effects has been questioned since the initial report. Furthermore, its poor druglike properties, together with the presence of a nitro-aromatic group, generally associated with toxicity issues, limit its utility as a drug candidate. Despite these drawbacks, CRT-0044876 and other molecular scaffolds designed on the basis of the shape of the APE-1 ligand binding site have been used in virtual screening studies.

Another specific APE-1 inhibitor that was shown in preclinical studies to sensitize cancer cells to DNA alkylating agents such as temozolomide is the antiparasitic drug lucanthone.[129] This compound and its derivative, hycanthone, inhibit APE-1 by direct binding.[130] However, due to the fact that the cell sensitization occurs at doses lower than those required to inhibit APE-1 activity *in vitro*, it has been postulated that these effects are mediated through the inhibition of topoisomerase II or other cellular proteins. In fact, lucanthone inhibits autophagy, a survival pathway that enables cancer cells to undergo self-digestion to generate ATP and other essential biosynthetic molecules to temporarily avoid cell death in cellular stress induced by nutrient deprivation, hypoxia, and exposure to many chemotherapeutic agents.[131] Due to its ability to cross the blood–brain barrier, lucanthone is currently in phase II clinical studies as a single agent or in combination with temozolomide and radiation in primary therapy for glioblastoma multiforme, although its potential could be expanded to the treatment of lung cancer.

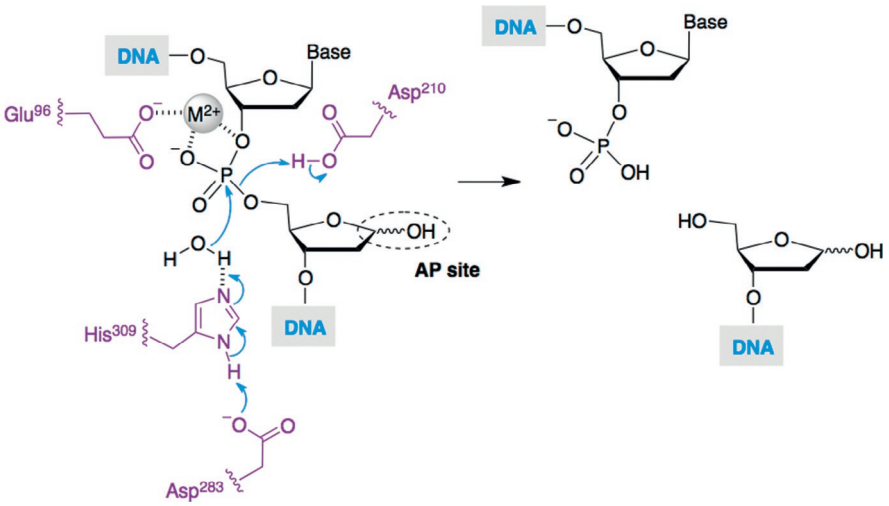
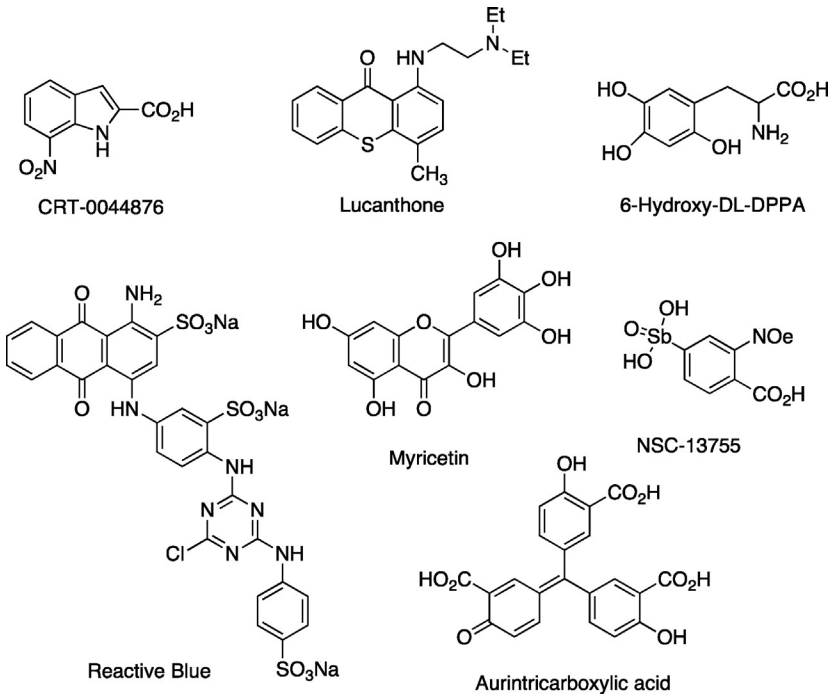


FIGURE 14.22

Mechanism of the reaction catalyzed by APE-1.

Since the reported effectiveness of CRT-0044876 came into question, novel direct inhibitors of APE-1 activity (i.e., 6-hydroxy-DL-DOPA, reactive blue 2, and myricetin) have been identified by screening the commercially available "library of pharmacologically active compounds" (LOPAC1280), a collection of well-characterized, druglike molecules representing all major target classes.[132] However, the three compounds have additional cellular targets, and chemical modification is difficult. In addition, 6-hydroxy-DL-DOPA is unstable in the presence of oxygen.

Another interesting compound discovered in this study is aurintricarboxylic acid (ATA), a potent inhibitor of APE-1 that, due to its promiscuity (it is a pan-selective inhibitor of DNA- and RNA-processing enzymes presumably because of its DNA-mimetic properties), is unattractive as a candidate agent for targeted combination therapy. The screening of a library of 2000 compounds showed that the arylstibonic acid derivative NSC-13755 has an significant APE-1 inhibitory effect.[133] However, its development is uncertain because in addition to the whole-organ toxicity potential of antimony-containing compounds, it may inhibit additional nucleic acid enzymes.



An indirect strategy to achieve inhibition of the APE-1 function is to chemically modify AP sites, making them unsuitable for APE-1 binding. For instance, methoxyamine (TRC102, MX) condensates with the tautomeric open-ring form of deoxyribose produced by DNA glycosylases blocks the BER pathway through a reaction that is faster than APE-1 binding (Figure 14.23).

Methoxyamine is mutagenic by itself because it converts cytosine bases into their N^4-methoxycytosine analogs that, due to the electron-withdrawing effect of the methoxy group, can exist as enamino or imino tautomeric forms (Figure 14.24a). While the enamino structure pairs with

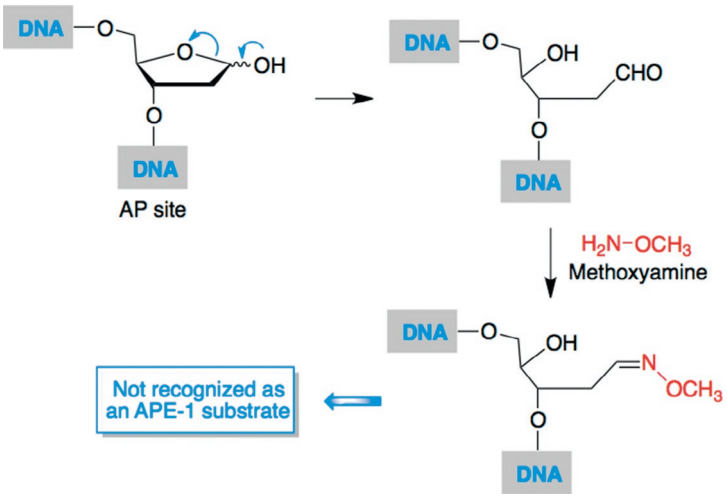


FIGURE 14.23

Inhibition of APE-1 following blockade of the AP sites by methoxyamine.

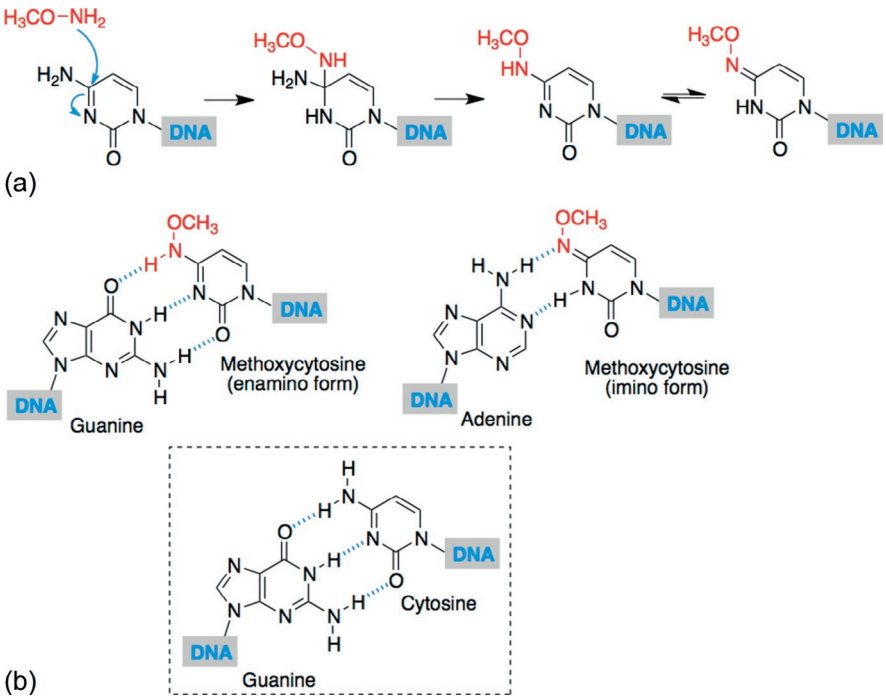


FIGURE 14.24

Chemical basis for the mutagenic activity of methoxyamine.

guanine forming three hydrogen bonds the same as unsubstituted cytosine, the imino tautomer is able to pair with adenine (Figure 14.24b).[134]

Methoxyamine potentiates the antitumor efficacy of alkylating agents such as temozolomide and carmustine in colon cancer and malignant glyoma xenogratfs[135] and entered phase I clinical assays in combination with temozolomide[136] or fludarabine in patients with solid tumors. It has been found that pretreatment with 5-iodouridine-deoxyribose (IUdR) and methoxyamine enhances the effects of ionizing radiation by causing a prolonged G_1 cell cycle arrest and by promoting stress-induced premature senescence.[137]

4.2.3 Inhibitors of Poly(ADP-Ribose) Polymerase

The last step of the BER pathway is a complex process that involves binding of DNA to PARP-1, which functions as a sensor of the strand breaks generated by APE-1. PARP-1 is an ubiquitous zinc finger DNA-binding enzyme that is activated by binding to DNA breaks and then catalyzes the synthesis of PAR branched polymers using NAD^+ as the building block. When PARP-1 binds to the nicked site, it becomes poly(ADP-ribosylated), and after some intermediate steps, it recruits enzymes involved in the repair process such as DNA Polβ.

PARP-1 catalyzes the reaction of Glu and Asp residues of nuclear proteins (including PARP itself, histones, or p53) with a NAD^+ molecule with displacement of nicotinamide to yield precursors **14.4**, which then undergo linear or branched polymerization to poly(ADP-ribose), as shown in Figure 14.25. In normal cells, the levels of these transient modifications are very low, but they increase 10–500 times after DNA damage.

Because of the negative charge of the ionized phosphate groups, these polymers help to open up the damaged DNA to allow access to DNA polymerases and DNA ligases.[138] In this way, the poly(ADP-ribosylation) of nuclear proteins by PARP-1 converts DNA damage into intercellular signals that activate either the BER pathway or cell death. If the BER process does not work properly because PARP is inhibited, the SSBs are accumulated with induction of DSBs, and the cell survival is more dependent on other repair pathways (i.e., HR). If the HR mechanism is also inhibited, for instance, because of mutation of breast cancer genes *BRC1* and *-2*, the cell survival is mostly dependent on the BER pathway and PARP inhibitors produce "synthetic lethality," which is defined as the result of simultaneous interaction of factors that would not be lethal if they were isolated (Figure 14.26).[139] Some PARP inhibitors have shown very interesting results in clinical trials when combined with cytotoxic drugs.[140]

PARP-1 activity is enhanced in many tumors, and its inhibition is associated with increased sensitivity to antitumor treatments that cause DNA strand breaks, including alkylating agents, topoisomerase I inhibitors, and ionizing radiation. For these reasons, PARP-1 seems to be pivotal in DNA repair processes and has become a target for anticancer therapy.[141,142] PARP-1 is believed to be one of the last effectors in the cascade of cell and tissue damage caused by ischemia and reperfusion injury. In situations of excessive DNA damage (e.g., acute exposure to a large pathological insult), overactivation of PARP results in cell-based energetic failure, leading to cellular necrosis, tissue injury, and organ damage or failure. Consequently, some PARP-1 inhibitors are being assayed for pathologies associated with damage caused by ROS.

Inhibitors of the first generation were designed on the basis of the NAD^+ binding site of this enzyme and are analogs of nicotinamide (e.g., 3-aminobenzamide), but they suffered from low activity and specificity. Nicotinamide, the second product of the PARP-catalyzed reaction, is itself a weak PARP inhibitor. A subsequent screening of 170 compounds allowed the identification of several heterocyclic

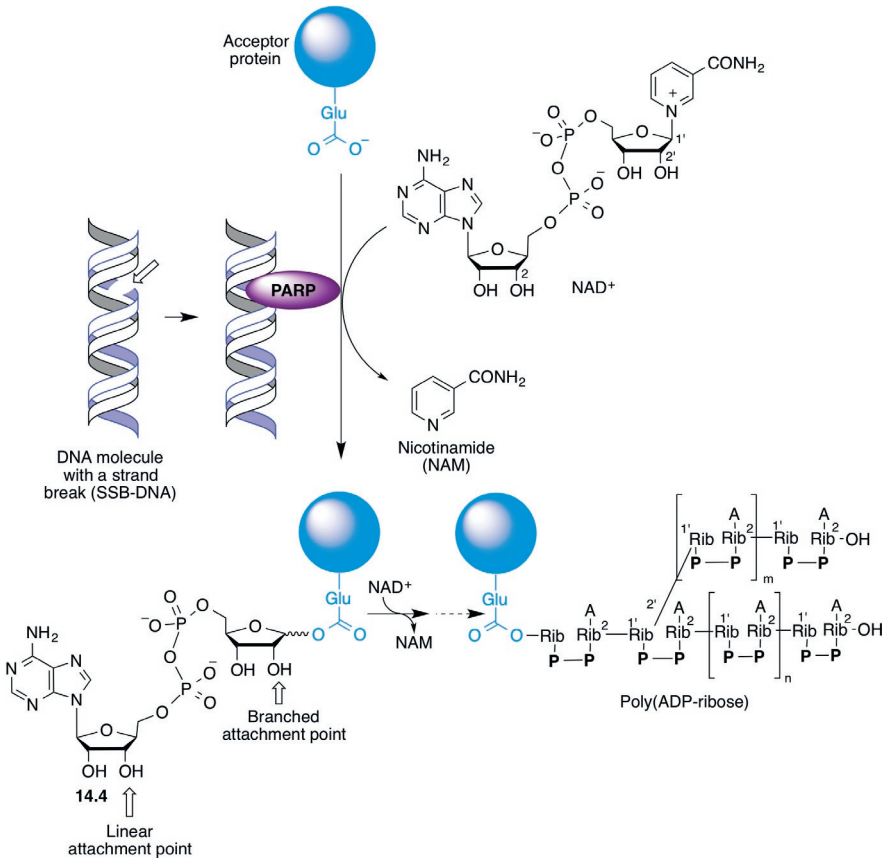


FIGURE 14.25

Poly-ADP-ribosylation of nuclear proteins.

systems that were used as leads for subsequent optimization and also to establish some of the structural features required for potent PARP inhibitory activity. These include an electron-rich aromatic or heteroaromatic system with a noncleavable bond at the position corresponding to C-3 of the benzamides, restricted rotation around the Ar–CO bond so that the carbonyl group is anti with respect to the C_1–C_2 bond of the aromatic ring, and one amide group for hydrogen bonding (Figure 14.27).[143] These SAR conclusions were rationalized by the subsequent resolution of the crystal structure of the PARP catalytic domain complexed with some inhibitors, which showed that the carbonyl oxygen forms two hydrogen bonds, with Ser-904 and Gly-863, and the amide nitrogen is a hydrogen bond donor to Gly-863. The knowledge gained from these studies led to the preparation of several fused tricyclic indoles, benzimidazoles, and other scaffolds containing a lactam function.

INO-1001[144] enhances the antitumor effects of doxorubicin in p53-deficient breast cancer and is in clinical trials for several types of cancer in combination with temozolomide.[145] Iniparib (BSI-201) is not really a PARP inhibitor, although it was first considered as such. Its development was discontinued

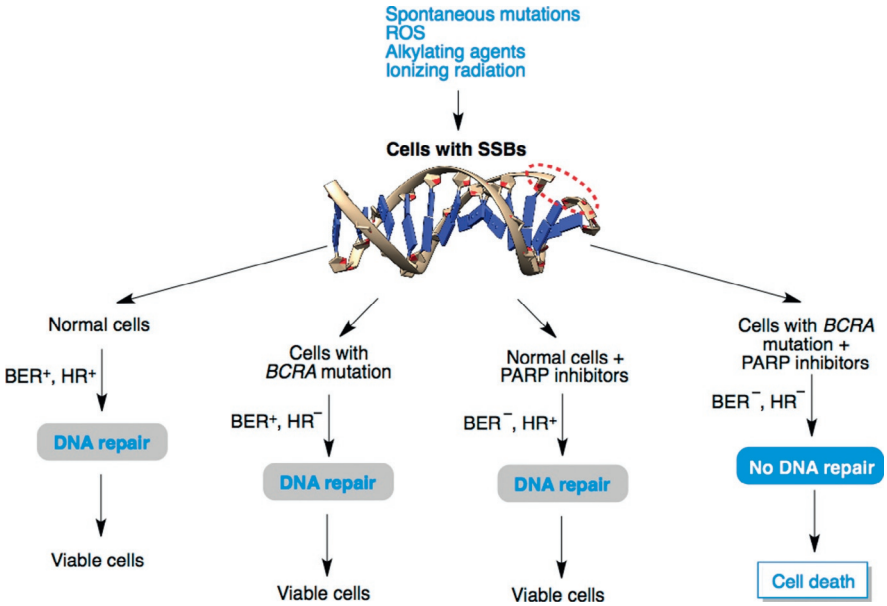


FIGURE 14.26

The role of PARP inhibitors in cancer therapy.

