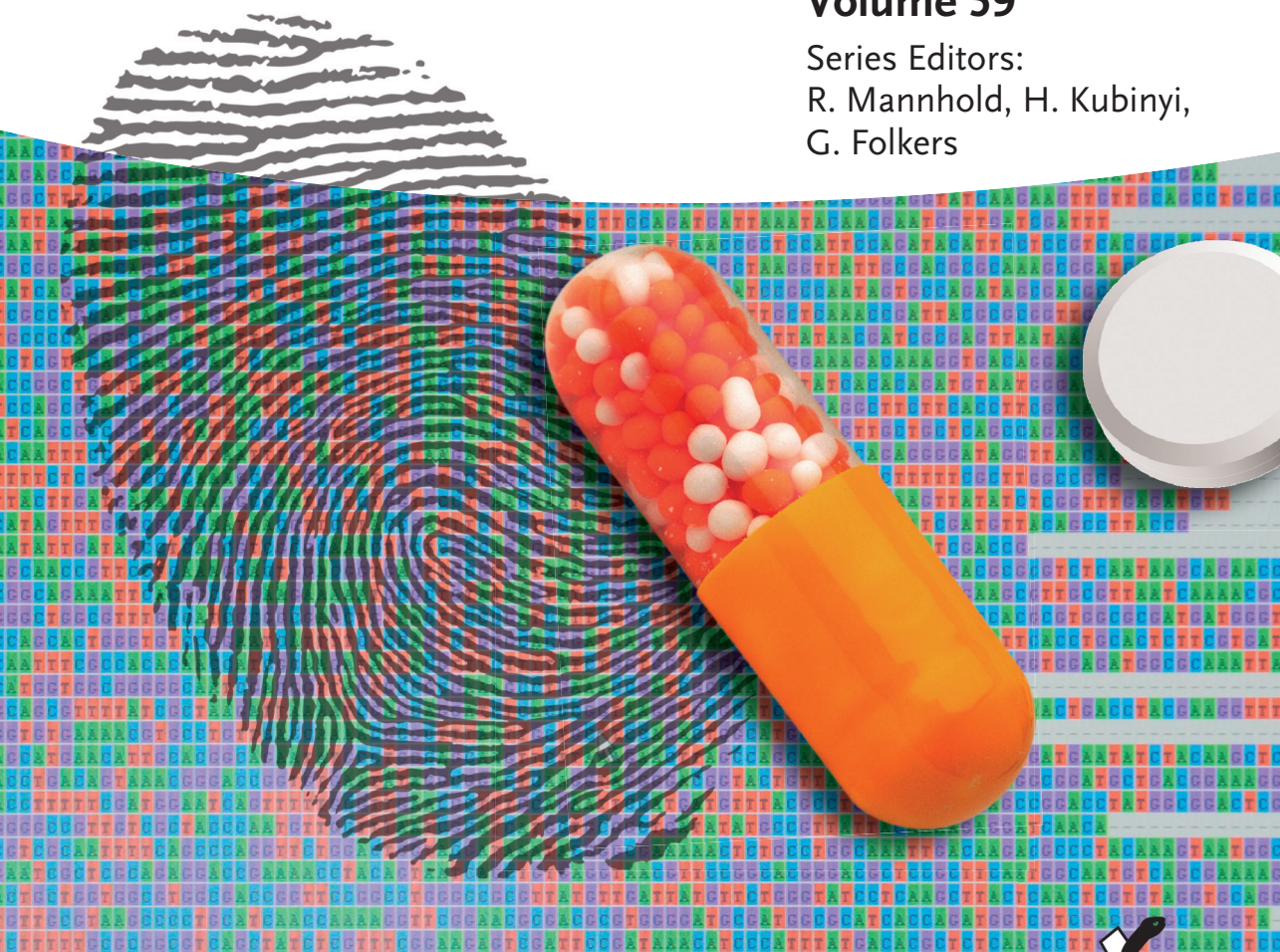


Edited by
Karen Lackey and Bruce Roth

Medicinal Chemistry Approaches to Personalized Medicine

Volume 59

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R. Mannhold, H. Kubinyi,
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Foreword

Over the past decade, major advances have been made in elucidating the pathophysiological processes involved in many human diseases, including solid and hematological malignancies, hepatitis C, asthma, Alzheimer's disease, Parkinson's disease, age-related macular edema, and even diabetes. We know more about the biology of human disease than ever before, yet most diseases are still classified by their clinical presentation, associated physical exam, imaging data, and laboratory abnormalities. Only a few diseases are defined by the molecular pathways that cause the disease.

Using a “clinically” oriented approach to medicine results in profound heterogeneity in the molecular underpinnings of a given disease. Compounding this problem is that this heterogeneity has traditionally not been taken into account when studies were designed to evaluate a new molecular entity in a given disease. As an example, in 2005, Peagram *et al.* performed a Medline literature search using the keyword “epidermal growth factor receptor” (EGFR) and found 13 569 citations. Despite this intense level of scientific investigation into the EGFR, it was not until 2004 that important mutations in the kinase domain of the EGFR that identifies patients who are particularly sensitive to the effects of small-molecule tyrosine kinase inhibitors such as gefitinib or erlotinib were first reported. This lack of insight contributed to the numerous failed studies in the frontline non-small cell lung cancer setting when these inhibitors were given to an all-comers population. The authors of this paper also performed simulations to model the impact of including patients in a clinical trial whose disease is not sensitive to a given drug's treatment effect. They simulated administering a highly effective treatment to women with newly diagnosed metastatic breast cancer and found that when a diagnostic was used to select those patients most likely to benefit, the clinical trial was robustly positive. When the percentage of patients who would not benefit was increased, the treatment effect waned. Importantly, if only 25% of patients benefited (as is roughly the case with Herceptin for women with Her2 overexpressing breast cancer), studying an unselected population in a clinical trial (i.e., where 75% are unlikely to benefit) would result in survival curves that are essentially overlapping. In other words,

without appreciating this heterogeneity in disease biology, a clinical trial evaluating a potentially important new therapy would be negative without a diagnostic to identify those most likely to benefit.

The pharmaceutical industry is under intense pressure to improve R&D productivity. This is in large part driven by increasing costs associated with conducting clinical trials compounded by very low success rates once a drug enters clinical testing. One cannot help but wonder how many of the over 90% of drugs that fail during clinical development would have succeeded had more attention been given to identifying the population most likely to benefit.

Fortunately, over the past decade and in particular the last several years, there has been a marked shift in the discovery and development process to incorporate these concepts. Advances in cellular and molecular biology, human genetics, translational medicine (including biomarkers and diagnostics), and innovative clinical trials designs have enabled us to enter the era of so-called personalized health care (PHC). This is leading to some of the most promising new therapies ever developed in the history of medicine. In oncology alone, this new era of medicine has resulted in numerous new drugs for patients. As of 2013, the NCI website has identified over 40 “targeted therapies,” although not all of these new medicines would meet the strict definition described above.

For some of these new therapies, we have observed treatment effects of almost unparalleled nature, a shorter time in clinical development, and although it is still in early days, it appears that the success rates are also likely to exceed industry averages.

It should also be pointed out that while the advances in personalized health care have been extremely impressive in oncology drug development, a similar targeted strategy is being embraced in the fields of immunology, neuroscience, and other areas of medicine. It should also be highlighted that while for most areas of medicine PHC is only recently being embraced, the field of infectious disease has adopted this concept for decades. The idea that all cases of “pneumonia” are not the same is today taken for granted. The technology for understanding the pathophysiology of this disease required much less sophisticated tools (i.e., the microscope and Petri dishes). This leads to subclassification of pneumonia by the causal agent with different treatments being prescribed based on the presumed organism responsible for the disease.

With the sequencing of the human genome over a decade ago and an increasingly sophisticated understanding of the pathophysiology of human disease-based metabolomics, proteomics, and other tools, we have clearly ushered in a new era in drug discovery and development. The end result is likely to have a very meaningful and lasting impact on academia, biotechnology and pharmaceutical companies, payers, health care providers, and most importantly patients.

Surprisingly, despite the importance of personalized health care in so many recent advances in drug therapy, there have been few attempts to collect the

success stories across industry and academia that have advanced research toward new, targeted therapies. This book, therefore, fills this gap in the literature and thus should be a useful resource for pharmaceutical and biopharmaceutical researchers for years to come.

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Preface

The notion of personalized medicine, in both the laity and the scientific community, is very often associated with screening, genetic profiling, and risk stratification. While it is unquestioned that genomics is the starting point of future “targeted medicine,” personal genomics and individual genetic testing for risk stratification are still under public debate, because of their ethical and legal implications. Therefore, an account of how all this collected genetic information translates into therapeutic practice and how it may do so in near future is of highest importance not only for the public dialogue but also for the experts in drug design and development.

This book provides such an account. Edited by Karen Lackey and Bruce D. Roth, both fundamentally involved in the topic, the book convenes experts from the medicinal chemistry field in the private sector and the academia to provide their perspectives on personalized medicine. Naturally, the scope is broad. The book consisting of 13 chapters covers a more general content on feasibility of medchem approaches, contrasted by those that describe case studies of successful implementations and also others that open up new field to explore. In addition to cancer – the therapeutic area one would expect to have been mainly covered, neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases as well as asthma have also been studied in this book. Methodological approaches and targets besides “chemistry” range from molecular profiling, G-quadruplexes, amyloid probes, and PET to histones, plaques in the brain, kinases, ubiquitination as a future target superfamily, and DNA repair pathways.

Of course, any book on this broad topic cannot be comprehensive or even encyclopedic. The translational process of personalized medicine is in full swing and many economical questions either for the private sector or for patients and social security systems remain to be solved.

The book parallels success stories – that have been long overdue to be reported – with recent and future developments in the field.

In this respect, it is not only at cutting edge in the field but also fulfills in an excellent way the requirement of this series to serve as a handbook for bench chemists, developers, and the academic realm of research and teaching. Especially teachers may feel encouraged to use the eminent expert information collected, to

challenge their students with this extension in medicinal chemistry to a medicine of the future.

The series editors are indebted to the authors and the editors who made it possible to cover this very essential issue.

We are also very much indebted to Heike Nöthe and Frank Weinreich, both at Wiley-VCH. Their support and ongoing engagement not only for this book but also for the whole series *Methods and Principles in Medicinal Chemistry* greatly contribute to the success of this excellent collection related to drug research.

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Weisenheim am Sand
Zürich
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Raimund Mannhold
Hugo Kubinyi
Gerd Folkers

A Personal Foreword

Personalized medicine and personalized healthcare have become virtual buzzwords used by the lay press and the pharmaceutical and biopharmaceutical industries in describing their current approaches to drug discovery and development aimed at providing patients with individualized therapies. Many established and emerging companies have even suggested that this is the foundation for their business strategy. Fundamentally, creating personalized medicine requires the integration of multiple disciplines, including medicinal chemistry, genetics, diagnostics, biochemistry, cellular biology, pharmacology, formulations, and clinical sciences, in order to ensure that patients have access to and are prescribed medicines with the highest likelihood of effectively treating their specific disease – and that patients unlikely to respond are not given drugs from which they will likely not receive benefit. The ultimate goal of the medical field is to have drugs that treat the underlying causes of the disease pathology. This approach has many benefits: to the companies, lower costs and higher success rates; for the patients, more effective therapies with better risk/benefit ratios. In fact, over the last several decades, many drugs, both small molecules and biologics, have been discovered and developed that would fall under this umbrella, especially in the treatment of cancer, where the emphasis on personalized medicine has led to greatly improved success rates in bringing new medicines to the market. Despite this emphasis on personalized medicine in the last decade, there has been no comprehensive treatment of this subject focusing specifically on the role of the medicinal chemist in this process, despite the fact that virtually all small-molecule drugs originate in the mind of the medicinal chemist.

In this book, we have attempted to bring together the collective experience of the pharmaceutical industry and academia, across multiple therapeutic areas and disciplines, in an attempt to capture the full spectrum of activities in implementing personalized medicine. Thus, we have chapters providing case studies of several recently approved “targeted therapies” in oncology where personalized medicine is most mature, but there are also chapters that cover developments in other therapeutic areas, development of diagnostics, imaging, and several on different aspects of new target discovery. Our hope is that this book will not only be a useful review of past practices in the discovery and development of personalized medicine but will also lay the foundation for future advances in

bringing life-changing, transformative medicines to patients. Ultimately, the goal of all of those who have committed their lives and energies to medicinal sciences is to bring benefit to the patients who are desperately waiting for the drugs that arise from the incredible scientific discoveries emanating from the work of these dedicated researchers.

Finally, we would like to thank all of the more than 40 authors and contributors to this book as well as the support and encouragement of Dr Heike Nöthe and Dr Frank Weinreich of Wiley-VCH. We are also greatly indebted to Ms Christine Cumberton for the finalization and compilation of chapters for submission to the publisher.

Nutley, NJ
South San Francisco, CA
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Karen Lackey
Bruce D. Roth

Acronyms

AChE(I)	acetylcholine esterase (inhibitor)
AD	Alzheimer's disease
ADC	antibody drug conjugates
ADME	absorption, distribution, metabolism, and excretion
AE	adverse events
AGC	protein kinase A, G, and C families
AHR	airway hyperresponsiveness
ALCL	anaplastic large-cell lymphoma
ALK	anaplastic lymphoma kinase
AP-1	activating protein 1
APC	adenomatous polyposis coli gene
APP	amyloid precursor protein
ATP	adenosine triphosphate
AUC	area under the curve
BBB	blood–brain barrier
BCC	basal-cell carcinoma
BCRP	breast cancer resistance protein
BER	base excision repair
BID	<i>bis in die</i> (Latin) meaning twice a day
BP	binding protein
CAD	coronary artery disease
CBD	corticobasal degeneration
CETP	cholesteryl ester transfer protein
CHMP	Committee for Medicinal Products for Human Use
CIA	collagen-induced arthritis
CI	confidence interval
CLR	clearance rate
CML	chronic myelogenous leukemia
CNS	central nervous system
CNV	copy number variations
COPD	chronic obstructive pulmonary disorder
CR	complete response
CRC	colorectal cancer

CSF	cerebral spinal fluid
CTC	circulating tumor cells
CUP	carcinoma of unknown primary
CDK	cyclin-dependent kinase
COMT	catechol-O-methyl transferase
DAG	diacylglycerol
DAT	dopamine transporter
DCR	disease control rate
DDR	DNA damage response
DECP	diethyl cyanophosphonate
DLB	dementia with Lewy bodies
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DR	direct repair
DUPA	(dicarboxypropyl)ureidopentanedioic acid
ER	estrogen receptor
ErbB2	erythroblastic leukemia oncogene homolog 2, also known as HER2/Neu
ERK	extracellular regulating kinase
FAM	6-carboxyfluorescein
FBDD	fragment-based drug discovery
FBLD	fragment-based ligand discovery
FDA	Food and Drug Administration
FDG	fluoro-deoxy-D-glucose
FFPET	formalin fixed paraffin embedded tissue
FISH	fluorescence <i>in situ</i> hybridization
FRET	fluorescence resonance energy transfer
FTD	frontotemporal dementia
GEMM	genetically engineered mouse model
GIM	genetic interaction mapping
GIST	gastrointestinal stromal tumors
GLUT	glucose transport proteins
GSK	glycogen synthase kinase
GTPase	guanine triphosphatase
GWAS	genome-wide association studies
HDAC	histone deacetylases
HDM	histone demethylases
HER2	human epidermal growth factor receptor 2
hERG	human ether-a-go-go related gene
HGF(R)	hepatocyte growth factor (receptor)
Hh	hedgehog
HIF	hypoxia inducible factor
HR	homologous recombinations
HSP	heat shock protein

HTS	high-throughput screening
IC ₅₀	concentration at 50% inhibition
ICGC	International Cancer Genome Consortium
ICS	inhaled corticosteroids
IGF(R)	insulin growth factor (receptor)
IHC	immunohistochemistry
IL-1	interleukin-1
IMT	inflammatory myofibroblastic tumors
INDEL	insertions or deletions of a short coding region
ITK	interleukin-2-inducible T-cell kinase
IV	intravenous
LABA	long acting beta-2 agonists
LE	ligand efficiency
LipE	lipophilic efficiency
LN	lymph node
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MBC	metastatic breast cancer
MBP	microprecipitated bulk powder
MCI	mild cognitive impairment
MCT	methylcellulose Tween
MGMT	O-(6)-methylguanine-DNA methyltransferase
MK	midkine
MLC	myosin light chain
MLK	mixed lineage kinase
MMR	mismatch repair
MMSE	minimental state examination
MOM	methoxymethyl
MP	molecular profiling
MPI	myocardial perfusion imaging
MRI	magnetic resonance imaging
MRT	mean residence time
MTD	maximum tolerated dose
MTEB	metabotropic glutamate receptor type
mTOR	mammalian target of rapamycin
NA	not applicable
NCI	National Cancer Institute
NER	nucleotide excision repair
NET	norepinephrine transporter
NFT	neurofibrillary tangles
NGS	next-generation sequencers
NHEJ	nonhomologous end joining
NHL	non-Hodgkin lymphoma
NIH	National Institute of Health
NK	natural killer

NME	new molecular entity
NMR	nuclear magnetic resonance
NOAEL	no adverse effect level
NPM	nucleophosmin
NRTK	nonreceptor tyrosine kinase
NSCLC	non-small cell lung cancer
OICR	Ontario Institute for Cancer Research
ORR	overall response rate
OS	overall survival
PARP	poly-ADP-ribose polymerase
PAS	peripheral anionic site
PBCA	poly(butyl-2-cyanoacrylate)
PCR	polymerase chain reaction
PD	pharmacodynamic or progressive disease or Parkinson's disease
PDAC	pancreatic cancer-ductal adenocarcinoma
PDB	Protein Data Bank
PDGF(R)	platelet-derived growth factor (receptor)
PEG	polyethyleneglycol
PET	positron emission tomography
PFS	progression free survival
PI3K	phosphoinositol 3 kinase
PiB	Pittsburgh compound-B
PK	pharmacokinetics
PLGA	poly(DL-lactide-co-glycolide)
PMD	protein misfolding diseases
PSMA	prostate-specific membrane antigen
PSP	progressive supranuclear palsy
PTM	posttranslational modifications
PTN	pleiotrophin
QSAR	quantitative structure-activity relationship
RECISTs	response evaluation criteria in solid tumors
RGD	arginine glycine asparagine
ROC	Ras/GTPase domain in complex proteins
ROCK	Rho-associated coiled coil containing protein kinase
RPLN	retroperitoneal lymph node
RTK	receptor tyrosine kinase
SAR	structure-activity relationship
SBS	sequencing by synthesis
SD	standard deviation
SF	scatter factor
SGA	synthetic genetic array
SGC	Structural Genomics Consortium
SiFA	silicon-based fluoride acceptors
siRNA	small interfering ribonucleic acid
SLAM	synthetic lethal analysis by microarray

SMI	small-molecule inhibitor
SMO	smoothened receptor
SNP	single-nucleotide polymorphism
SPECT	single-photon emission computed tomography
SphK	sphingosine kinase
SPR	surface plasmon resonance
STK	serine threonine kinase
Syk	spleen tyrosine kinase
TAC	time activity curve
TAMRA	6-carboxytetramethylrhodamine
TBAF	tetrabutylammonium fluoride
TBI	traumatic brain injury
TERRA	telomeric repeat-containing RNA
TET	ten-eleven translocation
ThT	thioflavin-T
TKI	tyrosine kinase inhibitor
TKL	tyrosine kinase-like
TNF	tumor necrosis factor
US	United States
UV	ultraviolet
VEGF(R)	vascular endothelial growth factor (receptor)
VMAT	vesicular monoamine transporter
W3C	World Wide Web Consortium
WES	whole-exome sequencing

1

Medicinal Chemistry Approaches to Creating Targeted Medicines

Bruce D. Roth and Karen Lackey

1.1

Introduction

Personalized medicines are therapies that maximize the biological effectiveness of treatment by targeting the molecular drivers of the disease through a deep understanding of disease biology, identifying and treating the patients most likely to respond based on personal genomics, metabolomics, proteomics, and perhaps epigenomics. This ability to very selectively target appropriate patient populations has become the foundation of much of drug discovery in the past decade due to the remarkable advances in molecular biology and diagnostics that have enabled the understanding of many diseases at the genomic level. Personalized medicine has become even more important, as healthcare costs continue to soar, such that creating the ideal situation where patients would only receive a potent, safe, and efficacious drug that treats their specific disease at a dose that is titrated for their metabolism has become an ethical, a societal, and an economic imperative. The state of personalized medicine today finds different therapeutic areas at very different stages of development. For oncology most of the personalized medicine approaches reflect attempts to design drugs that very selectively target the drivers of a patient's specific cancer. In diseases of neuroscience, current personalized approaches attempt to treat these complex diseases through polypharmacy. For inflammatory diseases, personalized medicine requires strategies for subsetting patients to ensure that the medicine is treating the underlying causes of the disease. In all of these therapeutic areas, the role of medicinal chemistry is to create drugs with very specific properties and biological activities to achieve the objectives of personalization of medical care. The techniques and strategies needed by medicinal chemists ranging from identifying active compounds to optimizing chemical series for the intended patient population, delivery route, and combination therapy required to enable personalized medicine will be discussed in this book. This book will cover the meaning of personalized medicine, its importance, how it is implemented, and how medicinal chemistry has evolved to facilitate it. Since drug discovery research to achieve personalized health care is being

conducted in academia, biotechnology companies, pharmaceutical companies, and research institutes, we have tried to ensure representation from all of these institutions as chapter authors. Because therapeutic areas are in such different stages of achieving personalized medicine, we have dedicated sections of the book to cover the state of the art in oncology, neurosciences, and inflammation to demonstrate the diversity of approaches. Gleevec will be showcased in this introductory chapter as the groundbreaking example of personalized medicine, highlighting the key issues involved, including identification of the intended molecular target and target patient population, expanding the patient population by understanding the drug profile, and the need for alternatively designed drugs to combat resistance and nonresponsive patients. More recent advances in combination therapy and drug delivery will be discussed to show how medicinal chemistry can impact the effectiveness of individualized medicine. Drug repurposing of clinical candidates and marketed medicines can utilize the medicinal chemistry approaches to rapidly achieve personalized medicine goals and will also be covered. We have also included a chapter focused on diagnostics in oncology, an essential aspect of patient identification, highlighting the advanced state of science in this therapeutic area, as well as chapters on approaches to patient stratification in other therapeutic areas and a chapter on imaging as a new diagnostic frontier. The book will conclude with a future perspective on how medicinal chemistry will continue to be the driving force behind translating human genomic information into personalized medicines. Although targeted biologics are an essential part of the armamentarium of drug treatment and have been foundational in the development of personalized medicine, they are beyond the scope of this book and will only be mentioned briefly in this introduction. We will, however, in this introduction touch on the impact of biologics, most notably Herceptin, on the development of personalized medicines, as well as highlighting some of the topics not specifically covered by other authors, such as drug targeting through antibody–drug conjugates and nanoparticles.

1.2

Role of Medicinal Chemistry in Drug Discovery

Medicinal chemistry plays a critical role in the early research essential for lead identification and chemical tool generation, which enables the marrying of small molecules with important protein targets key to allow a deeper understanding of disease biology. Lead identification methods have different requirements for different target classes, gene families, mechanisms of actions, and currently available knowledge and have helped to drive the evolution of medicinal chemistry. For example, high-throughput screening is a well-established tool that has taken advantage of advances in automation technology and creative biological assay systems to evaluate compound libraries of 100 000 to a several million high-quality starting points. This has required medicinal chemists to become skilled in data analysis, hit evaluation, and prioritization of active compound series based on the

physicochemical properties needed for the specific biological target as well as “drug-likeness.”