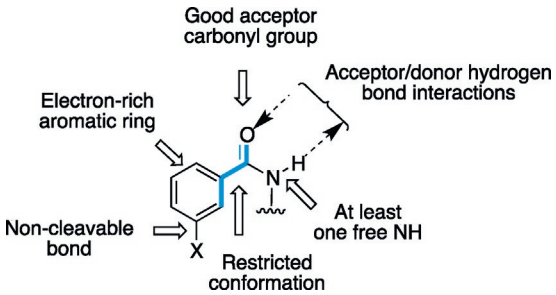
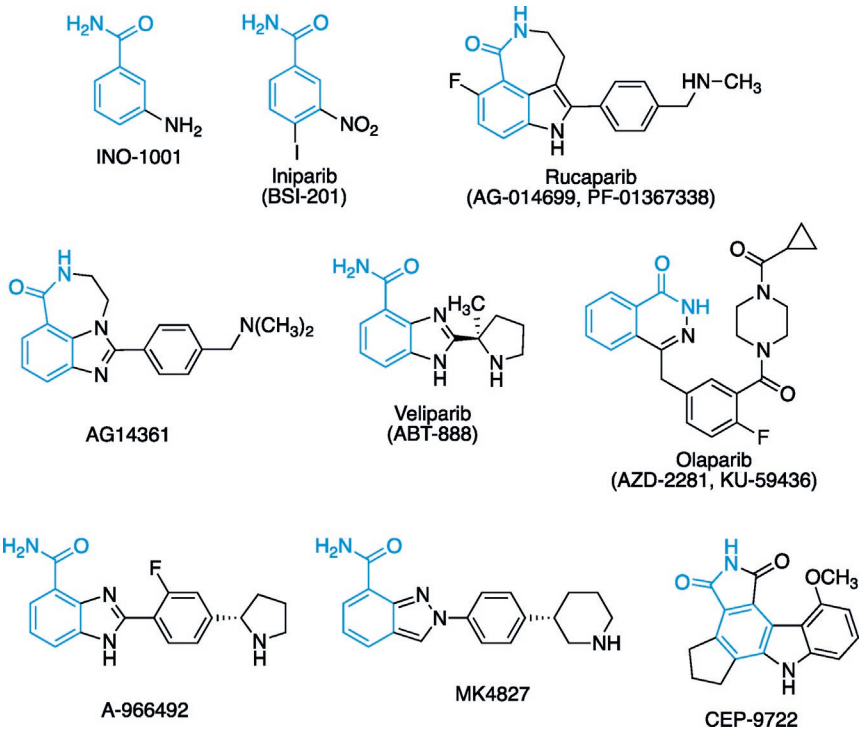


FIGURE 14.27

SAR conclusions for PARP inhibitors.

in 2011 after the disappointing results obtained from a phase III clinical study. Rucaparib (AG014699, PF-01367338), an analog of AG14361, was the first clinically studied PARP inhibitor in combination with temozolomide.[146] This combination is well tolerated, and PARP inhibition was observed at all dose levels studied, with increased SSBs in all patients with melanoma, pancreas and prostate cancer, among other tumors. Based on these results, a phase II study was conducted in patients with metastatic malignant melanoma.[147] Olaparib (AZD-2281) is an orally active PARP inhibitor lethal for BRCA-deficient cells that was tested in women with advanced ovarian cancer and showed mutation in *BRCA1* or *BRCA2* genes.[148] After very good results were obtained in phase I clinical studies, it entered phase II

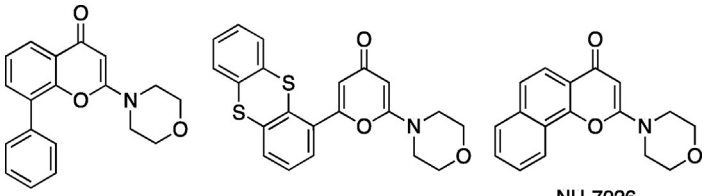
assays. However, due to its secondary effects, an advanced phase III study was interrupted in 2011. Veliparib (ABT-888) is another potent PARP inhibitor[149] that enhances the temozolomide effects in different cancer models, being a radiosensitizer in acute hypoxia conditions.[150] Unfortunately, it causes important myelosuppression. Another clinically evaluated compound is CEP-9722,[151] a prodrug that attenuated *in vivo* PARP activity and resulted in significant chemosensitization of temozolomide and irinotecan.[152]



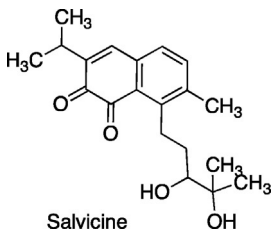
4.3 INHIBITORS OF ENZYMES INVOLVED IN DOUBLE-STRAND DNA BREAK REPAIR PATHWAYS

DSBs generated by ionizing radiation and ROS, or indirectly by DNA-damaging anticancer drugs such as alkylating agents and topoisomerase inhibitors, are repaired by either the HR or the NHEJ pathways. The enzymes involved in these pathways, members of the phosphatidylinositol 3-kinase (PI3K) superfamily, can be considered as molecular sensors of DSBs and have become anticancer targets because their inhibition confers radio- or chemosensitization to tumor cells.[153] The most relevant targets in this area[154] are the ATM, which is involved in the HR pathway and plays a critical role in the maintenance of genome integrity by triggering DNA damage sensors through phosphorylation of downstream targets such as p53,[155] and the DNA-PK, which is involved in the NHEJ pathway. The search for drugs that inhibit these pathways started from two nonselective PI3K inhibitors (see Chapter 10), namely

wortmannin (Wtmn) and LY-294002. Wortmannin (see Chapter 10, Section 5.4.1) is an effective radiosensitizer that failed in its clinical translation because of its poor water solubility and toxicity, although a nanoparticle formulation has recently established its potential.[156] LY-294002 significantly sensitizes ATM-proficient cells to ionizing radiation and DBS-inducing chemotherapeutics,[157] but its relatively low stability, fast metabolic degradation, and *in vivo* toxicity have prevented its clinical evaluation in humans as a radio- or chemosensitizer. The ATM-selective inhibitor KU-55933 was discovered by screening a combinatorial library based on LY-294002 structure.[158] This compound inhibits cancer cell proliferation by inducing G_1 cell cycle arrest through the downregulation of the synthesis of cyclin D1, and it has been proposed as a chemotherapeutic agent in cancers resistant to traditional chemo- or immunotherapy due to aberrant activation of AKT.[159] The structurally related NU-7026[160] impairs cellular DNA DSB repair through inhibition of DNA-dependent protein kinase[161] and decreases survival in cells exposed to ionizing radiation,[162] potentiating the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia.



Salvicine, a structurally modified diterpenoid quinone derived from *Salvia prionitis*, is a nonintercalative topoisomerase II poison (see Chapter 7, Section 6.3.2) that has entered phase II clinical trials and possesses a broad range of antitumor and antimetastatic activity. This compound simultaneously damages DNA and disrupts DNA repair by inhibiting DNA-PK activity through a mechanism that involves the generation of ROS probably associated with its *ortho*-quinone structure.[163]



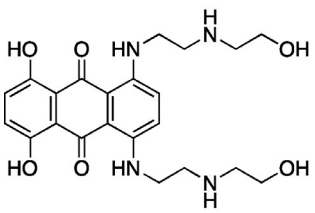
5 ANTITUMOR DRUG RESISTANCE RELATED TO CELLULAR ADHESION MOLECULES

Integrin-mediated signaling between tumor cells and the extracellular matrix plays a critical role in the resistance of certain leukemias to chemotherapy. Most adult patients with *de novo* AML achieve an initial complete remission after chemotherapy treatments. However, many of them relapse by emergence of acquired drug resistance probably induced by activation of the PI3K/AKT/Bcl-2 signaling

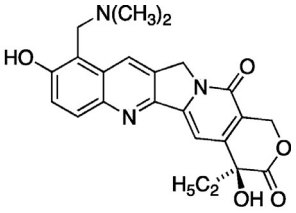
pathway (see Section 5.4 of Chapter 10 and Section 8.1 of Chapter 11), which is triggered by the attachment of α4β1 integrin to the glycoprotein of the extracellular matrix fibronectin on plasma membranes of leukemic cells. For this reason, the integrin antagonist AS101 (see Chapter 11, Section 4) could be combined with conventional cytotoxic drugs to target tumor cell resistance in AML.[164] This compound also sensitizes the human aggressive glioblastoma multiforme tumor to paclitaxel through inhibition of the cytokine interleukin-10.[165] Their radioprotective effects prevent the induction of DSBs in patients with radiation therapy overdose or accidental irradiation[166] by enhancing the ability of irradiated cells to repair their damaged DNA through an increase in the DNA polymerase activity.

6 ANTITUMOR DRUG RESISTANCE RELATED TO THE EXTRACELLULAR PH: TUMOR-ASSOCIATED CARBONIC ANHYDRASE AS AN ANTICANCER TARGET

In contrast to normal tissues (pH ≈ 7.4), most hypoxic tumors are acidic (pH ≈ 6). Tumor cells decrease their extracellular pH by two mechanisms that are enhanced in hypoxic conditions: production of lactic acid as a consequence of a higher glycolysis rate (the Warburg effect) and hydration of CO_2 catalyzed by the tumor-associated carbonic anhydrase IX isoform. Because drugs that are weakly ionized (e.g., mitoxantrone, paclitaxel, and topotecan) enter cells by passive diffusion in their nonionized form, variations in extracellular pH alter this ionization-dependent diffusion. Acidic extracellular pH values hamper the uptake of basic drugs into the tumor cells because their predominant ionized forms do not diffuse through cell membranes, and for this reason, enhancement of the extracellular pH by chronic ingestion of a sodium bicarbonate solution improves the cytotoxicity of these drugs.



Mitoxantrone



Topotecan

The carbonic anhydrase IX (CA IX) extracellular catalytic domain contributes to acidification of the tumor environment through hydration of carbon dioxide to bicarbonate and protons, hampering the absorption of drugs by passive diffusion (Figure 14.28). This enzyme is highly overexpressed in many types of cancer because it is regulated by the hypoxia inducible factor 1 (HIF-1), and these high levels correlate with chemoresistance to weakly basic anticancer drugs.

In addition, low extracellular pH values have other effects that include extracellular matrix breakdown, migration, invasion, induction of cell growth factors, and protease activation. CA IX inhibitors may overcome these effects and also inhibit the expression of aquaporin, a water channel protein that might be implicated in vascular permeability in tumors. The involvement of CA IX in alterations of the pH balance in tumor tissues explains the antitumor effects found for many carbonic anhydrase inhibitors.[167] The most advanced one is the sulfonamide indisulam (E-7070), which has a complex

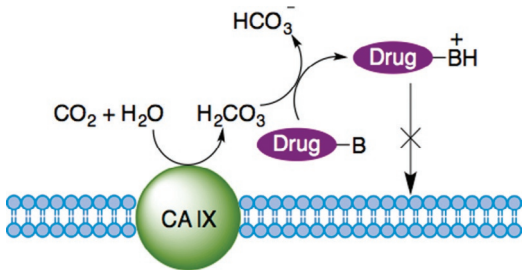
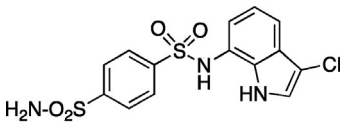


FIGURE 14.28

The role of carbonic anhydrase IX in anticancer drug resistance.

mechanism of antitumor action and is under clinical development for the treatment of solid tumors (see Chapter 10, Section 5.1).[168]



Indisulam (E-7070)

7 THE ROLE OF THE SPARC PROTEIN IN DRUG RESISTANCE

In addition to its role in cancer progression (Chapter 11, Section 8.2), the "secreted protein, acidic and rich in cysteine" (SPARC) also participates in sensitizing therapy-resistant cancers. The *SPARC* gene was identified as a putative resistance-reversal gene in colorectal cancer resistance to chemotherapy with 5-fluorouracil and irinotecan.[169] Following exposure to irinotecan and oxaliplatin, these cells also induce upregulation of the ATP-binding cassette family member ABCG2 and the component of the nucleotide excision repair ERCC1.[170]

Gene profiling has found that SPARC is a part of the invasion-specific cluster in breast, lung, pancreas, and prostate cancer, where SPARC expression is associated with invasiveness and metastasis. High levels of SPARC also indicate a worse prognosis in gastric cancer. To identify patients who are likely to become resistant to therapy, a nanoparticle-based imaging agent that targets SPARC has been developed as a molecular marker of prostate cancer metastatic potential. For this purpose, a SPARC-targeted peptide sequence was attached to a biocompatible nanoparticle that was also coupled to a fluorophore for *in vivo* imaging.[171]

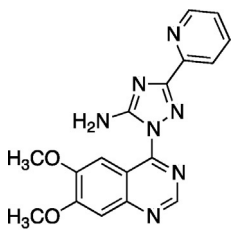
8 RADIORESISTANCE AND TUMOR RADIOSENSITIZATION

In several solid tumors, including lung, head and neck, gastrointestinal, and brain tumors, radiation is combined with standard cytotoxic chemotherapeutic agents. These radio-chemotherapy regimens provide local tumor control induced by radiation while chemotherapy is intended for metastases. However, the associated chemotherapy may also have a direct effect on the tumor cells by enhancing their

radiosensitivity, defined as an increase in cellular susceptibility to radiation-induced death. Some compounds used as radiosensitizers especially in hypoxic tumors have already been mentioned (see Chapter 4, Section 11). Here, a few examples of the main mechanism-based approaches to radiosensitization are discussed.[172]

One of these approaches is the combination of "anti-Ras" prenyl transferase inhibitors (PTIs) with radiotherapy. The causal link between aberrant Ras activity and tumor cell radioresistance has been well-established, but the specific mechanisms involved remain undefined because the analysis of clinical effects of this combination is complicated due to the fact that these drugs inhibit the prenylation of more than 100 proteins besides Ras.[173] Because radiation induces the immediate activation of all epidermal growth factor receptors, these may play a cytoprotective role in cellular radioresponse and may be a target for radiosensitization. In fact, the positive results obtained from a phase III trial comparing once-daily radiation to once-daily radiation plus the EGFR-targeting monoclonal antibody cetuximab (Erbitux®) led to FDA approval of this monoclonal antibody as a radiationsensitizer.[174] Heat shock protein 90 (Hsp90) also plays a critical role in establishing resistance to radiation therapy, and its inhibition sensitizes tumors to radiation.[175] Unfortunately, many of the Hsp90 inhibitors currently in clinical trials exhibit high toxicities, hindering their clinical use. Ganetespib (STA-9090), a second-generation Hsp90 inhibitor that has shown potent preclinical activity and is currently in several phase II trials across a broad range of indications (see Chapter 11, Section 8.2), offers a promising strategy for improving the outcome of radiotherapy in human cancers.[176]

The potential of epigenetic drugs as radiosensitizing agents has been centered in histone deacetylase inhibitors that, when administered before and after the radiation, induce tumor radiosensitization and may protect against normal tissue injury.[177] Some trials with SAHA or valproic acid combined with radiotherapy are ongoing. Mutant cells lacking components of the NHEJ repair pathway are very radiosensitive.[178] Accordingly, inhibitors of essential enzymes in this pathway enhance the radiosensitivity of tumor cell lines, although their selectivity is still unclear.[179] A similar situation occurs with PARP-1 inhibitors.[180] Because activation of the cell cycle checkpoint G_1 is an essential feature of the DNA damage response and provides a radioprotective effect, checkpoint kinase 1 (Chk1) inhibitors are radiosensitizers, and some of which are being clinically evaluated.[181] The ATM protein kinase activates signal transduction pathways in response to DNA damage that are essential for coordinating cell cycle progression with DNA repair. Accordingly, ATM is a potential target for novel inhibitors that could be used to enhance tumor cell sensitivity to radiotherapy. One of these inhibitors, CP466722, was suggested for potential clinical application, although whether this agent affects normal cell radiosensitivity remains a critical question.[182]



CP466722

Radiation can induce the expression of pro-angiogenic factors, including the potent pro-survival factor vascular endothelial growth factor (VEGF), which may lead to radiation resistance. Thus, delivery of

antiangiogenic agents that block the release of VEGF or its downstream effects may lead to enhanced tumor response to radiation. However, because oxygen is the most important molecule in stabilizing the DNA damage induced by radiation and angiogenesis inhibitors decrease oxygen levels, the combination of an angiogenesis inhibitor with radiation would seem contrary to common sense. Nevertheless, these combinations result in an improved antitumor effect, perhaps because improved sensitivity to radiation induced by angiogenesis inhibitors corresponds to the rapid phase of tumor growth and to the highest proliferation rates of the tumor vasculature.

9 INDUCED TUMOR CHEMORESISTANCE

In addition to the intrinsic nonresponsiveness of tumors to a given chemotherapy, they have the ability to induce or accentuate mechanisms that enable adaptive or evasive resistance after an initial response phase, leading to renewed tumor growth and progression. Several mechanisms participate in the generation of chemoresistance, which is one of the major problems in the fight against cancer. Unfortunately, an integrated understanding of acquired drug resistance involving intratumor or tumor microenvironment processes is still lacking.