Orthogonal screening approaches include fragment, virtual, and phenotypic screening. Fragment-based ligand discovery (FBLD) and fragment-based drug discovery (FBDD) have evolved fairly recently and involve screening small molecular weight compounds at high concentrations, usually employing biophysical techniques such as NMR or SPR, with the aid of protein crystallography. The aim of fragment-based discovery is to provide low molecular weight lead molecules that may provide better starting points for further functionalization. Alternatively, several differentially bound fragments can be connected in a way to rapidly increase ligand binding and potency. In some cases, specialized fragment sets can be created for particular target classes. For example, metal binding proteins make up a substantial number of potential drug targets, and fragment libraries can be designed that would preferentially bind to metals and pockets found in these proteins [1]. Virtual screening utilizes a variety of computational approaches (e.g., pharmacophore, shape, similarity searching) to identify potential active molecules for lower throughput assays or as a way to reduce assay screening costs by limiting the number of compounds evaluated. Success in these areas requires medicinal chemistry excellence in structure-based drug design, and the tools and skills to meet this need have evolved remarkably over the past two decades. Phenotypic screening is usually an efficacy assay of direct biological relevance to a disease, where the readout is the outcome desired for progression into *in vivo* assay systems. There is a resurgence of interest in this approach due to its historical success in translating early research to clinically useful drugs, albeit with the disadvantage of the difficulty in determining the precise mechanism of action in some cases [2]. The overall objectives of the lead identification techniques are to provide the medicinal chemist with options for starting points and tools for interrogating biologically important protein targets.

For targets that do not yield lead matter using these more traditional techniques, alternative approaches have been adapted to the lead-finding process. For example, although DNA encoded library technology has been around for over 20 years, only recently has it added significant value to drug discovery [3]. This technology entails creating libraries with tens to hundreds of millions of small molecules that can be pooled together and screened against protein targets under multiple conditions to obtain active compounds based on target affinity. The assay hits are decoded based on the DNA “bar code” of bound compounds, which can be sequenced after using PCR technology. Different families of compounds with a variety of mechanisms of modulating the protein can be found using this technology. This technology has forced medicinal chemists to expand the chemistries available in solvents compatible with DNA (e.g., water), while developing the informatics tools required in dealing with massive, complex data sets.

Ultimately, it is medicinal chemists who must generate the clinical drug candidate during the lead optimization phase of a research project. This requires optimizing the ability to potently modulate the biological target of interest both *in vitro* and *in vivo*, while controlling the physicochemical properties that govern

absorption, distribution, metabolism, and excretion required for the intended route of administration. For an oral drug, medicinal chemists optimize small molecules to be swallowed, to be absorbed into the blood stream, to be carried to the site of the diseased tissue without negative biological effects along the way (i.e., toxicity), and to modulate the intended biological target to restore the tissue to the fully effective and normal state (cure), with an exit from the body that is safe and timely. Each of these components requires specialized design criteria or in many cases, formulation science working with an efficacious compound to modulate properties through salt forms, crystallization techniques, and additives. Later, drug delivery advances in personalized medicine will be discussed and ways for medicinal chemists to make an impact highlighted. Other routes of administration require different properties to be built into the drug candidates. For example, asthma drugs may need to be inhaled or acute care drugs may need to be given intravenously. The medicinal chemist needs to incorporate specific properties that create extreme potency in lung and low systemic exposure for the inhaled drugs, while an IV drug needs to be highly soluble for low injection volumes. The route and dosing can play a role in personalized medicine by delivering the medicine to the diseased tissue in the most expedient manner, and by avoiding exposure to organs where toxicity could present a potential issue for treatment.

1.3

Evolution of Molecular Design for Subsets of Patients

The complexity of disease biology and human systems biology makes it seem impossible to believe that one treatment approach or one drug could achieve a cure for all patients with a particular disease. A small-molecule medicine would need to be absorbed systemically across diverse groups of patients and demonstrate specificity for the diseased cell or aberrant target or tissue, without exerting significant side effects along the way. Within the diseased tissue, the medicine needs to have specificity for the mechanism of action needed to reverse the pathology or to stop the progression. Most likely, there are a combination of mutations or aberrations responsible for the cause or progression of the disease, all of which are affected by genetics, epigenetics, the microenvironment, and as will be discussed later even the microbiome.

Just two decades ago, most projects worked on by medicinal chemists in oncology were variants of chemotherapeutics, where toxicity to the patient was accepted as part of the therapy. The objective was to kill tumor cells at a greater ratio than normal cells. No one expected to achieve oncology treatments without very significant toxicity. For example, camptothecin was shown to be a powerful anticancer agent in preclinical studies, with a mechanism of action of topoisomerase I inhibition. It went into clinical trials based on its ability to achieve a greater ratio of killing tumor cells as compared to normal cells, but with the assumption that the treatment for patients would be inherently toxic [4]. Many drug discovery projects continued throughout the pharmaceutical industry to improve the drug

properties of camptothecin, such as solubility, metabolic stability, and improved therapeutic window. Topotecan and GG211 progressed into the clinic, with incrementally improved drug profiles, but still were designed to treat solid tumors of all patients by killing tumor and normal cells, with just an improved ratio of the former. During this time in the late 1980s and early 1990s, the inhibition of kinases was being debated as a viable way to treat subsets of cancer patients based on protein expression patterns. The transition in oncology drug discovery began with the development of targeted biological agents such as Herceptin [5]. The recognition of Herceptin's exceptional efficacy in the 35% of breast cancer patients who overexpressed the erbB2 receptor first demonstrated the power of targeting therapy to a diagnostically defined patient population based on the mechanism of action of the therapeutic agent.

The landmark discovery of Gleevec (Glivec, STI571, imatinib), first synthesized in the mid-1990s and approved for marketing by the FDA in 2001, ushered in an era of targeted small-molecule anticancer drugs aimed at capitalizing on advances in the understanding of oncogenes and the key drivers of cancer. This event transformed medicinal chemistry in oncology to focus on targeted anticancer drugs, with the potential to be highly selective and much less toxic by preferentially killing tumor cells by attacking targets overexpressed or amplified in cancer, but not in normal cells. Only the highlights of the discovery of Gleevec will be discussed here, while more in-depth information can be obtained in Refs [6–9]. Chronic myelogenous leukemia (CML) is a blood disorder with excessive proliferation of cells (myeloid lineage) associated with a specific genetic abnormality: a reciprocal translocation between chromosome 9 and 22 (the so-called Philadelphia chromosome). The protein product of the aberrant gene, a fusion of the *abl* proto-oncogene and the *bcr* gene called *bcr-abl*, possessed significantly increased tyrosine kinase activity that was subsequently proved to be essential to cell transforming activity. Gleevec was designed to selectively inhibit this kinase activity, revolutionizing treatment of CML. This new paradigm for drug discovery and development was facilitated by having all of the tools required for a drug discovery project available, effectively linking preclinical models with disease in a clinical setting. Thus, the elevated kinase activity could be measured in a catalytic enzyme assay. Cells overexpressing *bcr-abl* could be used for *in vitro* and *in vivo* models. Clinical trials could be designed based on inhibiting a specific mechanism of action in a subset of cancer patients. These tools, combined with the molecular and genetic understanding of this disease, allowed the very rapid development of this molecule from bench to market, including a remarkably short 3 years of clinical trials prior to approval by the FDA for treatment of CML in the US. The ability to demonstrate safety and efficacy in humans in such a short period of time demonstrated the power of this paradigm to rapidly unite patients with therapies effective for their disease.

As the molecular targets of Gleevec became better understood, alternative indications were uncovered. Thus, two additional kinases potentially inhibited by Gleevec are c-Kit, a member of the type III group of receptor kinases, and the

PDGF receptor tyrosine kinase. Based on the work of Hirota *et al.* [10], which identified gain-of-function mutations in c-Kit in gastrointestinal tumors (GIST), clinical trials of Gleevec were initiated in these patients and such profound efficacy was demonstrated that it was approved by the FDA in 2002 based on phase II data [8]. Given the nature of tumor progression, a multitude of mutations have been identified, requiring second and third generation bcr-abl drug candidates along with drugs with unique mechanisms of action to treat CML patients [11].

Subsequent to the discovery of Gleevec, studies of aberrant cell signaling over the past two decades have demonstrated key roles for numerous protein kinases in proliferation, migration, apoptosis, and survival. It is common now to examine tumor tissue for overexpression, mutation, and constitutive activation of a driver-kinase protein, looking for correlation of the kinase activity and disease outcome. Relatively selective kinase inhibitors have been brought to the clinic and many have been approved for use as medicines, providing clear benefit to patients [12]. However, as mentioned earlier, drug resistance typically emerges with prolonged treatment.

1.4

Combinations for Effective Therapies

Targeted therapies also include the concept of combinations, but based on a deep understanding of biology. The most common combinations are where the treatment plan includes separate, selective drugs taken at prescribed intervals, allowing some flexibility in dosage for each medication. There can also be a single drug molecule with a built-in combination profile, where the modulation of more than one protein target makes the treatment more effective than a selective modulator. With diagnostics readily available, a personalized fixed-dose combination could also be possible with snap-together pills.

The identification of optimal drug combinations depends on many factors; however, deep understanding of disease biology is required to fully exploit available drugs in combinations to achieve personalized therapies. Medicinal chemistry has made great strides in creating molecularly targeted drugs with impressive selectivity. Treatments for individual cancer patients need to be designed for their tumors' complex signaling network, with consideration of feedback and compensation phenomena when driver pathways are inhibited. By way of example, Iadevaia *et al.* developed computational approaches for predicting effective combinations using IGF-1-stimulated breast cancer cells (MDA-MB231) as their model system [13]. Without going into the complexity of the modeling and experimental data, we will focus on how a medicinal chemist can use the approach to designing more effective medicines. Much of the signaling data in the literature is difficult to compare due to the effect of the diversity of experimental procedures on the quantitative values and the often, qualitative nature of the information. These authors chose the IGFR network to create a computational model because there exists a large body of data that can be analyzed to create and test a consensus

network, and with clinical candidates progressing in cancer trials, the outcome of the work is highly relevant to patients. The trained model was used to predict the effect of new perturbations in the signaling network and then tested experimentally to validate the model. They wanted to identify the most influential proteins responsible for the aberrant cell signaling to determine the best combinations of inhibitors and siRNAs. While the tool compounds they used were not selective for the protein targets studied, the model could be used for evaluating the next generation of signaling inhibitors, with more advanced designs.

The IGFR signaling network in the MDA-MB231 cell line included node points, activating and inactivating proteins, and the protein interactions. To illustrate the complexity, their formulation included 77 chemical reactions to describe the consensus IGFR network. A simplified subset of 41 reactions was used in the model based on inclusion of the most relevant interaction mechanisms in the network. Results of the model suggested that targeting one protein in the signal cascade at a time might activate nontargeted proteins, thus making ultrasensitive drugs or the use of single signaling inhibitors insufficient to block aberrant signaling. In order to determine the right combinations of target molecules, perturbing all molecules in the network simultaneously would help identify the optimal combinations needed to effectively block proliferation signaling. Their research conclusion was that optimal inhibition could be achieved by inhibition of both MAPK and PI3K pathways by correlating it to decreased cell viability. In an important contrast, nonoptimal combinations led to inadequate inhibition of the network and increased cell viability. The computational procedure is one example of many emerging algorithms and data analysis tools, rapidly advancing the field of personalized medicine. The goal is to have tools available to rapidly generate experimentally testable drug intervention strategies, allowing patients to receive optimized combination therapies and to discover novel signaling targets for medicinal chemists to design effective candidate drugs for future more effective combinations.

A cautionary example to counter the apparent success of the IGF signaling computational analysis outcome are the lessons for designing combination therapy with dasatinib reported by Park *et al.* [14]. Dasatinib is an oral, small-molecule src/abl tyrosine kinase inhibitor that received FDA approval in 2006 for CML patients who developed resistance to Gleevec. Disappointingly, phase II clinical trials with dasatinib as monotherapy were not encouraging, although preclinical studies with diverse agents suggested dasatinib combinations would be synergistic, although there appeared to be no clear rationale for the synergism. Park *et al.* concluded that molecularly targeted agents like dasatinib should be effective in combinations, but the trial designs and combination therapies may remain empiric. For medicinal chemistry, creating effective mechanism-based components of the therapeutic options remains a high priority, but the sheer number of empiric possibilities to be investigated by translational medicine experts is daunting. Deeper biological understanding and better *in silico* methods for cost-effective, timely, and predictive combinations for personalized medicine, taking into account the genetic heterogeneity and

plasticity of tumors, are urgently needed. These investigators felt that the critical hubs of tumorigenesis were likely to be determined, but felt that modeling of the compensatory pathways or genetic instability was too difficult with the current state of the art.

Achieving personalized medicine in autoimmune and inflammatory diseases is an emerging field of science that holds great promise, but the identification of mechanism-based, diagnostically identified subtypes of patient populations to increase the likelihood of individual response to treatments is still developing. Virgin and Todd recently reported on the concept of understanding disease metagenomics, defined as the sum of the genetic elements of the patient (host) plus all of the genetic elements in all of the microorganisms (bacteria, viruses, and parasites) that live in or on the host [15]. The relationship between genotype and phenotype in complex, chronic diseases such as type 1 diabetes and inflammatory bowel disease were shown to be determined by host gene–microbe interactions and the immune system damaged tissues. Information from genome-wide association studies (GWAS) and analysis of the microbiome can help define mechanisms for inflammatory diseases. The genes (and gene products) identified in the analyses of genotype–phenotype relationships, which lead to pathogenesis should provide validated biomarkers and druggable pathways for medicinal chemistry to discover tool compounds and ultimately drugs for specific subsets of patients.

“A diagnosis may be “clinically” precise but “mechanistically” imprecise . . . Over many decades, pathologists have lumped patients with similar but non-identical clinical and pathological signs and symptoms into diagnostic categories that predict outcomes and complications. Indeed, this has enormous value clinically, but it emphasizes similarities between patients in outcome rather than the differences in pathways that lead to a common endpoint” [15]. The key learnings making an analysis metagenetic, and not just genetic, are the disease diagnostics, the sum of multiple mechanism subsets, and the interactions of individual microorganisms and their genomes with specific host genes and pathways, all critical for understanding the genotype–phenotype relationships in complex diseases. For the medicinal chemist, this approach of subsetting patients by pathways and/or mechanisms of action, despite the complexity of many diseases, aids in the development of selective medicines or combinations. Moving away from diseases as a single pathological mechanism to diseases as multiple mechanism-based subtypes may require the chemist to work across normally separated therapeutic areas (e.g., antibacterial agents and immunomodulation). Since the microbiome, and thus the metagenomics, varies from person to person and affects the development of the immune system, understanding the host gene–microbe interactions is essential to improve drug outcomes. To devise a patient stratification strategy and uncover novel therapeutic opportunities, Virgin and Todd [15] proposed an iterative process of evaluating candidate pathways followed by mechanistic studies in animal models and microbial genetic studies to define the mechanism-based disease subtype with inherent biomarkers that distinguish between patients based on mechanism. The medicinal chemist can play a key role in the iterative cycle by designing drug candidates that target the subtypes.

1.5

Biomarkers in Targeting Patients

Biomarkers, as defined by the NIH, are “a characteristic objectively measured and evaluated as an indicator of normal biological and pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Biomarkers can be divided into two types: diagnostic biomarkers used in patient identification and stratification and pharmacodynamic (PD) biomarkers used to measure therapeutic response. These can be the same or different. For drug discovery and development, the best situation is continuity in the PD biomarker used preclinically to discover and optimize the drug for maximum efficacy and in therapeutic safety margins and the biomarker used clinically to evaluate response in patients. Diagnostic biomarkers are more involved because they are directly linked to the disease pathology and/or progression, and typically distinguish between normal and diseased tissues and patients. The promise for patients is that only those identified as having that diagnostic biomarker will receive the treatment and only responding patients will continue receiving the medicine. Many of the kinase inhibitors that have been successfully launched as drugs were designed to treat specific, diagnostically identified patients and were facilitated by codeveloped drug efficacy (PD) biomarkers that were used preclinically to validate the target and clinically to assess initial clinical response.

Drug efficacy (PD) biomarkers are important for a variety of reasons, but are most critical in developing the relationship between drug exposure and pharmacologic response. The availability of PD biomarkers in early clinical development ensures that only drugs that engage the target adequately are advanced into efficacy trials, greatly increasing the potential for success and reducing the cost of clinical trials, especially when combined with a diagnostic biomarker that has identified the patients most likely to respond. As an example, in the development of the dual erbB1 and erbB2 tyrosine kinase inhibitor (Tykerb), measuring the inhibition of autophosphorylation of the protein in the tumor tissue of patients allowed the early assessment of pharmacological activity. During clinical trials, the biologically effective dose was determined (rather than the maximally tolerated dose) based on target engagement using as the PD biomarker pathway inhibition as measured by the reduction in phosphorylated erbB2, or a downstream protein such as MAPK [16].

1.6

Emerging Field of Epigenetics

Epigenetics is an emerging field, still in the early stages of medicinal chemistry input, but worthy of mention in personalized medicine approaches. Budiman *et al.* recently reported studies on DNA methylation in personalized medicine [17]. To understand how the signature of DNA methylation can inform patient care, we will provide a brief background in epigenetics. While the human genetic code is

relatively static, epigenetics involves heritable changes that affect gene expression and phenotypes. Unfortunately, there is no known single baseline reference for the epigenome to make comparisons between normal and diseased tissues. The epigenome can vary among healthy, normal cellular populations as well as in disease cellular contexts. The way these changes in gene expression, and thus their related protein production, occur is through molecular modifications of histone proteins and the effect these marks have on cooperating partners. In perhaps an oversimplified model of epigenetics, modifications such as methylation, acetylation, ubiquitination, and the reverse (e.g., deacetylation) cause genes to be turned on or off, thus changing the cellular processes. These changes can be positively adaptive (i.e., they are good outcomes of gene expression changes) or they can cause aberrations that lead to disease.

For epigenetics to impact personalized medicine, the pattern of the histone or DNA modifications would need a diagnostic biomarker that meets the NIH definition. Budiman makes the case that patterns of loss of DNA methylation as well as acquired methylation can play a role in an individual's response to therapy and susceptibility to age-related diseases. It is fair to say that these are still very early days in the role of epigenetics in personalized medicine; however, biomarker development is technically feasible as long as the signatures of DNA methylation can be decoded. Once the field matures, it should be possible for a medicinal chemist to create drug candidates that modulate the epigenetic signature. There are several global public-private partnerships involved in the precompetitive research space, working on creating chemical probes and biological reagents to fully annotate the epigenome (e.g., <http://www.thesgc.org/scientists/epigenetics>). Ideally, a few prominent modifications will be linked to disease progression, similar to the computational algorithms being developed for complex signaling networks, and patients will be treated with personalized combinations that reverse the epigenetic modifications to restore healthy cellular processes.

1.7

Systems Chemical Biology

All of the examples given thus far have been reliant on analyzing a subset of data in the context of a single target or pathway, or taking things that were discovered in single pathways and combining them for the desired effect. David Wild *et al.* have defined systems chemical biology as the integration of chemistry, biology, and computation to generate an understanding about the way small molecules affect biological systems as a whole [18]. Chemical genomics builds models based on effects of compounds on multiple biological targets and pathways by studying relationships between chemical compounds and genes and their protein products. Systems chemical biology involves a broader view of analyzing networks of many kinds of data, including compounds, targets, genes, diseases, side effects, clinical data, metabolic data, and more. Thus, these are heterogeneous data sets that are very difficult to integrate, but for the future of personalized medicines, it is critical

that the scientific community taps into all of the information that is being generated in separate public data sources (combined with proprietary databases) to create knowledge about the entire biological system and how the components are differentially affected by treatments. The phrase semantic web refers to “a shared understanding of meaning and accessibility to tools across the data sets” [18]. Thus, a semantically integrated network of data would allow searches using common terminology across multiple databases with a single framework, and would allow the discovery of relationships that go across multiple data sets. The authors discuss a pathfinding algorithm that links drugs and side effects. The algorithm determined that a drug undergoing biological evaluation interacted with genes that had previously been found linked with older drugs with known, specific side effects (all with the gene in common). This information provides a testable hypothesis for a potential side effect. By analogy, one can discover potential risk factors for new drugs and uncover potential mechanisms causing side effects. Medicinal chemists can use this information as an opportunity to design out the side effect by adding the gene target as a selectivity assay in their lead optimization campaign. The World Wide Web Consortium (W3C) is responsible for making recommendations for components of the semantic web. For scientists, the desire is for a straightforward way to integrate heterogeneous data sets between organizations or data silos. This effort is important for the future of medicinal chemistry, since public databases, open access to clinical trial data, and proprietary databases need to be accessible for optimally determining drug efficacy and patient benefit, side effect profiles, stratification of patients, drug differentiation, appropriate combination therapies, unmet medical needs, and potential disease associations for new compounds. The ultimate objective in realizing systems chemical biology is in integrating diverse data resources, building knowledge and using existing computational approaches like homology modeling, QSAR, and virtual screening to enhance our drug design capabilities. The underlying question in this entire approach will be the quality and relevance of the data.

The ability to take a systems biology approach may allow the treatment of complex diseases, such as traumatic brain injury (TBI), where there have been over 200 clinical drug trials, but no successes and thus no FDA approved drugs [19]. For systems biology, as a mimic of complex disease, to result in personalized medicines, it must be integrated with diagnostic or biomarker-based codiscovery. TBI causes physical and chemical perturbations of brain cells, which activate certain targets and signaling pathways resulting in cell injury. As in systems chemical biology described in this section, Zhang *et al.* [19] state that to successfully treat a patient’s brain damage and functional deficit, a holistic approach utilizing and integrating diverse databases is necessary – proteomics, genomics, interactome, literature, text mining, experimental data, and more. The goals of systems biology in TBI is to better understand the mechanisms of disease to uncover targets, biomarkers, and diagnostic tools, and to create models to predict the interrelated functions of the system to discover the proteins that regulate cellular decisions. With the dearth of available treatments, TBI seems like a rich area for medicinal chemistry impact. The authors list over a dozen general, relevant public

databases that could be mined for a theranostic approach with an aim to create a TBI medicine that combines the diagnosis, treatment, and monitoring of patient response in one entity. Because nonbiomarker-based trials have resulted in failure, it is highly unlikely that future investment in the area will be supported without them.

Systems biology combined with structural bioinformatics equals systems medicine. Systems biology combines and analyzes diverse data sets to predict the outcomes of system perturbations, using network models. Structural bioinformatics has made significant progress in enabling the science of identifying protein–drug off-targets based on analyzing ligand binding sites to either predict potential toxicities, polypharmacy, or repurposing opportunities. To this end, Chang *et al.* have developed a novel *in silico* drug testing approach for systems medicine with the aim to maximize benefits to patients with treatment and identify risk factors (off-target mechanisms or genetic polymorphisms) that may preclude treatment [20]. Chang *et al.* used their integrative computational approach on predictions for the failed clinical candidate torcetrapib – a cholesteryl ester transfer protein (CETP) inhibitor. This drug candidate was designed to treat cardiovascular diseases by raising high-density lipoprotein cholesterol, but failed due to an increase in mortality in the torcetrapib-treated patients. Because one side effect observed in patients receiving torcetrapib was hypertension, the authors performed context-specific kidney metabolic modeling. The complexity of this approach is such that the authors used 336 explicitly predicted active metabolic genes, 1587 active reactions in the model, and 333 active reactions to develop a submodel for the pathways in the specified renal objectives. They also found different binding affinities for off-targets, using their structural analysis of the three CETP inhibitors that have reached clinical trials (torcetrapib, anacetrapib, and dalcetrapib), suggesting that there will likely be differences in the drug response phenotypes, especially with regards to side effects. Of course, there are limitations to the models because of the subsetting of complex data and the need to test the *in silico* predictions with real clinical data. However, it is important for a medicinal chemist embarking on a drug discovery project to understand potential off-targets to avoid as well as the design features needed to maximize the effectiveness of the drug.