In this section, chemoresistance to anti-VEGF, EGFR-targeted therapies, and ALK mutations is briefly discussed.

9.1 CHEMORESISTANCE TO ANTI-VEGF THERAPIES

Adaptive or evasive resistance in response to antiangiogenic therapy after an initial response phase involves mechanisms that enable neovascularization or reduce dependence of tumor growth with respect to new blood vessels. The clinical achievements with bevacizumab, sunitinib, and sorafenib constitute a milestone for the field of angiogenesis research, with survival benefits in many aggressive tumors, but these VEGF pathway inhibitors have failed to produce enduring clinical responses in most patients.[183]

9.2 CHEMORESISTANCE TO EGFR-TARGETED THERAPIES

Therapies targeting EGFR signaling are part of the arsenal of agents that are used to treat lung, colorectal, pancreatic, and head and neck cancers, and they were discussed in Chapter 10, Section 4.1. The initial enthusiasm over substantial clinical responses to EGFR tyrosine kinase inhibitors (TKIs), including monoclonal antibodies, has been tempered by the identification of an ever-increasing number of *de novo* and acquired resistance mechanisms. In fact, all patients with metastatic lung, colorectal, pancreatic, or head and neck cancers who initially benefit from EGFR-targeted therapies eventually develop resistance.

Mechanisms of resistance to EGFR small-molecule inhibitors or antibodies may be grouped into four categories that have been validated in patients with resistant lung adenocarcinoma (Figure 14.29). Some of these mechanisms, such as T790M mutation, occur in both acquired and innate resistance.[184]

Mutations of EGFR confer a drug-resistant state that does not diminish the kinase activity of the receptor but, rather, enhances its affinity for ATP while decreasing its affinity for the EGFR inhibitors. Among them, mutation T790M is found in approximately 60% of patients with acquired resistance and abrogates the activity of gefitinib or erlotinib. The third-generation, irreversible inhibitors AZD-9291

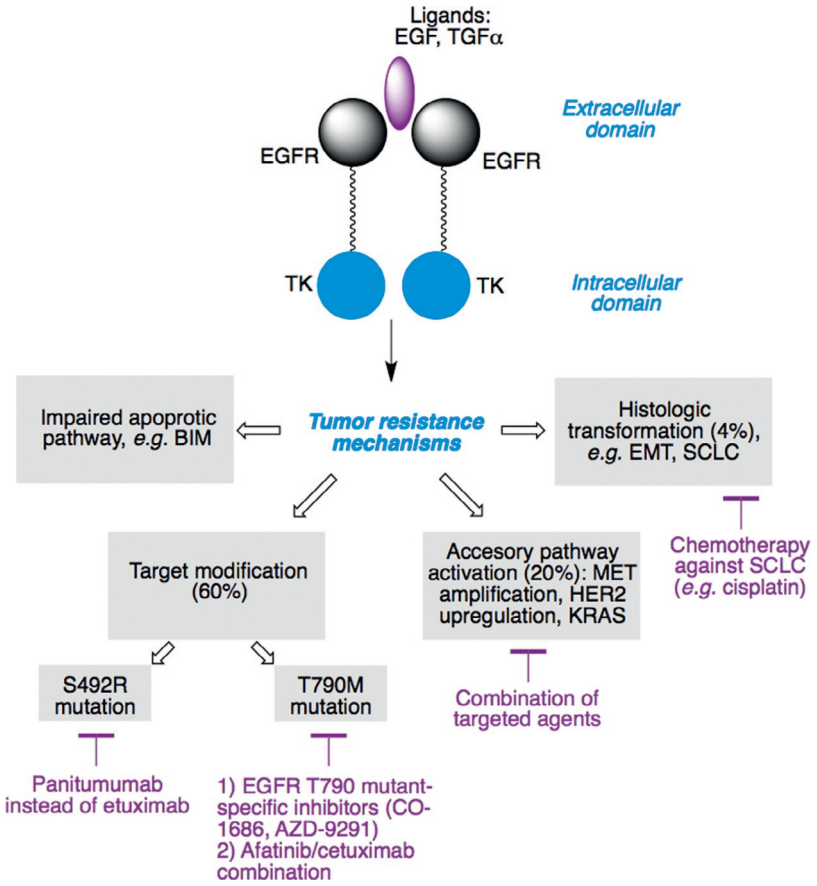
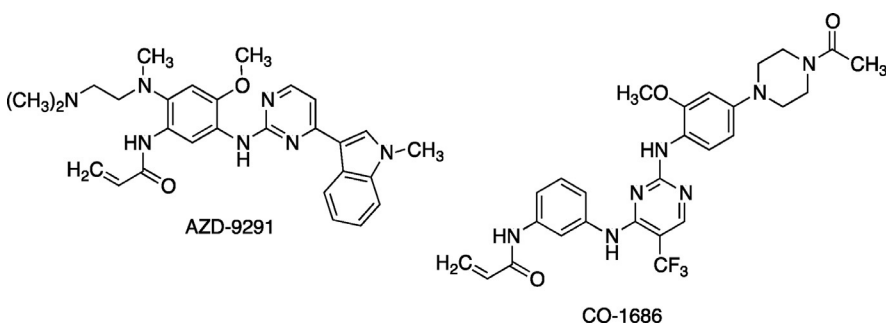


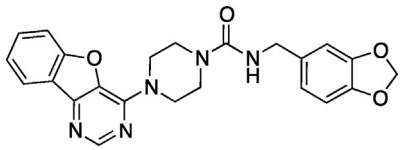
FIGURE 14.29

Mechanisms of resistance to EGFR inhibitors.

and CO-1686 are active against T790M-mutated EGFR.[185] AZ-5104, a metabolite of AZD-9291, shows the same activity.[186]



Other mutations, such as S492R, abrogate the activity of cetuximab but do not affect panitumumab. The epithelial-to-mesenchymal transition (EMT) is an EGFR resistance mechanism that was found in lung adenocarcinoma patients treated with EGFR TKIs. This process may be mediated, among other pathways, by the activation of the AXL tyrosine kinase receptor along with AKT activation. The name AXL is derived from the Greek word *anexelekto*, which means "uncontrolled." The AXL inhibitor MP-470 and the multitarget kinase inhibitor foretinib (discussed in Chapter 10, Section 4.7.1) restore the sensitivity to erlotinib in those patients.[187]

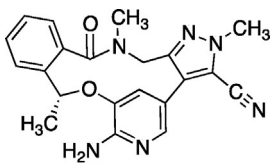


MP-470

Despite EGFR inhibition, because loss of one node in signaling pathways that stimulate cell growth diverts prosurvival or proliferation stimuli through other nodes, a bypass signaling pathway such as MET, HER-2, or *KRAS*, is often activated or upregulated leading to persistent activation of downstream signaling. To overcome this resistance, it would be necessary to combine differently targeted agents. Another important resistance mechanism in this pathway is the germline intronic deletion that removes the BH3 domain of Bcl-2 interacting protein (BIM), which precludes apoptosis. Resistance through histologic transformation may also occur in a minority of patients. Patients in which EGFR TKI-resistant lung adenocarcinomas are transformed into small cell lung cancers (SCLC) may benefit from treatment with etoposide and cisplatin, which is a standard chemotherapy regimen for this type of lung cancer.

9.3 CHEMORESISTANCE TO ALK MUTATIONS

Anaplastic lymphoma kinase (ALK) F1174L38 mutations, similarly to EGFR T790M mutations, increase the kinase affinity for ATP by approximately fivefold, which decreases sensitivity to ATP-competitive reversible inhibitors such as erlotinib and crizotinib. Pfizer's investigational agent PF-06463922, developed for patients with ALK-mutated non-small cell lung cancer who have developed resistance to crizotinib, demonstrated strong activity and selectivity in preclinical research and will be studied in a phase I clinical trial.



PF-06463922

REFERENCES

1 Gottesman MM, Fojo T, Bates SE. *Nature Rev Cancer* 2002;**2**:48.
2 (a) Morris PG, McArthur HL, Hudis CA. *Expert Opin Pharmacother* 2009;**10**:967; (b) Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, et al. *Cell* 2012;**150**:165.
3 Yadav VK, Kumar A, Mann A, Aggarwal S, Kumar M, Roy SD, et al. *Nucleic Acids Res* 2014;**42**:764.
4 Kerr D, Middleton M. *Curr Opin Pharmacol* 2006;**6**:321.
5 Sperinde J, Jin X, Banerjee J, Penuel E, Saha A, Diedrich G, et al. *Clin Cancer Res* 2010;**16**:4226.
6 For a review, seeLage H. *Cell Mol Life Sci* 2008;**65**:3145.
7 Flügge UI, van Meer G. *FEBS Lett* 2006;**580**:997.
8 For a review on the ABC efflux pump-based resistance to chemotherapy drugs, see Eckford PDW, Sharom FJ. *Chem Rev* 2009;**109**:2989.
9 Gottesman MM, Ling V. *FEBS Lett* 2006;**580**:998.
10 Dantzig AH, Alwis DP, Burgess M. *Adv Drug Deliv Rev* 2003;**55**:133.
11 Modok S, Mellor HR, Callaghan R. *Curr Opin Pharmacol* 2006;**6**:350.
12 Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, et al. *Science* 2009;**323**:1718.
13 Jin MS, Oldham ML, Zhang Q, Chen J. *Nature* 2012;**490**:566.
14 Schmid D, Ecker G, Kopp S, Hitzler M, Chiba P. *Biochem Pharmacol* 1999;**58**:1447.
15 Sharom FJ. *Front Oncol* 2014;**4**:article 41.
16 Ferte J. *Eur J Biochem* 2000;**267**:277.
17 Eytan GD. *Biomed Pharmacother* 2005;**59**:90.
18 Urbatsch IL, Sankaran B, Weber J, Senior AE. *J Biol Chem* 1995;**270**:19383.
19 Karwatsky J, Lincoln MC, Georges E. *Biochemistry* 2003;**42**:12163.
20 For representative reviews, see. (a) Avendaño C, Menéndez JC. *Curr Med Chem* 2002;159; (b) Avendaño C, Menéndez JC. *Med Res Rev Online* 2004;**1**:419; (c) Teodori E, Dei S, Martelli C, Scapecchi S, Gualtieri F. *Curr Drug Targets* 2006;**7**:893; (d) Falasca M, Linton KJ. *Expert Opin Invest Drugs* 2012;**21**:657.
21 Dantzig AH, Alwis DP, Burgess M. *Adv Drug Deliv Rev* 2003;**55**:133.
22 Zhou SF, Zhou ZW, Li CG, Chen X, Yu X, Xue CC, et al. *Drug Discov Today* 2007;**12**:664.
23 Bailey D, Spence J, Muñoz C, Arnold J. *Lancet* 1991;**337**:268.
24 Choi JS, Shin SC. *Int J Pharm* 2005;**292**:149.
25 Shin SC, Choi JS, Li X. *Int J Pharm* 2006;**313**:144.
26 Ohtani H, Ikegawa T, Honda Y, Kohyama N, Morimoto S, Shoyama Y, et al. *Pharm Res* 2007;**24**:1936.
27 Bansal T, Jaggi M, Khar RK, Talegaonkar S. *J Pharm Pharmaceut Sci* 2009;**12**:46.
28 Zhang S, Morris ME. *J Pharmacol Exp Ther* 2003;**304**:1258.
29 Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. *Cancer Res* 1981;**41**:1967.
30 Garrigues A, Loiseau N, Delaforge M, Ferté J, Garrigos M, André F, et al. *Mol Pharmacol* 2002;**62**:1288.
31 Pleban K, Ecker GF. *Mini Rev Med Chem* 2005;**5**:153.
32 Klopman G, Srivastava S, Kolossvary I, Epand RF, Ahmed N, Epand RM. *Cancer Res* 1992;**52**:4121.
33 Seelig A, Eur J. *Biochem* 1998;**251**:252.
34 Seelig A, Gatlik-Landwojtowicz E. *Mini Rev Med Chem* 2005;**5**:135.
35 Seelig A, Landwojtowicz E, Fischer H, Blatter X-L. van de Waterbeemd H, Lennernäs H, Artursson P, editors. *Drug bioavailability*. Weinheim, Germany: Wiley-VCH; 2003 [chapter 20].
36 Klopman G, Shi LM, Ramu A. *Mol Pharmacol* 1997;**52**:323.
37 Wang RB, Kuo CL, Lien LL, Lien EJ. *J Clin Pharm Ther* 2003;**28**:203.
38 Hendrich AB, Michalak K. *Curr Drug Targets* 2003;**4**:23.
39 Castaing M, Loiseau A, Dani M. *J Pharm Pharmacol* 2001;**53**:1021.
40 Broccatelli F, Carosati E, Neri A, Frosini M, Goracci L, Oprea TI, et al. *J Med Chem* 2011;**54**:1740.

41 Palmeira A, Rodrigues F, Sousa E, Pinto M, Vasconcelos MH, Fernandes MX. *Chem Biol Drug Des* 2011;**78**:57.
42 Palmeira A, Vasconcelos MH, Paiva A, Fernandes MX, Pinto M, Sousa E. *Biochem Pharmacol* 2012;**83**:57.
43 (a) Palmeira A, Sousa E, Vasconcelos MH, Pinto M, Fernandes MX. *Curr Pharm Des* 2012;**18**:4197; (b) Lutful A. *Drug Target Insights* 2013;**7**:27.
44 For a review, see Palmeira A, Sousa E, Vasconcelos MH, Pinto MM. *Curr Med Chem* 2012;**19**:1946.
45 Ekins S, Kim RB, Leake BF, Dantzig AH, Schuet EG, Lan LB, et al. *Mol Pharmacol* 2002;**61**:974.
46 Bates S, Kang M, Meadows B, Bakke S, Choyke P, Merino M, et al. *Cancer* 2001;**92**:1577.
47 Kreis W, Budman DR, Calabro A. *Cancer Chemother Pharmacol* 2001;**47**:78.
48 Kolitz JE, George SL, Marcucci G, Vij R, Powell BL, Allen SL, et al. *Blood* 2010;**116**:1413.
49 Yanagisawa T, Newman A, Coley H, Renshaw J, Pinkerton CR, Pritchard Jones K. *Br J Cancer* 1999;**80**:1190.
50 Toppmeyer D, Seidman AD, Pollak M, Russell C, Tkaczuk K, Verma S, et al. *Clin Cancer Res* 2002;**8**:670.
51 Nokihara H, Yano S, Nishioka Y, Hanibuchi M, Higasida T, Tsuruo T, et al. *Jpn J Cancer Res* 2001;**92**:785.
52 Naito M, Matsuba Y, Sato S, Hirata H, Tsuruo T. *Clin Cancer Res* 2002;**8**:582.
53 Smith BJ, Doran AC, McLean S, Tingley III FD, O'Neill BT, Kajiji SM. *J Pharmacol Exp Ther* 2001;**298**:1252.
54 For a review, see Fox E, Bates SE. *Expert Rev Anticancer Ther* 2007;**7**:447.
55 Sorbera LA, Castaner J, Silvestre JS, Bayes M. *Drugs Future* 2003;**28**:125.
56 Tang R, Faussat AM, Perrot JY, Marjanovic Z, Cohen S, Storme T, et al. *BMC Cancer* 2008;**8**:51.
57 Guns ES, Denyssevych T, Dixon R, Bally MB, Mayer L. *Eur J Drug Metab Pharmacokinet* 2002;**27**:119.
58 Chi KN, Chia SK, Dixon R, Rewman MJ, Wacher VJ, Sikic B, et al. *Invest New Drugs* 2005;**23**:311.
59 http://www.clinicaltrials.gov/ct/gui/show/NCT00028873.
60 Moins N, Cayre A, Chevillard S, Maublant J, Verrelle P, Finat-Duclos F. *Anticancer Res* 2000;**20**:2617.
61 Coley HM. *Methods Mol Biol* 2010;**596**:341.
62 For a monograph, seeMorphy JR, Harris CJ. *Designing Multi-Target Drugs*. London: Royal Society of Chemistry; 2012.
63 Colabufo NA, Contino M, Berardi F, Perrone R, Panaro MA, Cianciulli A, et al. *Toxicol In Vitro* 2011;**25**:222.
64 Tosi P-F, Madoulet C, Nicolau C, Hickman DT. U.S. Patent 8,409,580. August 30, 2010.
65 Polgar O, Bates SE. *Biochem Soc Trans* 2005;**33**:241.
66 Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. *Nature Rev Drug Discov* 2006;**5**:219.
67 Sonneveld P. *J Intern Med* 2000;**247**:521.
68 Raub TJ. *Mol Pharm* 2005;**3**:3.
69 Patel VA, Dunn MJ, Sorojin A. *J Biol Chem* 2002;**277**:38915.
70 Plo I, Lehne G, Beckstrom KJ, Maestre N, Bettaieb A, Laurent G, et al. *Mol Pharmacol* 2002;**62**:304.
71 Jin S, Gorfajn B, Faircloth G, Scoto KW. *Proc Natl Acad Sci U S A* 2000;**97**:6775.
72 Wu H, Hait WN, Yang JM. *Cancer Res* 2003;**63**:1515.
73 Dassow H, Lassner D, Remke H, Preiss R. *Int J Clin Pharmacol Ther* 2000;**38**:209.
74 Capella LS, Alcantara JSM, Moura-Neto V, Lopes AG, Capella MAM. *Tumor Biol* 2000;**21**:54.
75 Binkhathian Z, Lavasanifar A. *Curr Cancer Drug Targets* 2013;**13**:326.
76 Brigger I, Dubernet C, Couvreur P. *Adv Drug Deliv Rev* 2002;**54**:631.
77 Gaber MH. *J Biochem Mol Biol Biophys* 2002;**6**:309.
78 Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, et al. *Nature Rev Drug Discov* 2010;**9**:215.
79 Agarwal S, Arya V, Zhang L. *J Clin Pharmacol* 2013;**53**:228.
80 Wang W, Ballatori N. *Pharmacol Rev* 1998;**50**:335.
81 Zejia Y, Faustino PJ, Andrews PA, Monastra R, Rasmussen AA, Ellison CD, et al. *Cancer Chemother Pharmacol* 2000;**46**:255.