1.8

Theranostics and Designing Drug Delivery Systems

An extensive review of the concept and state of the art of theranostics, materials that integrate therapy and diagnostic imaging, was reported recently by Kelkar and Reineke [21]. While much of the details are beyond the scope of this medicinal chemistry perspective, a good understanding of the aims, the components that a medicinal chemist could impact and the current limitations of theranostics are critical for considering its application to personalizing medicine. Kelkar and Reineke state, “the ultimate goal of the theranostic field is to gain the ability to image and monitor the disease tissue, delivery kinetics, and drug efficacy with the

long term hope of gaining the ability to tune the therapy and dose with heretofore unattainable control" [21]. It is also possible that theranostic agents could impact all stages of drug discovery and development because they help to develop biomarkers of diseases both preclinically and clinically, greatly assisting in target validation, fine-tuning drug efficacy, and determining the final construction of the medicine. One clear limitation is the understanding or even the ability to optimally image and dose drug simultaneously (i.e., stoichiometry and issues with drug mechanism not interfering with imaging). In Section 1.9, two types of theranostics will be described to show areas of potential medicinal chemistry involvement.

Several examples in the recent literature demonstrate the concept of using a delivery system to construct an integrated system for personalizing medicines. Nanoparticles have some unique advantages beyond the design of conjugates, carrier materials, and payloads. For example, nanoparticles are not cleared by kidney, thus they could theoretically attain longer circulating blood levels. In addition, due to tumor tissue characteristics, nanoparticles selectively accumulate near tumors. Dual targeted nanoparticles with the potential to act as both a diagnostic and a therapeutic are particularly advantageous, as diagnostics and targeted therapies could benefit from effective and specific delivery to the site of disease tissue. Nanotechnology, via nanoparticles, could offer drug delivery methods that meet these requirements. Kluza *et al.* reported on a highly functionalized system, whereby they attached two ligands to a liposomal layer, surrounding a nanoparticle carrier with a diagnostic contrast agent [22]. While this sounds complicated, a medicinal chemist may be able to impact the optimization of such systems to create personalized medicines. The concept that these researchers pursued took advantage of the differential expression of specific molecules in the endothelium of newly formed versus normal vasculature. Thus, potentially a medicine could image blood vessels, while treating tumors via an antiangiogenic mechanism. Furthermore, the liposomal nanoparticles were considered bimodal because they were detectable via magnetic resonance imaging (MRI) as well as by fluorescence. MRI contrast agents are used to differentially image normal and diseased tissue; like therapeutic drugs, they must possess the desired properties of high contrast, stability, and acceptable pharmacokinetic properties. The two angiogenic biomarkers that Kluza *et al.* used were based on two receptors: $\alpha_v\beta_3$ integrin and galectin-1. The cyclic peptide cRGD (extensively studied $\alpha_v\beta_3$ inhibitor) and a designer 33-mer peptide Anginex (galectin-1 inhibitor) were conjugated to the bimodal liposomes [23,24]. The dual targeted liposomes were compared with single targeted liposomes, the peptides alone, the liposomes alone, and controls. The investigators found that all types of targeted liposomes were internalized and efficacy was observed for each of the single targeted liposomes. When they were mixed (i.e., like fixed-dose combinations) and examined for cellular uptake and cell cycle analysis, additive effects were observed. However, the dual targeted liposomes demonstrated synergistic effects. This outcome seems particularly important, where the mechanism of the disease has multiple pathways and numerous angiogenic factors to compensate with when a single pathway is blocked. A medicinal chemist could make an impact in this type of system by

optimizing the peptides as targeting agents attached to the nanoparticle carrier. Also, a medicinal chemist could use small-molecule inhibitors instead of peptides or target mechanisms beyond angiogenesis. In all of these targeting strategies, the connection to the nanoparticle carrier needs to be optimized through medicinal chemistry (further explained in the next example).

Liao *et al.* described a nanoscale platform to take an effective, but toxic drug like Doxorubicin and deliver it with greater tissue specificity in combination with an MRI agent [25]. The concept of a cancer therapy nanocarrier is to create a drug delivery system to reduce side effects by encapsulating the anticancer drug until it reaches the tumor and releases the cytotoxic agent. These researchers designed a hydrophobic core with high loading capacity, using a polymer of lactide and glycolide termed PLGA for poly(DL-lactide-co-glycolide). The ratio of the monomers was adjusted to vary the drug release rate and to avoid drug leakage in route to the tumor. A hydrophilic PEGylated lipid shell, similar to the one described earlier, was made paramagnetic by chelating diethylenetriamine pentaacetic acid–gadolinium [Gd(DTPA)(H₂O)]₂⁻ and targeted by linking to folic acid. The idea combines multimodal imaging, simultaneous diagnosis and therapy, specific targeting, and controlled release of therapeutics. Medicinal chemistry changes to the system could include folate replacement for alternative targeting or for creating dual target enhancement by linking folate plus an additional targeting agent. The release rates of the drug may need to be modified for specific tumor types and the payload could be two synergistic drugs that block cell signaling rather than a cytotoxin. Of course, a strong partnership between a medicinal chemist and a materials science expert would be needed to ensure that the nanoparticle morphology, stability, size distribution, and pharmacokinetic properties were optimized along with the target potency and efficacy.

A prodrug strategy can be employed to take effective, nonpersonalized medicines with toxicities or dose-limiting side effects and convert them to targeted medicines with fewer side effects and greater efficacy if properly targeted. Examples include monoclonal antibody–drug conjugates, aptamers, receptor agonists and antagonists, peptide hormones, and vitamins to name a few. The definition of a prodrug is a biologically inactive form of a drug that can be converted into the active parent molecule before or at the site of action. Focusing on ligand-targeted prodrug therapeutics, Kularatne *et al.* reported a method for targeting highly potent cytotoxic agents to prostate cancer tumors via PSMA (prostate-specific membrane antigen)-targeted prodrugs [26]. These researchers started with the design of the targeting agent to be attached to the cytotoxic drug; (dicarboxypropyl)ureidopentanedioic acid (DUPA) binds to cell surface glycoprotein PSMA and enters via endocytosis. The medicinal chemist's role is synthesis, design of the linker, optimization of the targeting agent, improving binding affinity, ensuring appropriate stability, and water solubility. Kularatne surveyed several cytotoxic agents and found eight suitable candidates for prodrug attachment with IC₅₀ values as nontargeted agents in the LNCaP (prostate) cancer cell line less than 10 nM, a threshold determined from their experience in the field. These cytotoxic agents were modified with linkers that preserved their cytotoxicity and terminated in a

moiety to allow facile disulfide linkage to DUPA. Thus, the design could be described as warhead – linker – DUPA.

The advantages of this approach are therapeutic flexibility, potential diagnostic value, and improved cell permeability. Therapeutic flexibility was demonstrated by taking an optimized targeting agent DUPA, attaching eight different cytotoxic agents via a common linker connection, and obtaining enhanced efficacy and cellular selectivity. Presumably, a similar approach could be taken to target any drug to pathological tissue with an appropriate linkage plus targeting ligand. As with the nanoparticle technology described in this section, a diagnostic agent could be cognate to the targeting agent to more easily identify responders to the new drug entity. Receptor-mediated endocytosis could be utilized for enhanced cellular uptake for the targeted agents, thus potentially improving cell permeability. All of these advantages also convey challenges. For example, many diseases do not have sufficiently potent compounds to fill the warhead role. The medicinal chemistry needed to create a linker of sufficient stability to reach the site of action, yet labile enough to release the drug, can be a challenge. A disadvantage of the whole approach is both the small quantity of molecules that enter the cell via endocytosis and the requirement of drug release into the cytosol once inside the cell. There are many components to the system that need to be optimized for it to work for personalized medicine. However, considerable success has been achieved with the antibody–drug conjugate approach as evidenced by the recent approval of T-DM1 for treatment of Her2+ breast cancer [27].

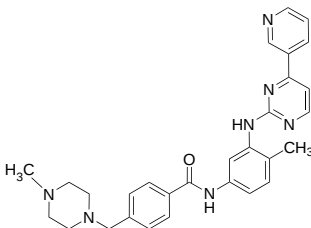
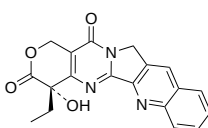
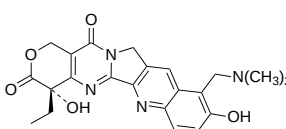
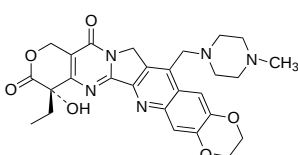
1.9

Rapid Progress in Further Personalizing Medicine Expected

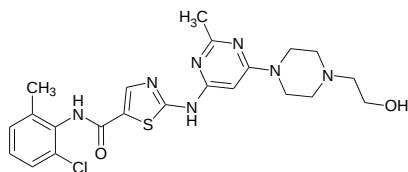
While the science of drug delivery and antibody–drug conjugates is beyond the scope of this book, it is tantalizing to believe that biomarker-induced drug release or tissue-specific distribution (chronobiology) could make personalized medicine safer and more efficacious within the next decade. With smart phones to collect clinical trial data and adaptive clinical trial design progressing, we are rapidly approaching a world of electronic information allowing incremental adjustments in dosing and combination. You can imagine a patient ingesting a multisegmented pill that had the ability to disperse the correct amount of each drug, titrated at a level for maximum patient benefit. Polypharmacy controlled by a patient and doctor's understanding of their physical well-being would be a huge advance in medicine. For example, Parkinson's disease and rheumatoid arthritis can be present in the same elderly patient. Finding devices or triggers based on patients' real-time data could allow optimized dopamine release and anti-inflammatory cotreatment. For these combinations to be effective, the medicinal chemist would need to design exquisitely selective compounds, with little or no drug–drug interactions. Fixed-dose combinations are probably more feasible in the near future, but the ideal would be to reach a state where pharmacogenomic/proteomic

feedback drives dosing. This need is especially true where tolerance, resistance, and comorbidity exist in patients.

Achieving true personalized medicine will necessarily require personalized administration. The traditional definition of drug delivery involves optimized devices and formulations. However, Florence and Lee reported “personalized medicine involves the correct diagnosis, the correct choice of drugs, the choice of optimal dose, the calculation of the dose for specific individuals, and drug administration at the appropriate time and, as with intravenous medication and implanted pumps, the proper rate” [28]. The authors describe the current standards in healthcare, where patients with chronic diseases typically have more than one diagnosis, and patients over 65 years take multiple medications (the authors quote an average of 13 per patient!). Patient compliance issues are significant with the complexity of multiple medications and different coexisting chronic diseases. Individualized dosage forms are needed for key patient parameters such as tissue distribution, metabolism, and avoiding drug–drug interactions. Advances in biotechnology, genomics, proteomics, and pharmacology have positioned medicinal chemists to design and create remarkable lifesaving medicines that will continue to push the frontiers of personalized drug therapy.