82 Voehringer D. *Free Radical Biol Med* 1999;**27**:945.
83 Yao KS, Godwing AK, Johnson SW, Ozols RF, O'Dwyer PJ, Hamilton TC. *Cancer Res* 1995;**55**:4367.
84 Sipos EP, Witham TF, Ratan R, Burger PC, Baraban J, Li KW, et al. *Neurosurgery* 2001;**48**:392.
85 Lai GM, Moscow JA, Álvarez MG, Fojo AT, Bates SE. *Int J Cancer* 1991;**49**:688.
86 Rudin CM, Yang Z, Schumaker LM, Vander Weele DJ, Newkirk K, Egorin MJ, et al. *Cancer Res* 2003;**63**:312.
87 Chen X, Carystinos GD, Batist G. *Chem Biol Interact* 1998;**111**:263.
88 Griffith OW. *J Biol Chem* 1982;**257**:13704.
89 Fojo T, Bates S. *Oncogene* 2003;**22**:7512.
90 Lee HR, Cho J-M, Shin D, Yong CS, Choi H-G, Wakabayashi N, et al. *Mol Cell Biochem* 2008;**318**:23.
91 Hamilton D, Wu JH, Batist G. *Mol Pharmacol* 2007;**71**:1140.
92 Nagata J, Kijima H, Hatanaka H, Asai S, Miyachi H, Takagi A, et al. *Biochem Biophys Res Commun* 2001;**286**:406.
93 For a review, see Oakley A. *Drug Metab Rev* 2011;**43**:138.
94 Rebbeck TR, Walker AH, Jaffe JM, White DL, Wein AJ, Malkowicz SB. *Cancer Epidemiol Biomarkers Prev* 1999;**8**:283.
95 Stoehlmacher J, Park DJ, Zhang W, Groshen S, Tsao-Wei DD, Yu MC, et al. *J Natl Cancer Inst* 2002;**94**:936.
96 Morales GA, Laborde E. *Annu Rep Med Chem* 2007;**42**:321.
97 Oakley AJ, Lo Bello M, Mazzetti AP, Federici G, Parker MW. *FEBS Lett* 1997;**419**:32.
98 Van Zanden JJ, Ben HO, van Iersel ML, Boeren S, Cnubben NH, Lo BM, et al. *Chem Biol Interact* 2003;**145**:139.
99 Townsend DM, Tew KD. *Oncogene* 2003;**22**:7369.
100 Raza A, Galili N, Callander N, Ochoa L, Piro L, Emanuel P, et al. *J Hematol Oncol* 2009;**2**:20.
101 Raza A, Galili N, Smith SE, Godwin J, Boccia RV, Myint H, et al. *Cancer* 2012;**118**:2138.
102 Raza A, Galili N, Mulford D, Smith SE, Brown GL, Steensma DP, et al. *J Hematol Oncol* 2012;**5**:18.
103 Galili N, Tamayo P, Botvinnik OB, Mesirov JP, Brooks MR, Brown G, et al. *J Hematol Oncol* 2012;**5**:20.
104 For a review, see Sancar A, Lindsey-Boltz LA, Unsal-Kaçmaz K, Linn S. *Annu Rev Biochem* 2004;**73**:39.
105 Bentle MS, Bey EA, Dong Y, Reinicke KE, Boothman DA. *J Mol Histol* 2006;**37**:1567.
106 Madhusudan S, Hickson ID. *Trends Mol Med* 2005;**11**:503.
107 Ding J, Miao Z-H, Meng L-H, Geng M-Y. *Trends Pharmacol Sci* 2006;**27**:338.
108 Curtin N. *Expert Opin Ther Targets* 2007;**11**:783.
109 For a review, see Ljungman M. *Chem Rev* 2009;**109**:2929.
110 Gerson SL. *J Clin Oncol* 2002;**20**:2388.
111 Sabharwal A, Middleton MR. *Curr Opin Pharmacol* 2006;**6**:355.
112 Bobola MS, Silber JR, Ellenbogen RG, Geyer JR, Blank A, Goff RD. *Clin Cancer Res* 2005;**11**:2747.
113 Gerson GL. *Nature Rev Cancer* 2004;**4**:296.
114 (a) Ranson M, Middleton MR, Bridgewater J, Lee SM, Dawson M, Jowle D, et al. *Clin Cancer Res* 2006;**12**:1577; (b) Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, Rich JN, et al. *J Clin Oncol* 2009;**27**:1262.
115 Khan OA, Ranson M, Michael M, Olver I, Levitt NC, Mortimer P, et al. *Br J Cancer* 2008;**98**:1614.
116 Apisarnthanarax N, Wood GS, Stevens SR, Carlson S, Chan DV, Liu L, et al. *Arch Dermatol* 2012;**148**:613.
117 (a) Friedman HS, Keir S, Pegg AE, Houghton PJ, Colvin OM, Moschel RC, et al. *Mol Cancer Ther* 2002;**1**:943; (b) Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, Friedman AH, et al. *J Neurooncol* 2009;**95**:393.
118 Paranjpe A, Srivenugopal KS. *Cancer Res* 2012;**72**:4685.
119 (a) Silvani A, Eoli M, Salmaggi A, Lamperti E, Maccagnano E, Broggi G, et al. *J Neurooncol* 2004;**66**:203; (b) Zustovich F, Lombardi G, Della Puppa A, Rotilio A, Scienza R, Pastorelli D. *Anticancer Res* 2009;**29**:4275.

120 Seo Y, Kinsella TJ. *Cancer Res* 2009;**69**:7285.
121 Robertson AB, Klungland A, Rognes T, Leiros I. *Cell Mol Life Sci* 2009;**66**:981.
122 Dinner AR, Blackburn GM, Karplus M. *Nature* 2001;**413**:752.
123 Vik ES, Alseth I, Forsbring M, Helle IH, Morland I, Luna L, et al. *DNA Repair* 2012;**11**:766.
124 Deng L, Schärer OD, Verdine GL. *J Am Chem Soc* 1997;**119**:7865.
125 Ide H, Kotera M. *Biol Pharm Bull* 2004;**27**:480.
126 Schärer OD, Jiricny J. *Bioessays* 2001;**23**:270.
127 For a review, see Wilson DF, Simeonov A. *Cell Mol Life Sci* 2010;**67**:3621.
128 Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, et al. *Nucleic Acid Res* 2005;**33**:4711.
129 Luo M, Kelley MR. *Anticancer Res* 2004;**24**:2127.
130 Naidu MD, Agarwal R, Pena LA, Cunha L, Mezei M, Shen M, et al. *PLoS One* 2011;**6**:e23679.
131 Carew JS, Espitia CM, Esquivel JA, Mahalingam D, Kelly KR, Reddy G, et al. *J Biol Chem* 2011;**286**:6602.
132 Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, et al. *PLoS One* 2009;**4**:e5740.
133 Seiple LA, Cardellina JH, Akee R, Stivers JT. *Mol Pharmacol* 2008;**73**:669.
134 Gdaniec Z, Ban B, Sowers LC, Fazakerley GV. *Eur J Biochem* 1996;**242**:271.
135 Liu L, Gerson SL. *Curr Opin Invest Drugs* 2004;**5**:623.
136 Savvides PS, Xu Y, Liu L, Rogers L, Bokar J, Silverman P, et al. *Cancer Res* 2012;**72**:1743.
137 Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, Kinsella TJ. *Mol Cancer Ther* 2006;**5**:893.
138 Plummer ER. *Curr Opin Pharmacol* 2006;**6**:364.
139 Malini G. *Nature Biotechnol* 2011;**29**:373.
140 For reviews, see (a) Underhill C, Toulmonde M, Bonnefoi A. *Ann Oncol* 2011;**22**:268; (b) Davar D, Beumer JH, Hamieh L, Tawbi H. *Curr Med Chem* 2012;**19**:3907
141 Curtin NJ. *Expert Rev Mol Med* 2005;**7**:1.
142 Haince J-F, Rouleau M, Hendzel MJ, Masson J-Y, Poirier GG. *Trends Mol Med* 2005;**11**:456.
143 Banasik M, Komura H, Shimoyama M, Ueda K. *J Biol Chem* 1992;**267**:1569.
144 (a) Wang C, Bedikian A, Kim K, Papadopoulos N, Hwu W, Hwu P. *J Clin Oncol* 2006;**24**:12015; (b) Mason KA, Valdecanas D, Hunter NR, Milas L. *Invest New Drugs* 2008;**26**:1.
145 Bedikian AY, Papadopoulos NE, Kim KB, Hwu WJ, Homsi J, Glass MR, et al. *Cancer Invest* 2009;**27**:756.
146 Plummer R, Lorigan P, Evans J, Steven N, Middleton M, Wilson R, et al. *J Clin Oncol* 2006;**24**:A8013.
147 Wang YF. *Drugs Future* 2009;**34**:177.
148 (a) Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al. *Lancet* 2010;**376**:205; (b) Chan SL, Mok T. *Lancet* 2010;**376**:211.
149 Penning TD, Zhu GD, Gandhi VB, Gong J, Liu X, Shi Y, et al. *J Med Chem* 2009;**52**:514.
150 Liu SK, Coackley C, Krause M, Jalali F, Chan N, Bristow RG. *Radiother Oncol* 2008;**88**:258.
151 Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. *Nature Rev Cancer* 2010;**10**:293.
152 Miknyoczki S, Chang H, Grobelny J, Pritchard S, Worrell C, McGann N, et al. *Mol Cancer Ther* 2007;**6**:2290.
153 Lord CJ, Garrett MD, Ashworth A. *Clin Cancer Res* 2006;**12**:4463.
154 Collis SJ, DeWeese TL, Jeggo PA, Parker AR. *Oncogene* 2005;**24**:949.
155 Bakkenist CJ, Kastan MB. *Cell* 2004;**118**:9.
156 Karve S, Werner ME, Sukumar R, Cummings ND, Copp JA, Wang EC, et al. *Proc Natl Acad Sci U S A* 2012;**109**:8230.
157 Jiang H, Fan D, Zhou G, Li X, Deng H. *J Exp Clin Cancer Res* 2010;**29**:34.
158 Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NMB, Orr AI, et al. *Cancer Res* 2004;**64**:9152.
159 Li Y, Yang D-Q. *Mol Cancer Ther* 2010;**9**:113.
160 Nutley BP, Smith NF, Hayes A, Kelland RL, Brunton L, Golding BT, et al. *Br J Cancer* 2005;**93**:1011.
161 Amrein L, Loignon M, Goulet A-C, Dunn M, Jean-Claude B, Aloyz R, et al. *Pharmacol Exp Ther* 2007;**321**:848.

162 Veuger SJ, Curtin NJ, Richardson CJ, Smith GCM, Durkacz BW. *Cancer Res* 2003;**63**:6008.
163 (a) Lu HR, Zhu H, Huang M, Chen Y, Cai Y-J, Miao ZH, et al. *Mol Pharmacol* 2005;**68**:983; (b) Zhou J, Chen Y, Lang J-Y, Lu J-J, Ding J. *Mol Cancer Res* 2008;**6**:194.
164 http://clinicaltrials.gov/show/NCT01010373.
165 Sredni B, Weil M, Khomenok G, Lebenthal I, Teitz S, Mardor Y, et al. *Cancer Res* 2004;**64**:1843.
166 Kalechman Y, Shani A, Albeck M, Sotnik BI, Sredni B. *Radiat Res* 1993;**136**:197.
167 Thiry A, Dogné J-M, Mesereel B, Supuran CT. *Trends Pharmacol Sci* 2006;**27**:566.
168 Oratz R, Blum J, Rowland K, Cunningham C, Jacobs S, Arseneau J, et al. *J Clin Oncol* 2005;**23**:685.
169 Tai IT, Dai M, Owen DA, Chen LB. *J Clin Invest* 2005;**115**:1492.
170 (a) Shirota Y, Stoehlmacher J, Brabender J, Xiong Y-P, Uetake H, Danenberg KD, et al. *J Clin Oncol* 2001;**19**:4298; (b) Candeil L, Gourdier I, Peyron D, Vezzio N, Copois V, Bibeau F, et al. *Int J Cancer* 2004;**109**:848.
171 Thomas S, Waterman P, Chen S, Marinelli B, Seaman M, Rodig S, et al. *J Nanomed Nanotechnol* 2011;**2**:2157.
172 For a review of mechanism-based approaches to radiosensitization, see Tofilon PJ, Camphausen K. *Chem Rev* 2009;**109**:2974.
173 For a review, see Rengan R, Cengel KA, Hahn SM. *Cancer Metast Rev* 2008;**27**:403.
174 Magne N, Chargari C, Castadot P, Ghalibafian M, Soria JC, Haie-Meder C, et al. *Eur J Cancer* 2008;**44**:2133.
175 Noguchi M, Yu D, Hirayama R, Ninomiya Y, Sekine E, Kubota N, et al. *Biochem Biophys Res Commun* 2006;**351**:658.
176 He S, Smith DL, Sequeira M, Sang J, Bates RC, Proia DA. *Invest New Drugs* 2014;**32**:577.
177 Camphausen K, Cerna D, Scott T, Sproull M, Burgan WE, Cerra MA, et al. *Int J Cancer* 2005;**114**:380.
178 Pierce AJ, Stark JM, Araujo FD, Moynahan ME, Berwick M, Jasin M. *Trends Cell Biol* 2001;**11**:S52.
179 Shinohara ET, Geng L, Tan J, Chen H, Shir Y, Edwards E, et al. *Cancer Res* 2005;**65**:4987.
180 Schreiber V, Dantzer F, Ame JC, de Murcia G. *Nature Rev Mol Cell Biol* 2006;**7**:517.
181 Janetka JW, Ashwell S, Zabludoff S, Lyne P. *Curr Opin Drug Discov Dev* 2007;**10**:473.
182 Rainey MD, Charlton ME, Stanton RV, Kastan MB. *Cancer Res* 2008;**68**:7466.
183 Bergers G, Hanahan D. *Nature Rev Cancer* 2008;**8**:592.
184 Chong CR, Janne PA. *Nature Med* 2013;**19**:1389.
185 Walter AO, Sjin RTT, Haringsma HJ, Ohashi K, Sun J, Lee K, et al. *Cancer Discov* 2013;**3**:1404.
186 Cross DAE, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. *Cancer Discov* 2014;**4**:1046.
187 Qi W, Cooke LS, Stejskal A, Riley Ch, Della Croce K, Saldanha JW, et al. *BMC Cancer* 2009;**9**:142.

CHAPTER

CANCER CHEMOPREVENTION 15

CONTENTS

1 INTRODUCTION

Blood pressure and cholesterol levels were known risk factors for cardiovascular disease from the early 1960s, but their chemopreventive modulation was not generally accepted until the 1970s for blood pressure and the 1980s for cholesterol. Currently, chemoprevention of cardiovascular diseases by using antihypertensive agents or statins has resulted in an approximately 50% reduction in their associated mortality.

In contrast, cancer chemoprevention is a highly controversial topic. The focus of cancer therapy has been on curing advanced tumors, despite the fact that cancer is a process that usually takes decades to develop and most cancer patients die because their tumors were not treated or removed before

Medicinal Chemistry of Anticancer Drugs. http://dx.doi.org/10.1016/B978-0-444-62649-3.00015-6

metastasis. Each metastasis is established by a single cell or small group of cells with a set of founder mutations that make them susceptible to antitumor agents, but with each cell division, the metastasis acquires new mutations, thus inducing drug resistance. Metastat®ic lesions of a size visible on medical imaging are already resistant to virtually every drug; for this reason, recurrence is often a matter of time that is predictable on the basis of known mutation frequencies and tumor cell growth rates.

This situation can be circumvented by the use of combined therapies because it is unlikely that a single tumor cell will be resistant to multiple drugs that act on different targets. However, despite major advances in the understanding of carcinogenesis and in bringing potent new drugs to the clinic, to achieve a significant positive change in the current mortality from the common forms of cancer, a much greater emphasis is necessary on preventing cancer before the complex series of genetic and epigenetic events that result in metastasis have occurred.[1]

2 CANCER BIOMARKERS, MOLECULAR MEDICINE, AND INDIVIDUALIZED TREATMENTS

A common and limited set of driver genes and pathways is responsible for most common forms of cancer, offering potential for early diagnosis. The genes themselves, the proteins encoded by these genes, and the end products of their pathways are in principle detectable in many ways, including analyses of relevant body fluids such as urine for genitourinary cancers, sputum for lung cancers, or stool for gastrointestinal cancers. Genetic, epigenetic, proteomic, glycomic, and imaging biomarkers can be used for cancer diagnosis, prognosis, and epidemiology. Some gene- and protein-based biomarkers, such as HER-2 (breast cancer),[2] BRCA1/BRCA2 (breast/ovarian cancer),[3] Bcr-Abl (chronic myeloid leukemia), or BRAF V600E (melanoma/colorectal cancer),[4] have an special clinical relevance. However, other cancer biomarkers have proven controversial due to their low specificity.[5] This is the case with the serum prostate-specific antigen (PSA) that, despite its low specifity, has guided prostate cancer diagnosis and management. New prostate cancer biomarkers that may supplement or replace PSA are emerging in large part from new genomic technologies, but this research has centered on disease diagnostics rather than on prognosis and prediction.[6]

Industry invests much more in research on cures for advanced cancers than on prevention or early detection, in part because new drugs offer greater financial returns than new diagnostic tests, but also because the development of new and improved methods for early detection and prevention of cancer is a very difficult task. Furthermore, achieving regulatory approval in cancer prevention is still challenging, which makes this area unattractive for pharmaceutical development because the reimbursement status for such investment is uncertain. It is still necessary to develop credible validated predictive biomarkers to change this situation.

The use of individualized cancer treatments is based on interpatient heterogeneity, one of the major obstacles to designing uniformly effective treatments for cancer. The dramatic responses to agents such as vemurafenib (Zelboraf®) and dabrafenib (Taginlar®), which target mutant *BRAF* in melanomas, or crizotinib (Xalkori®), which targets mutant *ALK* in lung cancers, show that interference with even a single mutant gene product is sufficient to stop cancer at least transiently. Unfortunately, mutations of tumor anti-oncogenes predominate over oncogene-activating mutations, and there are no drugs to replace the function of anti-oncogenes.

3 CANCER CHEMOPREVENTION

Cancer chemoprevention is a prophylactic method that uses "nontoxic" natural or synthetic compounds to reverse, inhibit, or prevent the development of cancer by inhibiting specific molecular steps in the carcinogenic pathway. The goal of cancer chemoprevention is to decrease cancer incidence while simultaneously reducing treatment-related side effects and mortality. In other words, the aim of chemotherapy is to kill cancer cells in the hope of preventing further cancer progression, whereas chemoprevention is the attempt to use natural or synthetic chemical agents to avoid cancer.

Tumor suppressor genes and proto-oncogenes encode proteins that affect three different carcinogenesis stages: initiation (in which carcinogens bind to DNA), promotion (in which epigenetic mechanisms lead to premalignancy), and progression to cancer.[7] As previously mentioned, with rare exceptions, the first stage is initiated decades before promotion and progression stages. The primary prevention requires strategies to prevent *de novo* malignancies in healthy populations, especially in individuals with high-risk features, such as patients with familial adenomatous polyposis (who are almost certain to develop colon cancer) or women who have a *BRCA1* or a *BRCA2* mutation. *BRCAs* are tumor suppressor genes whose hereditary high-risk mutations disable the homology-directed DNA repair process, increasing the risk of developing breast, ovarian, and certain other cancers. Obviously, the success of primary prevention will depend on a correct determination and analysis of biomarkers. Secondary prevention focuses on the progression of a premalignant lesion such as a colon adenoma into cancer. Tertiary prevention is directed at reversing, suppressing, or preventing carcinogenic progression to invasive cancer in patients who have been cured of an initial cancer or premalignant lesion but develop a second primary tumor. As previously mentioned, metastatic tumors are the leading cause of mortality in several cancers. Even if total cure of advanced malignancy cannot be achieved because an absolute prevention is not possible, extension of the latency period of carcinogenesis so that patients can have a higher quality of life is highly desirable.

The paradigm for developing new chemopreventive agents has changed markedly in approximately the past decade and now involves extensive preclinical mechanistic evaluation of agents before clinical trials are instituted. It also focuses on defining biomarkers of activity that can be used as early predictors of efficacy. Protection may be achieved as a consequence of decreased cellular uptake and metabolic activation of pro-carcinogens, enhanced detoxification of reactive electrophiles and free radical scavenging, or induction of repair pathways. Downregulation of chronic inflammatory responses and production of reactive oxygen and nitrogen species may also contribute to the prevention of cancer initiation. Other protective processes include modulation of DNA methyl transferases to prevent or reverse the hypermethylation-induced inactivation of tumor suppressor genes and inhibition of histone deacetylases, which affects some epigenetic mechanisms of carcinogenesis.

The major reported mechanisms of chemopreventive agents, often termed "suppressing agents," involve the inhibition of signal transduction pathways (e.g., by targeting NF-κB) to perturb the effects of tumor promoters that would otherwise lead to cell proliferation. In some cases, hormones may promote tumor progression, and antiestrogens such as tamoxifen may be used to block this effect. Recent reports suggest that interference with cancer cell metabolism and energy homoeostasis, via effects on pathways such as AMPK (see Chapter 10, Section 8) and mTOR signaling, may be an attractive goal to achieve chemopreventive agents. Other mechanisms of chemoprevention include the induction of apoptosis and inhibition of angiogenesis.[8]

In 2013, the editors of *Science* selected the application of immunotherapy to the prevention or treatment of cancer as the most relevant biomedical advance of the year; these important topics are only partially considered here because anticancer vaccination and immunostimulatory regimens were previously discussed (see Chapter 12, Sections 3 and 4).

4 CHEMOPREVENTIVE AGENTS

In contrast to the hundreds of drugs that have been developed and tested for the treatment of cancer and for its palliative care, very few agents have been approved for treating precancerous lesions or for reducing cancer risk (Table 15.1). However, during the past three decades, many classes of compounds, such as nonsteroidal anti-inflammatory drugs (NSAIDs), vitamins, food components, antidiabetic drugs, and ω-3 fatty acids, have been tested for their cancer-preventive potential,[9] and four major classes of drugs have produced positive results: retinoids, inhibitors of hormone action, cyclooxygenase-2 (COX-2) inhibitors, and cancer-related vaccines.[10]

Development of chemopreventive agents differs from that of anticancer agents mainly in that chemoprevention trials are most often conducted with asymptomatic healthy individuals, which requires extra vigilance to avoid harm. These individuals may have an average risk to develop a cancer, as is the case of prostatic cancer, or have an increased risk due to a genetic predisposition, a personal history of cancer, or evidence of pre-neoplastic lesions such as colorectal adenomas or actinic keratosis. Chemoprevention trials are under the same regulatory rigor as treatment trials, but the definition of "clinical

Table 15.1 Examples of FDA-Approved Cancer Chemopreventive Drugs

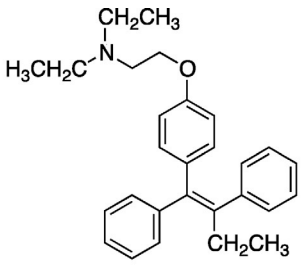
Drug	Cancer Type	Approval	Target/Mechanism
Tamoxifen (Nolvadex®, Istubal®, Valodex®)	Breast	1998	Selective estrogen receptor modulator (SERM)
Raloxifene (Evista®)	Breast	2007	SERM
Photodynamic therapy (PDT) with photofrin (Photofin®)	Esophageal	2003	Located in precancerous cells; upon exposure to certain light, produces an active form of oxygen that kills nearby cancer cells
Celecoxib[a]	Colorectal	1999	Multiple mechanisms
Valrubicin (Valstar®)	Recurrent urinary bladder cancer	1999	DNA topoisomerase II inhibitor
Fluorouracil (Efudex®, Fluoroplex®, Carac®)	Skin	1970	Interferes with DNA synthesis and leads to cell death
Diclofenac sodium (Solaraze®)	Skin	2000	Unknown mechanism
PDT with 5-aminolevulinic acid (Levulan®)	Skin	1999	Kills precancerous cells when exposed to light
Imiquimod (Zyclara® 3.75%, Aldara® 5%)	Skin	2004	Enhances immune response and promotes apoptosis
Masoprocol[b] (Actinex®)	Skin	1992	Antioxidant that may block certain enzymes needed for tumor growth
Ingenol mebutate (Picato®)	Skin	2012	Unknown mechanism

[a] *Withdrawn for this indication in 2011 upon request by its manufacturer.*
[b] *Withdrawn from the market.*

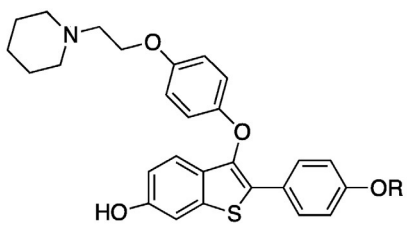
benefit" is not clearly established and long-term benefits or harms may remain unknown for years. In 2006, a Cancer Prevention Research Summit identified five barriers to research and development of chemoprevention drugs: uncertain reimbursement for new agents, limitations in current patent law and intellectual property protection, limitations in emerging prevention science, evolving designs of clinical trials and processes of drug approval, and limited public participation in clinical trials.[11,12] Despite these drawbacks, the findings derived from these studies have contributed to the general maturation of cancer prevention approaches.

4.1 ESTROGEN RECEPTOR MODULATORS AND ANTIANDROGEN COMPOUNDS

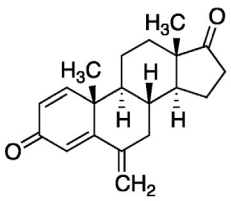
U.S. Food and Drug Administration (FDA) approval of the selective estrogen receptor modulator (SERM) tamoxifen for breast cancer prevention in 1998[13] was a landmark in chemoprevention research,[14] although reports indicating a strong relationship between estrogens and some breast cancers date back almost 100 years (see Chapter 3, Section 3.1).[15] The chronic administration of antiestrogens might be useful in breast cancer prevention because estrogens enhance the growth of approximately 70% of breast cancer cells during early stages of carcinogenesis. However, tamoxifen has been rarely utilized because of its associated risk of endometrial cancer and thromboembolic events. Clinical trials with raloxifene, another SERM modulator,[16] showed a lower risk of secondary effects, and it was approved by the FDA for breast cancer risk reduction in 2007. However, raloxifene also encountered resistance to acceptance for reasons that are less clear than those involving tamoxifen.[17] Good clinical results were also obtained with arzoxifene[18] and with some third-generation aromatase inhibitors, such as exemestane, anastrozole, and letrozole (see Chapter 3, Section 4.7), alone or alternated with tamoxifen.[19] It is relevant to remark that no evidence exists establishing whether a reduction in breast cancer risk from either agent translates into reduced breast cancer mortality. The chemopreventive use of SERMs may be extended to other cancers such as prostate and colon, in which estrogenic receptors ERα and ERβ play a carcinogenic role.



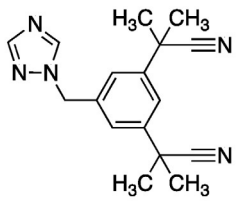
Tamoxifen



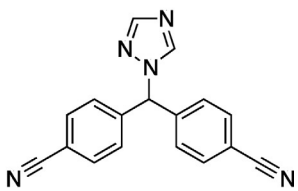
R = H Raloxifene
R = CH3 Arzoxifene



Exemestane

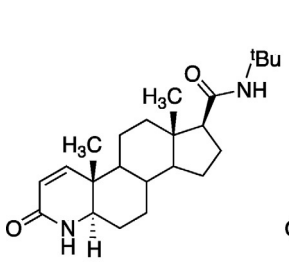


Anastrazole

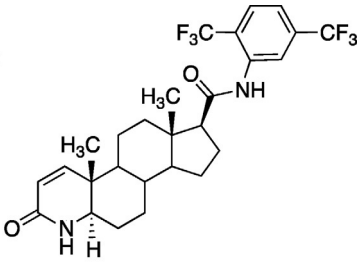


Letrozole

The interaction of ligands with androgen receptors (ARs) is especially involved in the development and progression of prostate cancer (see Chapter 3, Section 6). Some of the diagnosed cases of this cancer need no more than active surveillance instead of heavy-handed treatment modalities that tend to make cure of the disease worse than the disease itself, but the clinical dilemma is to identify markers with discriminant capabilities. The primary prevention appears to be an attractive strategy to eradicate this cancer by considering its high prevalence and the slow progressive development of healthy prostatic epithelium to dysplasia, prostatic intraepithelial neoplasia (PIN), locally invasive adenocarcinoma, and, finally, metastatic disease. In 2003, a prevention trial showed that the 5α-reductase inhibitor finasteride reduced prostate cancer overall by 25%, but apparently it also increased high-grade tumors, which obviated its acceptance. Although a subsequent analysis showed that the excess of high-grade tumors was probably due to biopsy artifacts, the initial concerns and patent limitations precluded the seeking of regulatory approval of finasteride for risk reduction. Recent results have shown that the finasteride analog dutasteride (GI-198745, Avodart®) reduced prostate cancer by 23% with no apparent increase in high-grade cancer.[20] This compound was approved for benign prostate hyperplasia and has been proposed for the chemoprevention of prostate cancer in men at high risk.[21] It has been recently shown that prostate cancers spread more quickly and are more often fatal in men who have the *BRCA2* mutation, which should be considered for tailoring their clinical management.[22]



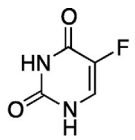
Finasteride



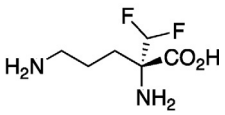
Dutasteride

4.2 5-FLUOROURACIL AND EFLORNITHINE

In the context of efforts toward identifying effective chemopreventive compounds active against carcinogenesis of the upper respiratory tract (URT) by using aerosol delivery, 5-fluorouracil (5-FU) and eflornithine were investigated. Because this combination resulted in a significant increase in the percentage of cancer-free animals, the two agents were proposed as chemopreventive agents in subjects at high risk for these types of cancer.[23] Eflornitine has also been used on its own for chemoprevention by oral administration.



5-Fluorouracil



Eflornithine

Eflornithine (difluoromethylornithine, DFMO) is a suicide inhibitor of ornithine decarboxylase, an enzyme important in cell proliferation that has pyridoxal phosphate as a cofactor. This inhibition takes place by the

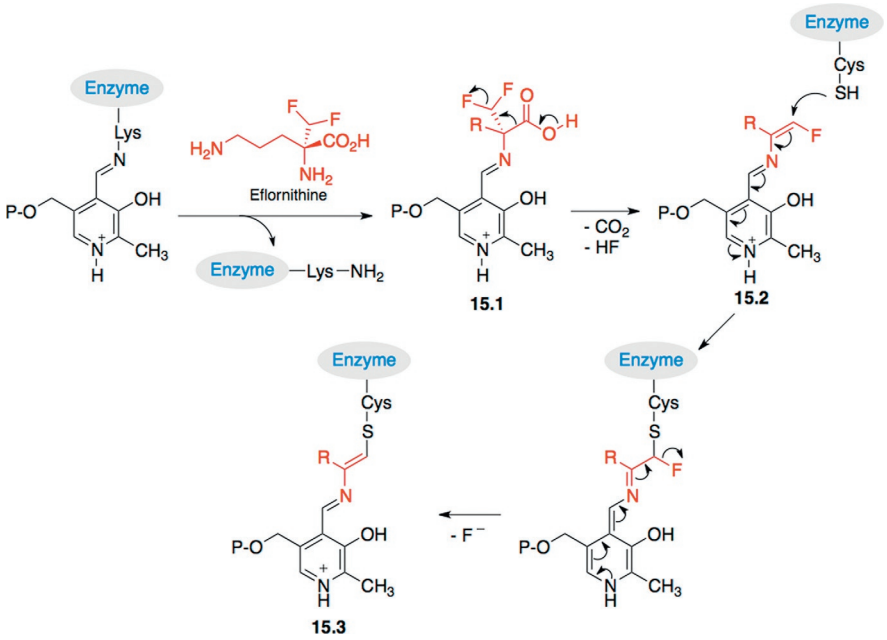


FIGURE 15.1

Suicide inhibition of ornithine decarboxylase by eflornithine.

mechanism shown in Figure 15.1, involving an initial transamination reaction that covalently attaches the α-amino group of eflornitine to the pyridoxal cofactor to give intermediate **15.1**. A decarboxylation reaction, which in this case is accompanied by the loss of a molecule of HF, gives **15.2**. The electron-withdrawing nature of the pyridinium moiety favours the subsequent addition of a cysteine residue of the enzyme, which is followed by loss of the second F atom as a fluorine anion to give **15.3**. The enzyme thus becomes covalently attached to the drug–cofactor complex and is therefore irreversibly inactivated.