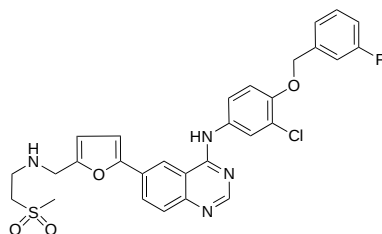
Drug	Structure	Target
Gleevec, STI571, imatinib		bcr-abl, PDGFR, c-Kit
Camptothecin, CPT		Topoisomerase I
Topotecan		Topoisomerase I
GG211		Topoisomerase I

Dasatinib,
Sprycel



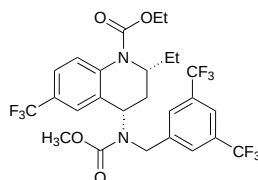
bcr-abl, src,
c-Kit, plus
other kinases

Tykerb,
GW572016,
lapatinib



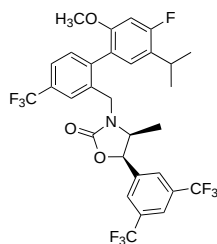
EGFR/erbB2

Torcetrapib,
CP529414



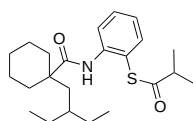
CETP

Anacetrapib,
MK0859



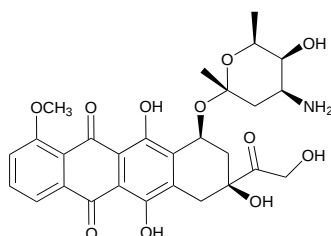
CETP

Dalcetrapib,
JTT-705



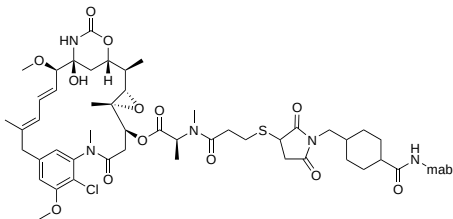
CETP

Doxorubicin



Cytotoxin

(Continued)

Drug	Structure	Target
TDM-1, trastuzumab emtansine		HER2

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2

Discovery of Predictive Biomarkers for Anticancer Drugs

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2.1

Introduction

Although the heterogeneous nature of human cancer has long been recognized by pathologists, surgeons, and medical oncologists, the relatively recent systematic effort to comprehensively annotate a large number of tumor genomes has yielded an even greater appreciation of the diverse nature of human cancers, including those that are histologically indistinguishable. At the heart of its pathogenesis, it is of course widely understood that human tumorigenesis is largely a consequence of genetic mutations and clonal evolution, and that an eventual loss of genome integrity can even lead to an acceleration of genomic “chaos” associated with substantial chromosomal aberrations and an accumulation of numerous mutations – some of which further drive disease progression and many of which constitute incidental “passengers.” As a result, no two cancer genomes look alike, complicating a “clean” categorization of disease states and imposing a formidable challenge to diagnosis and treatment paradigms. Indeed, as clinical trials of the novel so-called “rational, pathway-targeted” drug candidates proceed at an ever-accelerating pace, in many cases accompanied by biomarker studies, it is becoming increasingly apparent that an optimal therapeutic strategy demands a deep understanding of the relationship between the unique molecular profile of an individual patient’s tumor and its likely vulnerability to a particular drug treatment.

Such revelations have sparked substantial efforts to implement “personalized” cancer therapy strategies, based on measurable and predictive features of an individual patient’s cancer cells, with the goal of increasing the likelihood of delivering therapeutic benefit, while simultaneously sparing patients of (often toxic) treatments that are unlikely to impact their disease. This paradigm has played out clinically in no setting more than in the development of kinase-targeted drug treatments. The clinical activity of inhibitors of the HER2, ABL, BRAF, ALK, and EGFR kinases, for example, has been well correlated with a mutationally activated state of the genes encoding these oncogenic kinases – a phenomenon referred to as

“oncogene addiction” [1]. Fortuitously, it is also becoming clear that the simple *in vitro* models of established tumor-derived cell lines harboring such mutations often demonstrate a striking sensitivity to the corresponding drug treatments, thereby validating the use of such cell lines as a model system that can potentially enable the discovery of predictive biomarkers for novel oncology drugs [2]. Indeed, several groups have now reported studies involving drug sensitivity profiling with large panels of cancer cell lines that have revealed biomarker-correlated drug response findings, which have either been clinically validated or hold promise for potential future clinical application [3–5]. As the use of such cell line panels is expanded, accompanied by efforts to comprehensively define their molecular profiles, the ability to capture the diverse heterogeneity of the human cancer population with such a preclinical platform should grow considerably, providing an increasingly powerful model system for the discovery of candidate diagnostic biomarkers that can be implemented into clinical studies, thereby enabling appropriate patient subsetting and improved outcomes with novel investigational anticancer agents.

While much of the excitement around this new paradigm for matching cancer patients with appropriately targeted drugs stems from the recent experience with inhibitors of mutationally activated kinases in oncogene-addicted tumors, the majority of tumors do not display a clear dependency on a single mutationally defined state, thereby challenging the broader implementation of such a strategy. Consequently, there is a great interest in the identification of additional molecular “signatures” that can effectively guide the subsetting of cancer patients based on their likely response to treatment. For example, gene expression profiles are being used more routinely to stratify breast cancer patients, and additional preclinical studies have demonstrated the potential utility of gene expression signatures in the stratification of pancreatic cancers, gliomas, and a variety of other cancers [6,7]. Furthermore, recent advances in technologies that facilitate analysis of the tumor cell proteome, the metabolome, and the epigenome have made it possible to begin to explore the utility of these additional molecular profiles to subclassify tumor states and potentially link them to specific treatment responses.

This newly emerging paradigm shift toward personalized treatment with pathway-targeted drugs certainly represents a significant advance in cancer drug therapy. However, even when these agents produce clinical benefit in biomarker-defined patients, the inevitable acquisition of drug resistance substantially limits their overall utility [8]. As molecular mechanisms of acquired resistance are now being identified, it is becoming clear that many of these mechanisms can contribute to intrinsic resistance to treatment, and therefore may constitute predictive biomarkers that identify patients whose tumors are unlikely to respond to a particular drug. For example, the EGFR T790 M mutation, which is acquired during treatment with EGFR kinase inhibitors (erlotinib and gefitinib) in about half of all patients who initially respond, can also be detected in some patients prior to treatment, where it may predict innate drug resistance [9]. Since many such molecular mechanisms of acquired resistance can be discovered in cancer cell line models subjected to drug treatment and following the selection and molecular

characterization of drug-resistant clones, this approach to cell line modeling of acquired resistance provides a potentially powerful platform for the discovery of predictive biomarkers for a variety of investigational cancer drugs.

The steady accumulation of evidence supporting the molecular basis for treatment sensitivity and resistance has also provided a strong rationale for exploring a variety of drug combination strategies. Such combination approaches are often based on anticipated resistance mechanisms as well as on ever-increasing understanding of the nature of cross talk and redundancy among cellular pathways that impact cell proliferation and survival. Unfortunately, the challenges associated with identifying biomarkers predictive of the response to treatment combinations can be considerably greater than those that relate to single agent therapies. In part, this reflects the difficulties in modeling synergistic or even additive effects of drug combinations using preclinical cell line and *in vivo* models. In short, thus far, findings generated through such analyses have generally not been easily translatable to a clinical context, and there is an important need for alternative preclinical models that can potentially capture the synergistic benefit associated with combination therapies, and thereby potentially provide a platform for the discovery of biomarkers predictive of the response to drug combinations.

In addition to the rapidly accelerating biomarker discovery efforts associated with a variety of agents that target oncogenic pathways in tumor cells, efforts to identify predictive diagnostics for agents that target nontumor cells, especially antiangiogenic treatments, have similarly gained momentum in recent years. However, the discovery of clinically useful biomarkers to guide such treatments has been fraught with challenges. Thus, one of the most widely used anticancer drugs, Avastin, an antibody directed against the tumor angiogenesis-promoting factor vascular endothelial cell growth factor (VEGF), is currently approved for use in specific clinical indications; however, because a biomarker that could potentially enable prospective identification of patients likely to benefit from treatment is yet to be identified, it has been challenging to broadly expand the clinical development of Avastin [10]. This difficulty largely reflects the lack of robust preclinical models that faithfully recapitulate the complex biology associated with the tumor vasculature, as well as a remarkably limited understanding of the relative contribution of host and tumor cell functions in the variable response to Avastin treatment that has been observed clinically. Unfortunately, this issue is likely to similarly challenge the development of a large number of new antiangiogenic agents currently being investigated.

In the context of tumor–host interactions, cancer immunotherapy is another promising therapeutic strategy that has recently emerged, with provocative clinical findings being reported for agents targeting the ability of tumor cells to repel the immune response [11]. The discovery of predictive biomarkers in this setting may be equally challenging and will undoubtedly demand a substantially deeper understanding of the interplay between tumor cells and cells of the immune system that frequently infiltrate tumors to influence their growth.

In the following sections, we will review the various approaches currently being pursued to discover predictive biomarkers for cancer drug therapy – highlighting

the opportunities, challenges, and in some cases, successes associated with each. This is not intended to constitute a comprehensive review of the vast literature with relevance to predictive biomarkers in oncology that has accumulated in recent years. Rather, the intention is to briefly describe the various research strategies being employed to discover predictive diagnostics – from both the preclinical models and the evaluation of tumor specimens linked to drug-based clinical trials.

2.2

“Oncogene Addiction” as a Paradigm for Clinical Implementation of Predictive Biomarkers

The paradigm of oncogene addiction has provided the best examples thus far of the utility of predictive biomarkers for new anticancer drugs. Oncogene addiction refers to the observation that cancers can exhibit a strict dependency on a particular mutationally activated oncogene, which is required to maintain the malignant state [12]. Consequently, such cancers are exquisitely sensitive to the genetic disruption or pharmacologic inhibition of the relevant oncoprotein, unlike cells that lack the molecular aberration [12–14]. Since the target of the drug essentially defines the oncogenic pathway, the predictive biomarker can be the oncogene itself or associated proteins that function in the oncogenic pathway. Understanding this relationship between a specific tumor genotype and the response to a pathway-targeted drug can greatly increase the likelihood of a successful clinical trial. This section will highlight the limited but steadily growing examples of predictive biomarker success in oncology clinical studies associated with oncogene addiction.

Cancer is a molecularly heterogeneous disease, and there are very few examples of histologically defined tumor indications that are primarily driven by a single common oncogenic pathway, which would obviate the need for a predictive biomarker. One such example is chronic myelogenous leukemia (CML), which is uniformly defined by the presence of the BCR-ABL fusion gene that arises through chromosomal translocation. In CML, treatment with the small-molecule ABL tyrosine kinase inhibitor imatinib (Gleevec[®]/Imatinib; Novartis) has been highly effective [15]. Unfortunately, CML seems to be the exception rather than the rule, and most other well-studied malignancies are associated with more complex genetic backgrounds.

In a different oncogene addiction context, mutations in the hedgehog (Hh) pathway genes, predominantly loss-of-function *PTCH1* mutations and, less commonly, gain-of-function *SMO* mutations, have been found to result in constitutive hedgehog pathway signaling. Hedgehog is a key regulator of cell growth and differentiation during embryogenesis and development, but it is not essential in adult tissues. In basal-cell carcinomas, constitutive hedgehog pathway signaling drives proliferation of basal-cells of the skin, thereby contributing to malignant progression. This preclinical rationale led to a phase I trial [16] of the first-in-class *SMO* inhibitor vismodegib (GDC-0449; Genentech), which inhibits the hedgehog pathway in patients with advanced basal-cell carcinoma. Impressively, of the 33

treated patients, 18 experienced an objective response to vismodegib, according to radiological assessment ($n = 7$), physical examination ($n = 10$), or both ($n = 1$). The median duration of the study treatment was 9.8 months. Furthermore, a patient with metastatic medulloblastoma, a disease context similarly associated with mutational activation of the Hh pathway, who failed to respond to multiple prior therapies, experienced a rapid but transient tumor regression and symptomatic improvement. These preliminary findings provided critical proof of concept for targeting the hedgehog pathway, ultimately leading to FDA drug approval.

Arguably, the most famous example of successful clinical implementation of predictive biomarkers relates to the anti-HER2 receptor monoclonal antibody trastuzumab (Herceptin[®]; Genentech), which was FDA-approved in 1998 for its ability to shrink breast cancer tumors that overexpress HER2. HER2 is encoded by the *ERBB2* gene and amplification or overexpression is observed in approximately 30% of breast cancers and results in activation of cell proliferation and survival pathways. Thus, tumors with HER2 overexpression are “addicted” to and depend on HER2 signaling for viability. In a landmark study [17], it was shown that a biomarker (HER2-positive) could be used to stratify patients that showed a 40% increase in overall survival benefit when treated with a paclitaxel and Herceptin combination compared to treatment with paclitaxel alone. This trial also highlighted the importance of identifying the appropriate patients for treatment because the benefit of adding Herceptin to paclitaxel would not have shown statistical significance in an unselected population (see Figure 2.1). The companion diagnostic assay for Herceptin, now known as the Herceptin test, involves immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) to assess HER2 status.