4.3 NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AS CANCER CHEMOPREVENTIVE AGENTS

Acute inflammation is a localized protective reaction of tissue to irritation, injury, or infection characterized by the influx of inflammatory cells (e.g., macrophages and neutrophils), induction of vasoconstriction, edema, erythema, and sensitivity to pain. However, chronic inflammation contributes to the etiology of many diseases and has been linked to various steps involved in tumorigenesis. Epidemiological studies and clinical trials indicated that long-term use of NSAIDs can decrease the incidence of certain malignancies, including colorectal, esophageal, breast, lung, and bladder cancers, and the clinical evidence of their chemopreventive activity was first reported in 1983 for sulindac (Clinoril®). The best-known targets of NSAIDs are COX enzymes, which convert arachidonic acid to prostaglandins (PGs) and thromboxane. COX-2 is not expressed in most untransformed epithelial cells, but the earliest premalignant lesions that lead to most solid tumors display its expression, with an increase in enzyme levels parallel

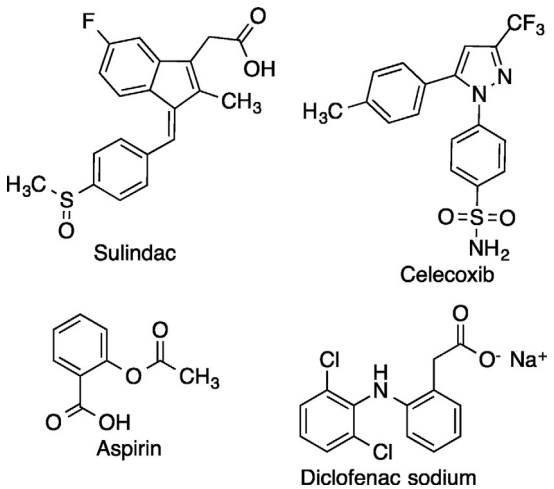
to cancer progression. Although these discoveries were initially made in colon, similar observations were reported in most solid tumors except ovarian cancer, in which COX-1 appears to be induced.[24]

COX-2-derived prostaglandin E2 (PGE2) can promote tumor growth by binding its receptors and activating signaling pathways that control cell proliferation, migration, apoptosis, and/or angiogenesis. The chemopreventive effects of COX-2 inhibitors seem to be mediated by their effects on stromal cells of the intestine, especially angiogenesis suppression.[25,26] Numerous anti-inflammatory agents, including those identified from natural sources, have shown chemopreventive activities[27,28] and could also be useful for cancer therapy. However, the prolonged use in high dosages of COX-2 selective inhibitors (COXIBs) is associated with unacceptable cardiovascular side effects (discussed below), which makes the development of more effective chemopreventive agents with minimal toxicity crucial.[29]

Because the selective COX-2 antagonist celecoxib prevented colon carcinogenesis in animal models[30] and reduced the number of colorectal polyps in humans, it received FDA approval in 1999 for reducing polyp burden in patients with familial adenomatous polyposis (a high-risk genetic condition).[31] However, its previously mentioned cardiovascular effects halted all clinical trials of COX-2-selective compounds for cancer prevention,[32] and celecoxib was withdrawn in 2011 for this indication upon request by its manufacturer.[33] A combination of low doses of eflornithine and sulindac produced dramatic reductions in some colorectal carcinomas with generally minimal toxicity but with a significant number of cardiovascular events. However, as in the case of celecoxib, subsequent analysis showed that these cardiovascular events were limited to individuals who previously had a high cardiovascular risk.[34]

Daily aspirin reduces the long-term incidence of some adenocarcinomas and prevents distant metastasis.[35] A prospective study of aspirin use and multiple myeloma supported an etiologic role for aspirin-inhibited pathways mediated by NF-κB or COX-2 and warranted further evaluation of aspirin for multiple myeloma chemoprevention.[36]

Solaraze® is a gel effective for the treatment of actinic keratosis (AK) that contains diclofenac sodium as the active agent. Although the cancer chemopreventive mechanism of this agent is unknown, a study on the chemopreventive action of diclofenac in lung cancer induced in female Wistar rats indicated that its effects are mediated by the induction of apoptosis.[37] The contribution to efficacy of individual components of the vehicle, especially hyaluronate sodium, has not been established.

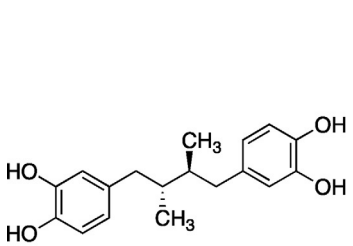


Other mechanisms independent of COX inhibition contribute to the chemopreventive activity of NSAIDs. Thus, sulindac sulfide and sulindac sulfone and its derivatives can inhibit certain cyclic guanosine monophosphate-degrading isozymes, causing an increase in intracellular cGMP levels that activate the cGMP-dependent protein kinase (PKG), which in turn activates pathways that lead to apoptosis.[38] Other mechanisms of apoptotic induction by NSAIDs may involve the peroxisome proliferator-activated receptors PPARα, -γ, and -δ; the retinoic X receptor-α (RXRα); NF-κB signaling[39]; 3-phosphoinositide-dependent kinase-1; the sarcoplasmic/ER Ca^{2+} ATPase; and the enzyme carbonic anhydrase.

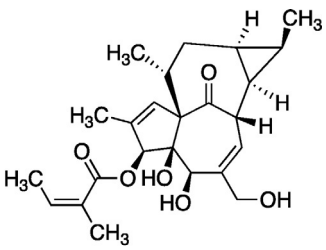
4.4 MASOPROCOL AND INGENOL MEBUTATE

The main types of skin cancers are nonmelanoma (basal cell and squamous cell cancers) and melanoma. Although the last type of cancer accounts for 5% of all skin cancer cases, it produces the vast majority of skin cancer deaths. Most of the preventive agents against squamous cell cancers are directed to actinic keratosis (AKs), which are precancerous lesions that may progress to this cancer if left untreated. One compound approved by the FDA in 1992 to be topically used for its treatment was the antioxidant dicatechol masoprocol (nordihydroguaiaretic acid, Actinex®), although it was later withdrawn from the market. It was first isolated from the creosote bush (*Larrea tridentata*), soon showing different activities and possible clinical aplications.[40] Because it may block certain enzymes needed for tumor growth, an oral form of nordihydroguaiaretic acid is being studied in the treatment of patients with nonmetastatic relapsed prostate cancer.[41]

In 2012, the FDA approved for the treatment of AKs a topical gel containing ingenol mebutate (ingenol 3-angelate, Picato®), a compound derived from the sap of the *Euphorbia peplus* plant. Ingenol mebutate induces apoptosis followed by immune reactions in target lesions, although its precise mechanism of action is unknown.[42]



Masoprocol



Ingenol mebutate

4.5 PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) was discussed in Section 12 of Chapter 4. In 1999, PDT with 5-aminolevulinic acid was approved for treatment of precancerous skin lesions, whereas PDT with photofrin received regulatory approval for esophageal cancer chemoprevention in 2003.

5 NUTRITIONAL SUPPLEMENTS

Cancer chemoprevention by naturally occurring dietary agents has received much interest because of the broad safety window of these compounds. In 1968, Pauling proposed for cancer treatment and prevention natural and nutritional substances, especially the antioxidants,[43] and defined this method as the "orthomolecular" (meaning "right molecule") approach. He subsequently expanded the list of diseases he believed could be influenced by orthomolecular therapy and the number of nutrients suitable for such use, but many medical and nutrition scientists do not share these views.

5.1 ANTIOXIDANTS IN CANCER CHEMOPREVENTION

Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent, which may produce free radicals that can start chain reactions in a cell, causing damage or death (see Chapter 4). Antioxidants are molecules that inhibit the oxidation of other molecules. Free radicals are generated by normal physiological processes, including aerobic metabolism and inflammatory responses to eliminate invading pathogenic microorganisms, but a chronic cell injury initiates an inflammatory response and activates cytoquines or receptor molecules to recruit mast cells and leukocytes. As previously mentioned, chronic inflammation deregulates cellular homeostasis and can drive carcinogenesis, because the "respiratory burst" leads to an increased uptake of oxygen and the subsequent release of free radicals from leukocytes.

These radicals are reactive oxygen species (ROS), such as hydroxyl and superoxide radicals, or nitrogen oxide reactive species (RNOS), such as nitric oxide, peroxynitrite, and nitrous anhydride. All of them can activate lipid peroxidation and the arachidonic acid cascade with the production of cell-proliferation-stimulating eicosanoids. ROS can also damage DNA, modifying its structure and function. Other DNA-damaging agents, such as malondialdehyde, are by-products of the arachidonic acid cascade.[44] The radicals effects include mutations in cancer-related genes, post-translational modification of cancer-related proteins, and activation of signal transduction pathways resulting in the transcriptional induction of proto-oncogenes (e.g., *c-FOS*, *c-JUN*, and *c-MYC*), increasing the cancer risk. In general, ROS might be carcinogenic, but they are also useful to treat cancer because apoptosis induced by radiotherapy and by many anticancer drugs involves their generation.

Antioxidants are reducing agents, frequently thiols or polyphenols, that terminate radical chain reactions by removing free radical intermediates. They are claimed to behave as chemoprotective agents and are found in foodstuffs, especially of a vegetal origin. Many patients being treated for cancer use antioxidants in the hope of reducing the toxicity of chemotherapy and radiotherapy, but mechanistic considerations suggest that antioxidants might reduce the effects of conventional cytotoxic therapies. For this reason, the use of nutritional supplements with antioxidant properties for cancer treatment or cancer chemoprevention is controversial;[45] in fact, some clinical studies have not found any benefit in these associations.[46] Furthermore, it has recently been reported that supplementing the diet with the antioxidants *N*-acetylcysteine (NAC) and vitamin E markedly increases tumor progression and reduces survival in mouse models of *B-RAF*- and *K-RAS*-induced lung cancer. Both compounds increased tumor cell proliferation by reducing ROS, DNA damage, and p53 expression in mouse and human lung tumor cells. Furthermore, because somatic mutations in p53 occur late in tumor progression, antioxidants may accelerate the growth of early tumors or precancerous lesions in high-risk populations such as smokers and patients with chronic obstructive pulmonary disease who receive NAC to relieve mucus production.[47]

The main antioxidants are ascorbic acid, ergothioneine, green tea polyphenols, and lycopene.

5.2 VITAMIN C (ASCORBIC ACID)

Vitamin C (ascorbic acid) is a very potent free radical scavenger that has attracted much attention. Its antioxidant properties are related to its ability to generate a stabilized radical because of the operation of the captodative effect (Figure 15.2), which allows it to react with harmful, more reactive species, particularly the hydroxyl radical, and prevent their interaction with biomolecules.

Ascorbic acid is a required nutrient for a variety of biological functions. Its health-promoting effects can be attributed to its activity as a water-soluble antioxidant and as a cofactor for a number of enzymes, most notably hydroxylases involved in collagen synthesis. Humans and other primates depend on the diet to prevent diseases such as scurvy, associated with lack of collagen due to vitamin C deficiency, and to maintain general health.

Ascorbic acid has cancer preventive and therapeutic potential, but its real value remains controversial.[48] Pauling reported that nearly 100 "terminal" cancer patients treated with a daily dose of 10 g of vitamin C survived three or four times longer than similar patients who did not receive this supplement.[49] To test this claim, three double-blind studies were conducted with advanced cancer, the last of them reported in 1985. These studies showed that patients given vitamin C did not progress better than those given a placebo.[50] Furthermore, a statistical study including more than 170,000 patients at risk of lung cancer showed no evidence of benefit.[51] Later studies found that vitamin C supplementation during cancer treatment may interfere with the effect of chemotherapy in humans.[52] A similar study had previously shown that β-carotene consumption increases the risk of lung cancer.[53] Despite these results, ascorbic acid has been recommended for many years to prevent gastrointestinal cancers. It has been suggested that

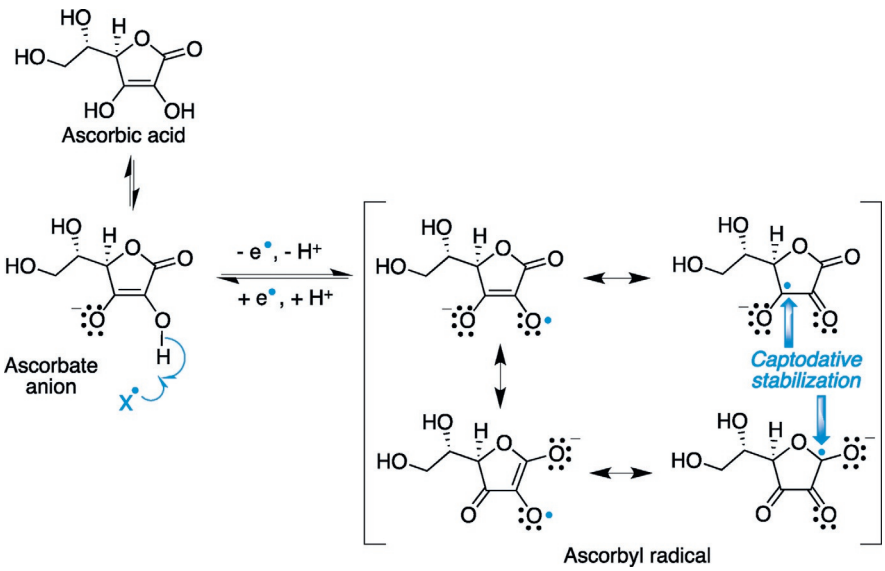


FIGURE 15.2

Ascorbate as an antioxidant.

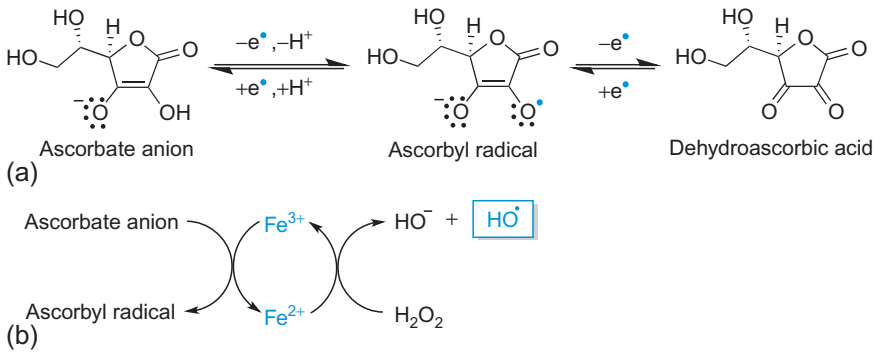


FIGURE 15.3

Ascorbate as a pro-oxidant.

patients with existing cancer may not benefit from vitamin C supplementation, but its deficiency is not likely to be beneficial.[54] Interestingly, it has recently been found that the combination of ascorbic acid with an inhibitor of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphataseisozyme 3 (PFKFB3) synergistically induces apoptosis in non-small cell lung cancer cells.[55]

The administration of vitamin C megadoses is also controversial due to the fact that ascorbic acid at low concentrations functions primarily as an antioxidant, whereas at higher concentrations, and depending on environmental conditions, it can act as a pro-oxidant that imposes oxidative stress and induces cell death.[56] Thus, vitamin C can transform Fe^{3+} into Fe^{2+}, and the ascorbate–Fe^{2+} chelate may catalyze the production of ROS via Fenton chemistry (Figure 15.3).[57] High concentrations of vitamin C are known to induce apoptosis in several tumor cell lines, which has been attributed to its pro-oxidant action.

Vitamin C-dependent proline hydroxylation plays a role in gene transcription mediated by HIF-1, which includes a number of cancer-related genes. Binding of HIF-1 to DNA requires dimerization of HIF-1α and HIF-1β subunits. Under normal oxygen concentration conditions, the HIF-1α subunit is degraded by HIF-specific prolyl hydroxylases that hydroxylate its proline residues 402 and 564, and these hydroxylated residues promote the binding of HIF to the von Hippel–Lindau tumor suppressor and its ubiquitin-dependent degradation, thereby repressing transcription of target genes. Under the hypoxic conditions characteristic of the fast-growing tumors, HIF-1α hydroxylation is repressed, resulting in an increase in the HIF-dependent gene transcription that promotes angiogenesis and tumor growth. Because HIF-1α prolyl hydroxylase (and the final ubiquitin-dependent HIF degradation) is stimulated by appropriate levels of ascorbic acid, these levels would inhibit tumor progression through inhibition of the HIF-1 pathway.[58] On the contrary, at low levels, the HIF-1α hydroxylation is reduced and thereby HIF-α-dependent gene transcription and tumor growth are promoted.

5.3 ERGOTHIONEINE

Ergothioneine (ERT) is a component of white button mushrooms that is considered as an antioxidant with cancer chemopreventive properties.[59] However, despite the wide interest in its antioxidant potential, the physiological function and role in disease of ergothioneine are limited.[60] Its antioxidant

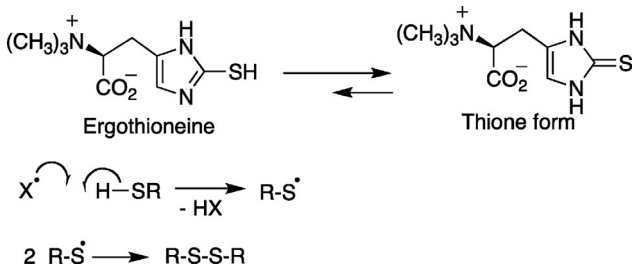


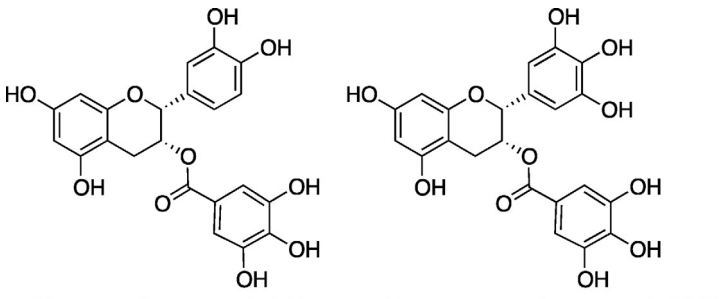
FIGURE 15.4

Ergothioneine as a radical scavenger.

properties appear to be related to at least four molecular activities.[61] One of them is the ability to scavenge free radicals, which can be proposed to arise from the easy one-electron oxidation of its mercapto group to a disulfide, similarly to the main water-soluble antioxidant thiol glutathione.[62] However, ERT chemistry differs from conventional sulfur-containing antioxidants mainly because it exists as a tautomer between its thiol and thione forms, with the latter predominating under physiological conditions. Consequently, ERT shows a peculiar stability and reactivity compared to other naturally occurring thiols because it does not auto-oxidize, forms disulfides with difficulty because the standard redox potential at pH 7 of the thiol–disulfide couple of ergothioneine is –0.06 V compared to –0.20 to –0.32 V for other natural thiols, requires a more severe oxidative stress to oxidize, and does not promote the classical Fenton reaction (Figure 15.4).[63] It also has chelating properties toward divalent metallic cations due to its α-amino acid moiety, activates antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (SOD), inhibits superoxide-generating enzymes such as NADPH–cytochrome *c* reductase, and affects the oxidation of various hemoproteins such as hemoglobin and myoglobin.

5.4 GREEN TEA

Green tea contains a large number of bioactive compounds, including catechins, flavonols, lignans, and phenolic acids. Polyphenols (e.g., epicatechin gallate and epigallocatechin gallate) are potent radical scavengers that have been extensively studied as chemopreventive agents.



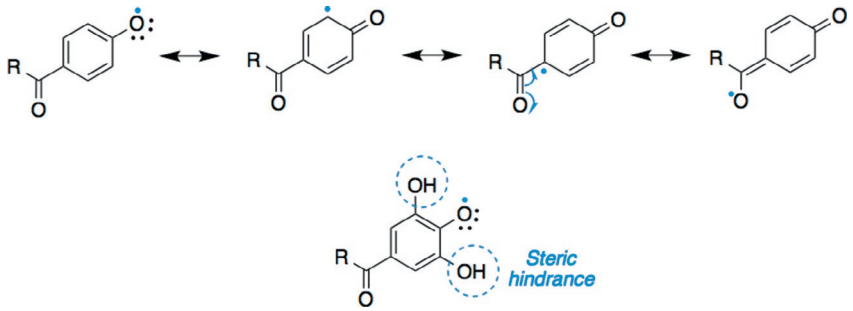


FIGURE 15.5

Green tea polyphenols as radical scavengers.

The radical-scavenging properties of these polyphenols are due to stabilization of the phenolic radical as a result of extensive delocalization of the unpaired electron around the aromatic ring and into the *p*-acyl substituent and also to the steric hindrance provided by the neighboring hydroxyl groups (Figure 15.5).

The results obtained from a phase II clinical assay that studied the modulation by these substances of the urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), an oxidative DNA damage biomarker, suggest that chemoprevention with green tea polyphenols is effective in diminishing this DNA damage.[64] A large and growing body of preclinical and clinical evidence suggests that the green tea has a protective effect across different types of cancer,[65] although the data are not definitive. In addition to angiogenesis and telomerase inhibition, important enzymes such as urokinase, ornithine decarboxylase, NADPH–cytochrome P450 reductase, protein kinase C, steroid 5-α reductase, tumor necrosis factor (TNF) expression, and nitric oxide synthase, appear to be implicated in the anticancer activity of green tea. Its component epigallocatechin-3-gallate (EGCG) and other polyphenols have shown effects on tumor signaling pathways and also have indirect effects on epidermal growth factor receptors (EGFRs), signal transducers and activators of transcription (STATs), and activator protein-1 (AP1). EGCG is also a potent inhibitor of NF-κB pathways.[66]

5.5 LYCOPENE

Lycopene is an open-chain carotenoid found in several fruits and vegetables, especially tomatoes. It may accumulate in high concentrations in several tissues reacting with hydroxyl radicals to give a stabilized, highly delocalized species (Figure 15.6).

Chemoprevention with lycopene gave positive results in prostate cancer.[67] This compound inhibits androgen receptor expression in prostate cancer cells, reduces their proliferation, modulates cell cycle progression, and affects the insulin-like growth factor intracellular pathway. However, clinical trials of lycopene in early stage, PSA relapse, or advanced prostate cancer patients have yielded inconsistent results. Furthermore, because prospective studies showed that lycopene levels are significantly lower in the serum and tissue of patients with prostatic cancer than in controls, the measurement of lycopene concentration was considered as a possible biomarker of this cancer; however, other studies have failed to demonstrate such a connection.[68,69]

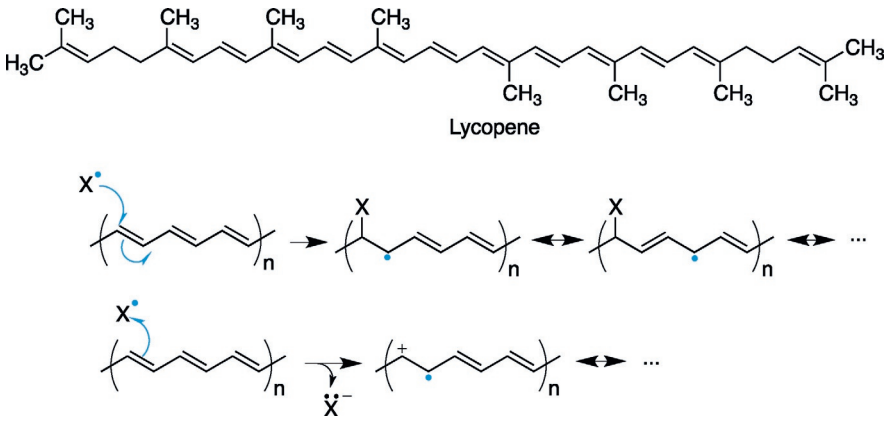


FIGURE 15.6

Lycopene as a radical scavenger.

5.6 NATURAL PRODUCTS WITH MISCELLANEOUS PROTECTIVE MECHANISMS

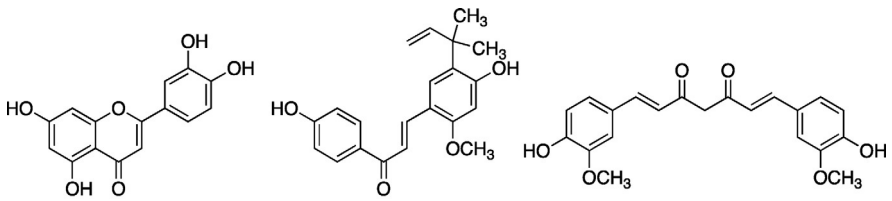
Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a common flavonoid in many fruits, vegetables, and medicinal herbs. Plants rich in luteolin have been used in Chinese traditional medicine for treating various diseases, such as hypertension, inflammatory disorders, and cancer. It is an active constituent of *Lonicera japonica* (Caprifoliaceae), and its anticancer activity is associated with the induction of apoptosis and inhibition of cell proliferation, metastasis, and angiogenesis. Luteolin sensitizes cancer cells to therapeutic-induced cytotoxicity through suppressing cell survival pathways such as phosphatidylinositol 3′-kinase (PI3K)/Akt, NF-κB, and X-linked inhibitor of apoptosis protein (XIAP). It also stimulates apoptosis pathways induced by the tumor suppressor protein p53. These properties suggest that luteolin could be an anticancer agent, but recent epidemiological studies have also attributed cancer-preventive properties to this compound,[70] with a significant inhibitory effect on the tumor growth of squamous cell carcinoma of head and neck (SCCHN).[71]

Like most flavonoids, luteolin can act as antioxidant or as pro-oxidant. It has been shown that it induces ROS, whose accumulation plays a pivotal role in suppression of NF-κB and potentiation of JNK, without involving mitochondrial electron transport and probably by the suppression of SOD activity. These effects sensitize cancer cells to undergo TNF-induced apoptosis.[72] As do many dietary compounds, luteolin displays low bioavailability because of its poor water solubility. To circumvent this problem, the development of luteolin nanoparticles has been considered.

Licochalcone A is an estrogenic flavonoid, originally derived from the licorice root (*Glycyrrhiza glabra*), that inhibits the c-Jun N-terminal kinase 1 (JNK1) activity, but it has little effect on JNK2-mediated c-Jun phosphorylation. This inhibition results in G_1 phase arrest and apoptosis. Because JNK1 is highly expressed in colon and pancreatic cancer cell lines, licochalcone A may have preventive or therapeutic potential against these tumors.[73]

Dietary components have also emerged as a promising source of new epigenetically active compounds able to regulate gene expression and molecular targets implicated in tumorigenesis.

The polyphenolic compound curcumin, isolated from the rhizome of *Curcuma longa*, has diverse biological activities (see Section 4.11 of Chapter 10 and Section 7.1 of Chapter 11). It blocks the activation or transcription activity of the transcriptional factor NF-κB and has both anti-inflammatory and anticarcinogenic activity, although suppression of NF-κB activity can increase susceptibility to infections[74] and induce primary tumors.[75] In addition, use of curcumin appears to be an attractive preventive and/or therapeutic approach against human cancer because it is a DNA hypomethylating agent that is able to restore the epigenetic regulation balance, modulating several microRNAs (mRNAs) and their multiple target genes.[76] It affects cell proliferation of androgen-dependent prostate cancer through the induction of cell cycle arrest in G_2 and modulation of Wnt signaling, which plays a central role in mammary stem cell homeostasis.[77] Interestingly, in androgen-independent prostate cancer cells, curcumin does not affect Wnt/β-catenin transcriptional activity, which suggests that curcumin may be an interesting chemopreventive agent for early stage prostate cancer.[78] From a chemical standpoint, structure-related activity studies on curcumin analogs revealed that the high number of *ortho*-methoxy substitutions and the high level of hydrogenation of the heptadiene moiety of curcumin are responsible for the high radical scavenging potential of curcuminoids. In contrast, their highest anti-inflammatory and antitumoral potentials are related to the lowest hydrogenation and to the highest level of unsaturation of the diketone moiety. A drawback of curcumin is its conversion in the intestinal tract into relatively inactive substances by the action of UDP-glucuronosyltransferase, sulfotransferase, alcohol dehydrogenase, and P450. Its combination with adjuvants and its delivery in vehicles such as liposomes are two of the several approaches developed to overcome this problem.



Luteolin Licochalcone A Curcumin

Some constituents of cruciferous vegetables with a five-membered cyclic sulfur-containing structure have antioxidant, chemotherapeutic, and chemoprotective activities. In this context, oltipraz, originally developed as an antischistosomal agent, was found to protect against chemically induced carcinogens in the lung, stomach, colon, and urinary bladder in animals.[79] Its utility as a cancer chemopreventive agent is thought to depend on the induction of enzymes involved in phase II xenobiotic detoxification.[80] Oltipraz stimulates glutathione *S*-transferase[81] and can be effective in patients with histories of *Schistosoma haematobium* bladder infections, who are at increased risk for developing bladder cancer.[82] This enzyme is important in the inactivation of compounds such as polycyclic aromatic hydrocarbons and *N*-nitrosamines, which produce electrophilic carcinogenic metabolites. For instance, the fungal toxic secondary metabolite aflatoxin B1 (AFB1), which may contaminate food, gives carcinogenic compounds by the P450 cytochrome-related epoxydation of its furane double bond. These carcinogenic metabolites may be inactivated by glutathione addition catalyzed by glutathione *S*-transferase stimulated by oltipraz (Figure 15.7).

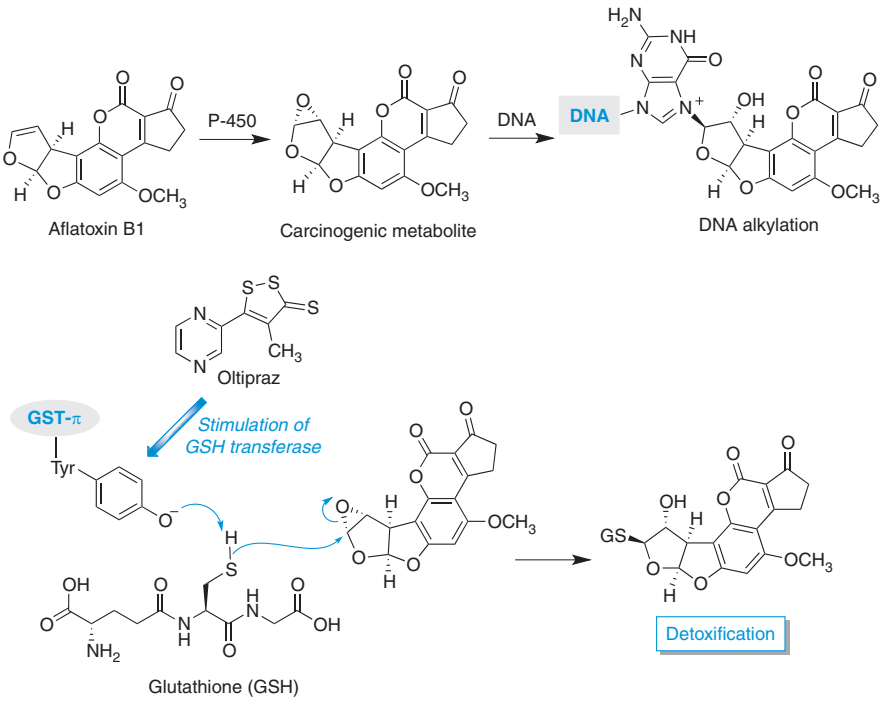


FIGURE 15.7

Carcinogenesis by aflatoxin B1 and its detoxification stimulated by oltipraz.

Sulforophane is another component of cruciferous vegetables (e.g., broccoli) that has been proposed as a chemoprotector. This compound bears an unusual isothiocyanate function and is generated from its precursor glucoraphanin by the myrosinase enzyme upon damage to the plant (e.g., from chewing), a process that involves hydrolysis of the heteroside followed by a Lösen rearrangement (Figure 15.8). Similarly to the previously mentioned oltipraz, sulforophane is an inducer of phase II-metabolizing enzymes. This compound induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells[83] and is being clinically studied as a chemopreventive agent in several cancers.[84]

Resveratrol is a phytoalexin that is found in many plants, especially in the skin of red grapes, and is therefore present in red wine. In addition to its antioxidative function, it induces quinone reductase, which is a phase II enzyme that metabolizes carcinogens. It has recently been found that resveratrol and aspirin reduced the frequency of tetraploid cells arising from primary epithelial cell cultures exposed to mitotic inhibitors. Also, in a mouse model of intestinal oncogenesis resembling familial adenomatous polyposis, both resveratrol and aspirin reduced the frequency of tetraploid cells accumulating in the gut, correlating with their chemopreventive action.[85] Clinical trials are in progress regarding the use of resveratrol in cancer chemoprevention.[86]

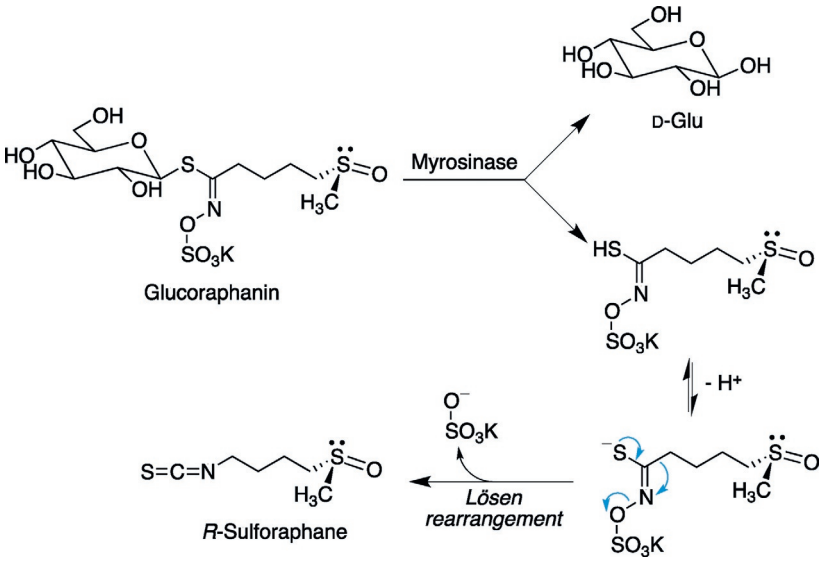
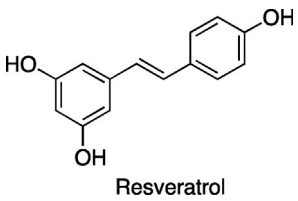


FIGURE 15.8

Bioactivation of glucoraphanin.

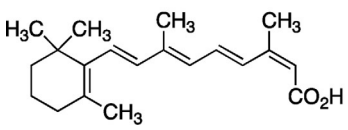


6 LIGANDS FOR NUCLEAR RECEPTORS IN CANCER CHEMOPREVENTION

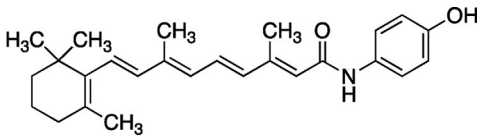
Nuclear receptors are transcription factors that regulate cell differentiation and proliferation in specific organs and are also important for carcinogenesis. They may be directly activated after the binding of specific ligands, but this binding may also trigger transcription in other cellular contexts because of the selective recruitment of other proteins, such as transcriptional coactivators and co-repressors that interact with transcription factors. Nuclear receptors are ideal targets for chemoprevention, the most studied of which are the estrogen receptors (ERα and ERβ); the androgen receptor (AR); the retinoic receptors RAR-α, -β, and -γ; the retinoid X receptors RXRα, -β, and -γ; the vitamin D receptor (VDR); and PPARγ.[87]

The development of a malignant phenotype frequently includes a block in the normal differentiation process, and numerous compounds, such as retinoids or vitamin D_3 analogs, have been studied with this

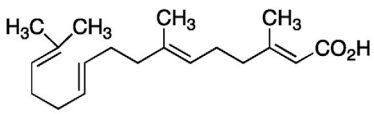
approach in mind. Vitamin A and its analogs, collectively known as retinoids, have profound effects in cell growth and differentiation, and the loss of retinoid function is linked to carcinogenesis in some cancers.[88] In addition to the success of retinoids in the therapy of acute promyelocytic leukemia, some of them are of interest in the prevention of several other cancers (oral cavity, head and neck, breast, skin, and liver); however, the first positive results of retinoids in the settings of oral intraepithelial and cervical neoplasias and in the prevention of second head and neck malignancies did not lead to regulatory approval because of toxicity problems. The retinoic receptor ligand 13-*cis*-retinoic acid (isotretinoin) is one of the standard treatments for the prevention of oral cancer,[89] but several atypical retinoids have also been assayed for cancer chemoprevention. Fenretinide, an amide of tretinoin that acts as a ligand of RAR-β and RAR-γ receptors, has entered clinical trials, showing a beneficial effect in the prevention of premenopausal breast cancer in combination with tamoxifen.[90] This treatment may cause the accumulation of ROS, resulting in cell death through apoptosis and/or necrosis. It is accumulated preferentially in fatty tissue such as the breast, which may contribute to its effectiveness against breast cancer. Polyprenoic acid, also called acyclic retinoid, has shown RAR, RXR, and PPAR activities and is useful in the prevention of hepatocellular carcinoma.[91] Finally, adapalene (Differin®), approved in 1996 for the treatment of acne, prevents cancer in patients with cervical intraepithelial neoplasia.