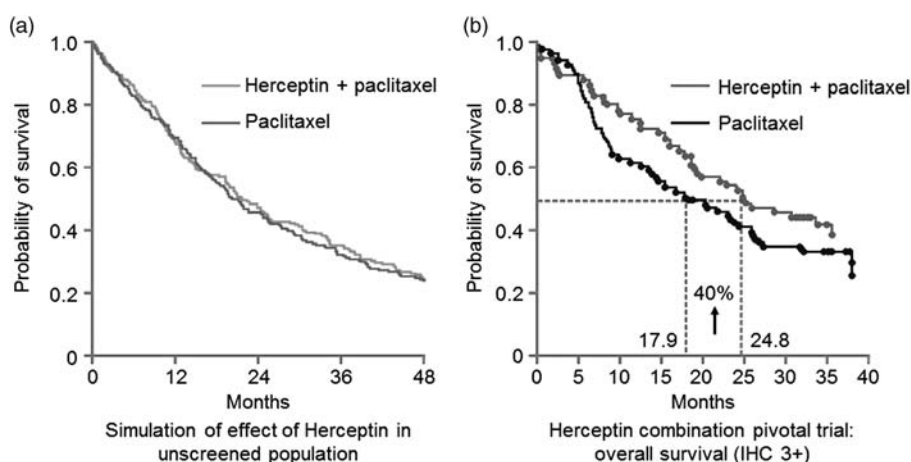


Figure 2.1 Kaplan–Meier estimates of overall survival, according to whether patients were randomly assigned to receive paclitaxel or paclitaxel plus trastuzumab (Herceptin) in an unselected population (a) or in Her2+ patients (b) [17].

In non-small cell lung cancer (NSCLC), a growing number of mutations affecting various receptor tyrosine kinases (RTKs) have been discovered, and effective therapies that target these RTKs have emerged. For example, two small-molecule inhibitors of the epidermal growth factor receptor (EGFR) kinase (IressaTM; AstraZeneca) [18] and erlotinib (Tarceva[®]; OSI Pharmaceuticals/Genentech) [19] have been approved for the treatment of NSCLC patients. Several studies have shown that *EGFR* mutation-positive patients experience an impressive 60% response rate with these agents, significantly exceeding the response rate for conventional chemotherapy [19,20]. Although tumors carrying these mutations are initially very sensitive to the targeted therapies, in most cases resistance arises within the first year of treatment. Two primary mechanisms of acquired resistance that have been identified are the T790 M gatekeeper mutation in the EGFR catalytic domain and amplification of the gene encoding the hepatocyte growth factor receptor (HGFR or MET) oncogene (discussed below).

The interplay between EGFR and MET signaling, and an apparent “coaddiction” in some settings, has prompted substantial interest in cotargeting these two pathways. In a recent phase II clinical study, a humanized monovalent monoclonal antibody, onartuzumab (MetMab), doubled progression-free survival (PFS) in patients with high Met-expressing NSCLC when used in combination with erlotinib, compared with placebo plus erlotinib [21]. The treated patient population did not experience overall a statistically significant improvement in PFS with the combination compared with erlotinib alone (HR = 1.09, $p = 0.687$, median PFS: 2.2 versus 2.6 months). However, in those patients with Met-positive tumors, onartuzumab plus erlotinib experienced a statistically significant doubling of PFS compared to those who received erlotinib alone (HR = 0.53, $p = 0.04$). The median PFS was improved from 1.5 to 2.9 months. The addition of onartuzumab to erlotinib also led to a statistically significant improvement in OS compared to erlotinib alone (HR 0.37, $p = 0.002$) in patients with Met-positive tumors. The improvement in median OS was tripled from 3.8 to 12.6 months. Although PFS and OS were improved in patients classified as having Met-positive tumors, those with Met-negative tumors had worse outcomes when treated with onartuzumab plus erlotinib compared to erlotinib alone. These findings highlight the importance of a companion diagnostic in evaluating the efficacy of experimental therapeutics to distinguish between patients who are likely to benefit from a new medicine and those who may in fact suffer consequences of a particular treatment.

Another recent biomarker-associated clinical success has been the development of the dual ALK/MET kinase inhibitor crizotinib (Pfizer) for the treatment of patients with ALK-rearranged NSCLC. Activating mutations in the form of chromosomal translocations affecting the ALK kinase are observed in 3–5% of NSCLCs [22]. Following the demonstration of the exquisite sensitivity of tumor cells with *ALK* abnormalities to crizotinib, a phase I clinical trial demonstrated the safety and impressive activity of crizotinib in patients with NSCLC that harbored *ALK* rearrangements [23]. All the responding patients harbored *ALK* gene rearrangements detected by FISH, but did not harbor *MET* amplification or *EGFR* mutations.

In malignant melanoma, recurrent mutations in the *BRAF* gene, specifically the V600E hotspot, cause hyperactivation of the kinase activity of BRAF and downstream activation of the MAP kinase (MAPK) cascade [24,25]. Based on abundant preclinical findings demonstrating that such tumors were likely to be addicted to BRAF signaling, clinical studies of a BRAF inhibitor (vemurafenib) in a cohort of patients with V600E *BRAF*-mutated melanoma were undertaken. This study revealed that vemurafenib doses that caused >80% inhibition of extracellular signal-regulated kinase (ERK) phosphorylation resulted in an impressive 81% RECIST response rate (unconfirmed responses), including complete responses [26,27]. The median PFS in this phase I trial was estimated to be at least 7 months compared with only 2 months for historical controls. Significantly, as a proof of concept for the role of oncogene addiction in this setting, tumor regression and disease control were observed in patients whose tumors harbored mutant, but not the wild-type *BRAF*. Although the response rates with vemurafenib in patients with V600E *BRAF*-mutated melanoma have been impressive and sometimes durable, lasting more than 18 months, acquired drug resistance is still inevitable. Moreover, a subset of BRAF mutant patients failed to respond to treatment, implicating underlying mechanisms of intrinsic and acquired resistance.

Accumulating evidence has pointed to an important role for the PI3 kinase signaling pathway in many human cancers, and there has been tremendous effort to discover and develop small-molecule inhibitors of PI3K signaling. The predictive biomarkers currently being explored for their potential to identify patients likely to benefit from treatment with PI3K pathway inhibitors include *PIK3CA* mutations, loss of PTEN expression, and *HER2* amplification, whereas certain *KRAS* mutations may confer resistance [28,29]. However, no single biomarker may be sufficiently robust or specific to be useful as a companion diagnostic in this context when considering the abundance of cross talk and feedback associated with PI3K pathway signaling. Collectively, the findings described above highlight recent successes as well as challenges associated with biomarker-guided patient selection for some of the recently developed kinase-targeted agents (Table 2.1).

Table 2.1 Listing of various FDA-approved and investigational kinase inhibitors and associated predictive biomarkers.

Drug	Target	Status	Cancer type	Diagnostic	Reference
Trastuzumab	HER2	FDA approved	Breast	HER2 IHC, HER2 copy number (FISH)	[17]
Imatinib	BCR-ABL	FDA approved	CML	BCR-ABL fusion (FISH)	[15]
Gefitinib	EGFR pathway	FDA approved	NSCLC	EGFR-mutated	[18]
Erlotinib	EGFR pathway	FDA approved	NSCLC	EGFR-mutated	[19]

(continued)

Table 2.1 (Continued)

Drug	Target	Status	Cancer type	Diagnostic	Reference
Crizotinib	ALK	FDA approved	NSCLC	ALK rearrangements (FISH)	[31]
Vemurafenib	BRAF V600E	FDA approved	Melanoma	BRAF V600E mutation	[26]
Onartuzumab	Met pathway	Phase III	NSCLC	Met overexpression (IHC)	[21]
PI3K inhibitors	PI3K pathway	Phase II	Multiple	PI3K mutation, PTEN loss	[28]
MEK inhibitor	MAPK pathway	Phase II	Multiple	KRAS mutation	[29]

2.3

Cancer Cell Lines as a Model System for Discovery of Predictive Biomarkers

2.3.1

Historical Application of Cell Lines in Cancer Research

Tumor-derived cell lines have been used for many years for drug discovery and development. Since the establishment of the first continuous culture of human cells – HeLa cells derived from Henrietta Lacks – by Gey *et al.* in 1951 [32], there are now more than 2000 tumor-derived cell lines available from public repositories. Much of our understanding of cancer biology and cellular signaling has been enabled by the study of cancer cells *in vitro*. Cell-based efficacy screening to identify anticancer drug candidates, as we know it today, was pioneered by researchers at the National Cancer Institute in the late 1980s [33]. They assembled a panel of 60 cell lines derived from diverse tumor types (the NCI60) and developed many of the protocols and technologies required for cell-based assays that are still in use today [34]. At that time, cancer therapies largely consisted of nonspecific chemotherapeutics. These cytotoxic agents yielded clinical responses in a broad range (25–70%) of patients [2]; thus, capturing this frequency in cell lines required a relatively small number of lines.

Over the last decade, technological advances have unveiled the true complexity and heterogeneity of cancer genomes, and the development of clinical therapeutics has shifted from chemotherapeutics to targeted inhibitors, thereby improving efficacy and reducing toxicity. As a result, the use of targeted anticancer therapeutics is often limited to smaller fractions of patients harboring a drug-sensitizing mutation; thus, a much larger panel of lines is required to capture these lower frequency features. Uncovering the complexity of the disease we are combating has provided an understanding of why clinical responses are generally poor and vary considerably from patient to patient, and has revealed the need to model genotype–phenotype relationships in order to better predict response. As such, clinical development has become more dependent on companion diagnostics to guide treatment decisions, increasing the need for improved preclinical model systems.

Based on observed clinical responses to targeted therapeutics, it is estimated that an ideal cell line profiling panel that covers the majority of tissues-of-origin would consist of 2000–6000 cell lines [2], which is beyond the scope of most currently available platforms. Studies using subsets of cell lines derived from melanoma [35], lung [36], and breast [37,38] as well as multiple other lineages [39,40] have supported the notion that genomically annotated cancer cell line panels are credible tools for preclinical drug target development and functional studies. Two seminal studies recently expanded this concept by employing panels of between 500 and 1000 cell lines [3,4]. These marked the first large-scale integration of cell-based screens with complex “omic” data to identify biomarkers that can guide rational therapeutic strategies, thereby taking us one step closer toward the ultimate goal of personalized medicine.

2.3.2

Biomarker Discovery Using Cell Line Models

In addition to the moral and ethical necessity to deliver efficacious, nontoxic therapies to the right patient, there is a strong financial incentive to decrease the time required for preclinical and clinical drug development to reduce costs for both researchers and patients. As described above, early successes in targeting oncogene-addicted tumors were supported by effective biomarkers. The small-molecule inhibitor, imatinib (Gleevec), targeting the c-ABL oncogene is the paradigm – tumors and cultured cell lines harboring the BCR-ABL oncogenic fusion in chronic myeloid leukemia (CML) show remarkable sensitivity to imatinib [41,42]. Similar observations have been made in other genotype response settings, including ALK and EGFR inhibition in genotype-defined subsets of NSCLC and BRAF inhibition in *BRAF* mutant melanoma. Efforts to predict such clinical efficacy using larger collections of *in vitro* tumor-derived models clearly show that such approaches can identify subsets of tumors (and hence patients), which may benefit from treatment [5,40,43]. Indeed, cell-based analyses of mutations, amplifications, deletions, and translocations have demonstrated genotype-associated sensitivity to a variety of kinase inhibitors, paving the way for a series of clinical successes [43–45]. Development of the Center for Molecular Therapeutics (CMT1000) at Massachusetts General Hospital was the first large-scale effort to show the degree with which tumor cell line response to therapeutics can be correlated with clinical responses. Cell lines with gene amplifications or activating mutations affecting EGFR, HER2, MET, or BRAF kinases show exquisite sensitivity to inhibitors of those kinases and have been confirmed in other cell-based studies [22,42,46–48]. As a result, NSCLC cell lines can now be genetically grouped into defined subsets based on activating mutations (Figure 2.2) [5]. Genomic amplification or rearrangement of one of these genes, ALK, defines sensitivity to an ALK inhibitor in a subset of lung cancers, lymphomas, and neuroblastomas resulting in remarkable clinical responses to the ALK inhibitor, crizotinib [23,42]. This approach also unveiled novel drug responses such as non-HER2 amplified lines responding to HER2-targeted therapies [49]. In these cases a NRG1-mediated