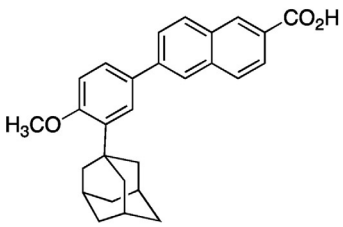
13-*Cis*-retinoic acid
(isotretinoin)



Fenretinide



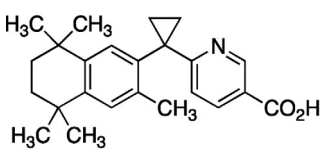
Polyprenoic acid



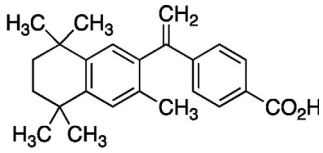
Adapalene

The ligands of RXRs, known as rexinoids, may modulate the activity of other transcription factors because their receptors form heterodimers with other nuclear receptors, such as RARs, VDR, and PPARγ.[92] Preclinical studies have shown that these compounds appear to maintain the cancer prevention potential of retinoids with less toxicity, and that when combined with a SERM, they can also effectively kill breast cancer cells.[93] Among them, the rigid analog of *cis*-retinoic acid LG100268 and bexarotene (Targretin®) selectively binds and activates the three retinoid X receptor subtypes (RXRα, RXRβ, and RXRγ), which are found all over the body, including neurons and other brain cells. Once activated, these receptors function as transcription factors that regulate the expression of genes that control cellular differentiation and proliferation. Bexarotene was approved as an oral antineoplastic agent indicated for cutaneous T cell lymphoma. Also, because it prevents multidrug resistance and inhibits angiogenesis and metastasis, it was considered as a promising chemopreventive agent against

cancer,[94] entering clinical trials for primary prevention of breast cancer after encouraging preclinical results.[95] Epidemiological studies demonstrated that diseases of the central nervous system, such as Alzheimer's disease, Parkinson's disease, and schizophrenia, protect against cancer. The most remarkable example is Alzheimer's disease, which can reduce the risk of suffering from cancer by up to 50%. In 2012, it was reported that bexarotene reduced amyloid plaque and improved mental functioning in a small sample of mice that exhibited Alzheimer-like symptoms and expressed apolipoprotein E (ApoE). It was thought that bexarotene stimulates expression of ApoE, leading to intracellular clearance of β-amyloid. In 2013, several studies did not find a reduction in amyloid plaques, but they showed that soluble forms of β-amyloid were reduced.[96] Although various theories have been put forward in an attempt to explain this relationship, recent work has shown that approximately 100 genes could be behind the relationship between these diseases: 74 genes that were less active in nervous system diseases were found to be more active in cancer, whereas 19 genes that were more active in nervous system diseases were found to be less active in cancer.[97] The clinical study of BEAT-AD (Bexarotene Amyloid Treatment for Alzheimer's Disease) is in progress.[98]

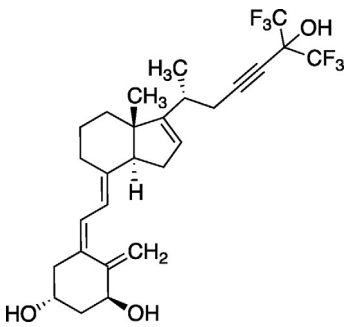


LG100268



Bexarotene

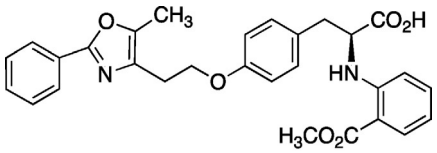
The VDR is another transcription factor that exerts a direct control of gene expression and interacts with regulatory pathways such as SMAD3, which is a component of the signal transduction pathway that regulates the cytokine transforming growth factor-β that helps to prevent carcinogenesis. Vitamin D intake diminishes the risk of colon cancer, protecting the colon from the carcinogenic effects of bile acids.[99] However, ingestion of large amounts of this vitamin results in hypercalcemia. Some synthetic analogs of vitamin D called deltanoids, such as the hexafluro-1α,25-dihydroxy vitamin D_3 derivative Ro24-5531, have shown potent differentiative and antiproliferative activities with less propensity to cause hypercalcemia.[100]



Ro24-5531

PPARγ modulators (SPARMs) are also important in cancer chemoprevention. Among them, the nonthiazolidinedione tyrosine-based PPARγ ligand GW7845 induces apoptosis and limits migration

and invasion of rat and human glioma cells, and it also inhibits mammary carcinogenesis in animal models.[101] Clinical trials to study its preventive activity against breast, colon, and prostate cancer have been announced.



GW7845

REFERENCES

1 Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Díaz LA, Kinzler KW. *Science* 2013;**339**:1546.
2 Orphanos G, Kountourakis P. *Hematol Oncol Stem Cell Ther* 2012;**5**:127.
3 Musolino A, Bella MA, Bortesi B, Michiara M, Naldi N, Zanelli P, et al. *Breast* 2007;**16**:28.
4 Dienstmann R, Tabernero J. *Anticancer Agents Med Chem* 2011;**11**:285.
5 Lamparella N, Barochia A, Almokadem S. *Adv Exp Med Biol* 2013;**779**:145.
6 (a) Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. *Sci Transl Med* 2012;**4**:127rv3; (b) Ajona D, Pajares MJ, Corrales L, Pérez-Gracia JL, Agorreta J, Lozano MD, et al. *J Natl Cancer Inst* 2013;**105**:1385.
7 Tsao AN, Kim ES, Hong WK. *CA Cancer J Clin* 2004;**54**:150.
8 For a review, see Steward WP, Brown K. *Br J Cancer* 2013;**109**:1.
9 For a review, see Patterson SL, Maresso KC, Hawk E. *Clin Chem* 2013;**59**:94.
10 Hong WK, Lippman SM, Hittleman WN, Lotan R. *Clin Cancer Res* 1995;**1**:677.
11 Herberman RB, Pearce HL, Lippman SM, Pyenson BS, Alberts DS. *Cancer Res* 2006;**66**:11540.
12 (a) Sporn MB, Suh N. *Nature Rev Cancer* 2002;**2**:537; (b) Gtavitz L. *Nature* 2011;**471**:S5.
13 Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. *J Natl Cancer Inst* 1998;**90**:1371.
14 Goss PE, Ingle JN, Alés-Martínez JE, Cheung AM, Chlebowski RT, Wactawski-Wende J, et al. *N Engl J Med* 2011;**364**:2381.
15 Lathrop AE, Loeb L. *J Cancer Res* 1916;**1**:1.
16 Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. *Breast Cancer Res Treat* 2001;**65**:125.
17 Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. *Cancer Prev Res* 2010;**3**:696.
18 Suh N, Glasebrook AL, Palkowitz AD, Bryant HU, Burris LL, Starling JJ, et al. *Cancer Res* 2001;**61**:8412.
19 Vogel VG. *Curr Drug Targets* 2011;**12**:1874.
20 Andriole GL, Bostwick DG, Brawley OW, Gomella LG, Marberger M, Montorsi F, et al. *N Engl J Med* 2010;**362**:1192.
21 Andriole G, Bostwick D, Brawley O, Gomella L, Marberger M, Tindall D, et al. *J Urol* 2004;**172**:1314.
22 Castro E, Goh C, Olmos D, Saunders E, Leongamornlert D, Tymrakiewicz M, et al. *J Clin Oncol* 2013;**10**:1748.
23 Wattenberg LW, Wiedmann TS, Estensen RD. *Cancer Res* 2004;**64**:2347.
24 Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN. *J Clin Oncol* 2005;**23**:254.
25 Gupta RA, Dubois RN. *Nature Rev Cancer* 2001;**1**:11.
26 http://clinicaltrials.gov/ct/show/NCT00033371;jsessionid=14A1950A717BDE8B1E1404757F3BBC82?order=6.

27 Aggarwal BB, Shishodia S. *Biochem Pharmacol* 2006;**71**:1397.
28 Aggarwal BB, Ichikawa H, Parodia P, Weerasinghe P, Sethi G, Bhatt ID, et al. *Exp Opin Ther Targets* 2006;**10**:87.
29 Wang D, Dubois RN. *Gut* 2006;**55**:115.
30 Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, et al. *Cancer Res* 2000;**60**:293.
31 Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, Gordon GB, et al. *N Engl J Med* 2000;**342**:1946.
32 Solomon SD, Wittes J, Finn PV, Fowler R, Viner J, Bertagnolli MM, et al. *Circulation* 2008;**117**:2104.
33 Phillips RK, Wallace MH, Lynch PM, Hawk E, Gordon GB, Saunders BP, et al. *Gut* 2002;**50**:857.
34 Zell JA, Pelot D, Chen WP, McLaren CE, Gerner EW, Meyskens FL. *Cancer Prev Res* 2009;**2**:209.
35 Rothwell PM, Wilson M, Price JF, Belch JFF, Meade TW, Mehta Z. *Lancet* 2012;**379**:1591.
36 Birmann BM, Giovannucci EL, Rosner BA, Colditz GA. *Cancer Prev Res* 2014;**7**:33.
37 Thakur P, Sanyal SN. *J Environ Pathol Toxicol Oncol* 2010;**29**:255.
38 Gurpinar E, Grizzle WE, Piazza GA. *Front Oncol* 2013;**3**:181.
39 Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. *Biochem Pharmacol* 2006;**72**:1605.
40 For example, see. (a) Luo J, Chuang T, Cheung J, Quan J, Tsai J, Sullivan C, et al. *Eur J Pharmacol* 1998;**346**:77; (b) Castro-Gamero AM, Borges KS, Moreno DA, Suazo VK, Fujinami MM, de Paula Gomes Queiroz R, et al. *Invest New Drugs* 2013;**31**:858.
41 https://clinicaltrials.gov/show/NCT00313534.
42 Lebwohl M, Swanson N, Anderson LL, Melgaard A, Xu Z, Berman B. *N Engl J Med* 2012;**366**:1010.
43 Strasser BJ. *Science* 1999;**286**:1488.
44 Hussain SP, Hofseth LJ, Harris CC. *Nature Rev Cancer* 2003;**3**:276.
45 Watson J. *Open Biol* 2013;**3**:120144.
46 D'Andrea GM. *Cancer J Clin* 2005;**55**:319.
47 Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P, Bergo MO. *Sci Transl Med* 2014;**6**:221ra15.
48 Gann PH. *J Am Med Assoc* 2009;**301**:102.
49 (a) Cameron E, Pauling L. *Proc Natl Acad Sci U S A* 1978;**73**:3685; (b) Cameron E, Pauling L. *Proc Natl Acad Sci U S A* 1978;**75**:4538.
50 Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM. *N Engl J Med* 1985;**312**:137.
51 Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. *Lancet* 2004;**364**:1219.
52 Heaney ML, Gardner JR, Karasavvas N, Golde DW, Scheinberg DA, Smith EA, et al. *Cancer Res* 2008;**68**:8031.
53 Forman D, Altman D. *Lancet* 2004;**364**:1193.
54 Telang S, Clem AL, Eaton JW, Chesney J. *Neoplasia* 2007;**9**:47.
55 Vuyyuri SB, Rinkinen J, Worden E, Shim H, Lee S, Davis KR. *PLoS One* 2013;**8**:e67081.
56 Traber MG, Stevens JF. *Free Rad Biol Med* 2011;**51**:1000.
57 Rietjens IMCM, Boersma MG, de Haan L, Spenkelink B, Hawad HM, Cnubben NHP, et al. *Environ Toxicol Pharmacol* 2002;**11**:321.
58 Kuiper C, Molenaar IG, Dachs GU, Currie MJ, Sykes PH, Vissers MC. *Cancer Res* 2010;**70**:5749.
59 Emani CS, Williams MJ, Wiid IJ, Hiten NF, Viljoen AJ, Pietersen R-DD, et al. *Antimicrob Agents Chemother* 2013;**57**:3202.
60 Creah IK, Halliwell B. *Biochim Biophys Acta Mol Basis Dis* 2012;**1822**:784.
61 Sotgia S, Zinellu A, Mangoni AA, Pintus G, Attia J, Carru C, et al. *PLoS One* 2014;**9**:e84918.
62 For a review of organosulfur compounds in cancer chemoprevention, see Moriarty RM, Naithani R, Surve B. *Mini Rev Med Chem* 2007;**7**:82.
63 Franzoni F, Colognato R, Galetta F, Laurenza I, Barsotti M, Di Stefano R, et al. *Biomed Pharmacother* 2006;**60**:453.

64 Luo H, Tang L, Tang M, Billam M, Huang T, Yu J, et al. *Carcinogenesis* 2006;**27**:262.
65 Yang G, Shu XO, Li H, Chow WH, Ji BT, Zhang X, et al. *Cancer Epidemiol Biomark Prev* 2007;**16**:1219.
66 Ramshankar V, Krishnamurthy A. *J Nat Sci Biol Med* 2014;**5**:3.
67 (a) Mohanty NK, Saxena S, Singh UP, Goyal NK, Arora RP. *Urol Oncol* 2005;**23**:383; (b) Kavanaugh CJ, Trumbo PR, Ellwood KC. *J Natl Cancer Inst* 2007;**99**:1074.
68 Mariani S, Lionetto L, Cavallari M, Tubaro A, Rasio D, De Nunzio C, et al. *Int J Mol Sci* 2014;**15**:1433.
69 Kristal AR, Till C, Platz EA, Song X, King IB, Neuhouser ML, et al. *Cancer Epidemiol Biomark Prev* 2011;**20**:638.
70 For a review, see Lin Y, Shi R, Wang X, Shen HM. *Curr Cancer Drug Targets* 2008;**8**:634.
71 Majumdar D, Jung K-H, Zhang H, Nannapaneni S, Wang X, Amin ARMR, et al. *Cancer Prev Res* 2014;**7**:65.
72 Ju W, Wang X, Shi H, Chen W, Belinsky SA, Lin Y. *Mol Pharmacol* 2007;**71**:1381.
73 Yao K, Chen H, Lee M-H, Li H, Ma W, Peng C, et al. *Cancer Prev Res* 2014;**7**:139.
74 Bharti AC, Aggarwal BB. *Biochem Pharmacol* 2002;**64**:883.
75 Attisano L, Wrana JL. *EMBO J* 2012;**31**:4486.
76 Teiten MH, Dicato M, Diederich M. *Mol Nutr Food Res* 2013;**57**:1619.
77 Monteiro J, Gaspar C, Richer W, Franken PF, Sacchetti A, Joosten R, et al. *Carcinogenesis* 2014;**35**:2.
78 Teiten MH, Gaascht F, Dicato M, Diederich M. *Int J Oncol* 2011;**38**:603.
79 Kensler TW, Groopman JD, Sutter TR, Curphey TJ, Roebuck BD. *Chem Res Toxicol* 1999;**12**:113.
80 Auyeung DJ, Kessler FK, Ritter JK. *Mol Pharmacol* 2003;**63**:119.
81 Kensler TW, Egner PA, Dolan PM, Groopman JD, Roebuck BD. *Cancer Res* 1987;**47**:4271.
82 Glintborg B, Weimann A, Kensler TW, Poulsen HE. *Free Rad Biol Med* 2006;**41**:1010.
83 Suppipat K, Park CS, Shen Y, Zhu X, Lacorazza HD. *PLoS One* 2012;**7**:e51251.
84 Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, et al. *Carcinogenesis* 2007;**28**:1485.
85 Lissa D, Senovilla L, Rello-Varona S, Vitale I, Michaud M, Pietrocola F, et al. *Proc Natl Acad Sci U S A* 2014;**111**:3020.
86 https://clinicaltrials.gov/ct/show/NCT00098969;jsessionid=9628876D173ADEF448DE62395C60762D?order=8.
87 Sporn MB, Suh N. *Carcinogenesis* 2000;**21**:525.
88 Okuno M, Kojima S, Matsushima-Nishigaki R, Tsurumi H, Muto Y, Friedman SL, et al. *Curr Cancer Drug Targets* 2004;**4**:285.
89 Lee JJ, Hong WK, Hittelman WN, Mao L, Lotan R, Shin DM, et al. *Clin Cancer Res* 2000;**6**:1702.
90 Decensi A, Costa A. *Eur J Cancer* 2000;**36**:694.
91 Muto Y, Moriwaki H, Saito A. *N Engl J Med* 1999;**340**:1046.
92 Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. *Science* 2001;**294**:1866.
93 Hede K. *J Natl Cancer Inst* 2004;**96**:1807.
94 Qu L, Tang X. *Cancer Chem Pharm* 2010;**65**:201.
95 (a) Arun B, Mohsin S, Miller A, Isaacs C, Saxton K, Hilsenbeck S, et al. *J Clin Oncol* 2005;**23**(16S):1002; (b) Howe LR. *Clin Cancer Res* 2007;**13**:5987.
96 Fitz NF, Cronican AA, Lefterov I, Koldamova R. *Science* 2013;**340**:924.
97 Ibáñez K, Boullosa C, Tabarés-Seisdedos R, Baudot A, Valencia A. *PLoS Gen* 2014;**10**:e1004173.
98 ClinicalTrials.gov identifier: NCT01782742.
99 Makishima M, Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, et al. *Science* 2002;**296**:1313.
100 Guyton KZ, Kensler TW, Posner GH. *Annu Rev Toxicol* 2001;**41**:421.
101 Suh N, Wang Y, Williams CR, Risingsong R, Gilmer T, Willson TM, et al. *Cancer Res* 1999;**59**:5671.

Index

Note: Page numbers followed by *f* indicate figures and *t* indicate tables.

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z