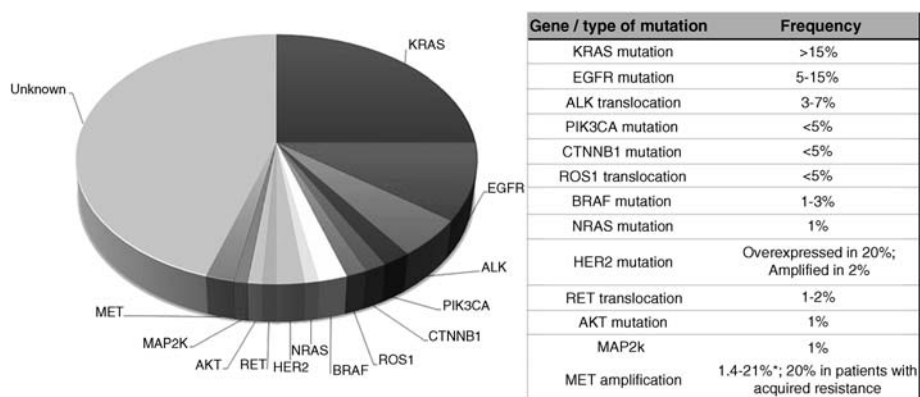


Figure 2.2 Mutation spectrum in non-small cell lung cancer (NSCLC). Frequencies of mutations measured in NSCLC tumor biopsies. Mutations depicted are ones that occur exclusively with the exception of

PIK3CA. Mutations in TP53 and LKB1 are found at frequencies of 35 and 10–38% of NSCLC cooccurring with other mutations. *MET amplification is variable due to differing methods of analysis.

autocrine loop engages the HER2 kinase, suggesting that patients with NRG1-driven tumors lacking *HER2* amplification may derive significant clinical benefit from HER2/HER3-directed therapies [49].

These early successes using relatively small collections of cell lines led to several efforts to systematically evaluate mutations in cancer cell lines to create a genotype–phenotype knowledge base for discovery research. The Cancer Genome Project at the Wellcome Trust’s Sanger Institute is resequencing the most common cancer-associated mutations in human cancer cell lines [50,51]. To date they have sequenced 64 genes across 770 cell lines, with the goal to increase the utility of cell lines to enable discovery research. In collaboration with the CMT1000, this was put into practice by integrating this information with cell line sensitivity profiling, copy number, gene expression, and DNA rearrangements, which confirmed known gene–drug relationships as well as new associations [4]. In parallel, researchers from the Broad Institute published what they termed the *Cell Line Encyclopedia* consisting of sequencing, expression, copy number, and drug response data across 947 publically available cell lines [3]. This work identified several gene–drug relationships that are likely to prompt clinical follow-up.

Moving forward, the public availability of these data sets holds great promise for numerous investigators to generate hypotheses. Integration of these and other data sets from cell line panels and cross-referencing with data from primary tumors is where untold potential lies. The greatest challenge is relating the cell-based analyses with primary tumors, drug efficacy in patients, and clinical outcome. Most notably the International Cancer Genome Consortium (ICGC) [52,53] and an NCI initiative termed “The Cancer Genome Atlas” are beginning to curate the spectrum of mutations across a broad range of tissue types [54,55], facilitating an assessment of how closely *in vitro* models reflect *in vivo* biology.

2.3.3

Cell Lines as Models of Human Cancer

The use of cancer cell lines as models for understanding tumor biology is still and most likely always will be a source of controversy [56]. Nevertheless, cell lines are being extensively used as *in vitro* models in biomedical science with the general belief that they recapitulate many of the characteristics of the tumor of origin. Whether cell lines truly capture the genomic diversity of primary tumors is a topic of ongoing debate [56,57]. Comparison of gene expression signatures of tumor-derived cell lines and primary tumors of the same tissue results in segregation of cell lines from the tumors [56,58–60]. While cell lines of common tissue origin invariably cluster together and segregate from cells of different origin, this does not mean they are representative of the original tumor. One argument could be that data derived from tumors often capture the presence of nontumor tissue (stromal cells, invading macrophages, and normal cells), whereas cell lines constitute a more pure population; however, this is unlikely to account for all the dissimilarities.

Primary cultures of tumor cells are certainly more reflective of *in vivo* tumors [61], although it is notable that for many tissue types cancer cells grow slower than normal cells [57,62]. This suggests that *in vitro* culture selects for only fast-growing tumor cells or enforces cellular changes amenable to high rates of proliferation. It is estimated that 30 cell divisions are required to form a 1 g tumor and only 10 further divisions to reach a lethal size [56]. Since most cell lines are derived from detectable, palpable masses resected from the patient, they may only represent a single time point quite late in the evolution of a tumor. This is supported by the observation that cancer cell lines do not represent the clinical spectrum of cancers at a specific site since tumors most likely to yield viable cell lines are fast growing, are of advanced stage, and poorly differentiated in most cases [57].

Several studies have looked in greater depth at the genomic characteristics of cell lines and *in vivo* tumors [35,36,38,59,63]. There are examples where specific pathways lose function *in vitro*, such as loss of Hh signaling in cultured medulloblastoma cells [64], loss of *EGFR* amplification in glioblastoma [65], and inability to culture glioma tumor cells with the recurrent *IDH1* mutation under standard conditions *in vitro* [66]. Overall, many of the recurrent genomic attributes of primary tumors are reflected in cell lines. While it is not surprising that any single cell line faithfully recapitulates a single tumor or class of tumors, the use of larger panels of cell lines does potentially capture the overall heterogeneity of tumors [67]. However, when utilizing cell line panels, it is certainly important to consider the differences between cell lines and tumors. For example, breast cancer cell lines are known to have a high frequency of high-level chromosomal amplification, contain more activating kinase mutations, and lack specific tumor subtypes [38,58,68,69]. However, the burden of evidence falls on the side of the cell lines being valid genetic surrogates of *in vivo* tumors.

2.3.4

Challenges and Limitations of Cell Line Models

Advantages of cultured cell lines are many: they provide a pure population of cells that can be used by multiple laboratories; most have limitless replicative capacity and some are amenable to *in vivo* growth; growth conditions can be adjusted to assess different phenotypes (growth on or in substrates, hypoxia, etc.); they can be easily observed in real time and extracts can be readily obtained for analysis; and they can be genetically modified and tested for drug sensitivities (Table 2.2). Disadvantages are also numerous: *in vitro* selection of subpopulations of cells not truly reflecting the primary tumor; gradual genetic “drift” over time due to increased genomic instability or poor handling; absence of the tumor microenvironment (stromal cells, immune cells, and inflammatory cells); absence of vascularization; attachment to plastic; and nonphysiological growth conditions (nutrient, oxygen, and hormonal levels) (Table 2.2).

Validity of data derived from cell lines also depends on cell line identity that historically has been somewhat questionable, but concerted efforts to demand proof of cell line identity in publications should reduce the significance of this issue in coming years [57,70]. The growth conditions of cells in culture are a constant source of debate – selection of media, serum concentration, supplements, environmental conditions, even vessel size in which the cells are grown, all influence baseline growth characteristics and assay outcomes. In efforts to more

Table 2.2 Comparison of properties of cancer cell lines with primary tumors.

	Feature	Cell line
Cancer hallmarks	Sustaining proliferative signaling	Yes
	Evading growth suppressors	Yes
	Avoiding immune destruction	?
	Enabling replicative immortality	Yes
	Tumor promoting inflammation	?
	Activating invasion and metastasis	Yes
	Inducing angiogenesis	No
	Genome instability and mutation	Yes
	Resisting cell death	Yes
	Deregulating cellular energetics	Yes
Microenvironment	ECM	Partial
	3D growth	Partial
	Tissue heterogeneity	No
	Oxygen tension	Variable
	pH	Variable
	Growth factors	Variable
	Hormones	Variable

All features listed are properties of primary tumors. The extent to which cell lines grown *in vitro* recapitulate these features is listed. Yes: feature is reflected *in vitro*. No: feature is not represented *in vitro*. ?: unknown. Partial: feature is partially represented *in vitro*. Variable: feature can change depending on culture conditions.

faithfully mimic *in vivo* growth *in vitro*, a number of technologies have been developed, such as 3D culture [71,72], tumor spheroids [73], and hollow fiber assays [74], with varying degrees of technical difficulty and outcomes. Numerous studies have compared 2D and 3D models [75,76], although a thorough assessment of drug sensitivities in these contexts has yet to be reported, and a clear understanding of which of these systems more faithfully model clinical responses may never be achievable. While working with cell lines is challenging, most can be managed by responsible shepherding of cell banks, good cell culture technique, and a consistent approach to assay design and implementation. Despite these challenges and limitations, cancer cell line models have unquestionably provided an important and now clinically validated preclinical model system for the discovery of predictive biomarkers for at least some types of anticancer drugs.

2.4

Modeling Drug Resistance to Discover Predictive Biomarkers

As described above, the identification of somatic alterations in the cancer genome that result in a tumor's dependency on a given oncogenic pathway (i.e., "oncogene addiction") has led to the successful clinical development of agents that specifically target these pathways [77]. These targeted therapies often elicit substantial clinical responses in a significant proportion of patients whose tumors harbor the respective mutation. However, cures are yet to be achieved, as disease progression following the initial response to therapy occurs due to the development of an *acquired* resistance. Furthermore, there remain patients who exhibit a primary refractoriness to therapy, likely due to an *innate* resistance to the targeted agent. Knowledge of the causative mechanisms of drug resistance utilized by tumor cells will be critical in guiding the development of rational therapeutic strategies to overcome or prevent resistance from occurring. In this section, we will discuss how the experimental modeling of drug resistance has facilitated this effort.

Several experimental approaches have been taken to elucidate the mechanisms underlying drug resistance. The *in vitro* generation of largely isogenic pairs of cancer cell lines that exhibit differential sensitivity to a given drug has represented one of the most common, as well as successful, approaches. This method involves culturing a cell line model that is exquisitely sensitive to a given targeted therapy to gradually increasing drug concentrations in order to select for subpopulations of cells that emerge and remain viable at high drug concentrations. Interrogation of the genomic and/or biochemical differences between these isogenic pairs can lead to the identification of the causal factors that drive resistance and, consequently, can reveal biomarkers that are predictive of resistance. Analogous approaches have been taken *in vivo* using either xenografts or genetically engineered mouse models (GEMMs) that develop cancer as a consequence of the constitutive or inducible expression of a given oncogene [78–80]. Intermittent drug dosing or inactivating the inducible oncogenic transgene can result in the emergence of drug-resistant tumors or tumors that are no longer dependent upon the driving oncogene for

their maintenance. Similar to the *in vitro* modeling method, the molecular and biochemical characterization of these tumors could provide mechanistic insights into resistance.

Systematic gain- or loss-of-function screens using genome-wide or targeted libraries expressing RNAi to knockdown, or open reading frames to overexpress, genes have represented another approach to identify modifiers of drug sensitivity [81–83]. Random mutagenesis screens have been shown to be useful in defining the spectrum of mutations in the drug target that might confer resistance to therapy, in anticipation of such a mechanism in the clinic [84,85]. Importantly, the mechanisms and biomarkers of resistance that have been identified through these experimental approaches have often been confirmed in drug-resistant clinical specimens, hence affirming the validity of these approaches.

Experimental modeling studies have led to the identification of multiple modes in which cancer cells evade targeted therapy (Table 2.3). One of the most common mechanisms is through the selection of genetic variants in the drug target itself. Secondary mutations in a critical “gatekeeper” residue found within the catalytic cleft of oncogenic tyrosine kinases can alter drug accessibility through either steric hindrance or increasing the affinity for ATP. These mutations have been validated as clinical resistance markers for multiple targeted therapies, including imatinib-resistant CML (ABL^{T315I}), imatinib-resistant GIST (KIT^{T670I}), erlotinib/gefitinib-resistant NSCLC (EGFR^{T790M}), and crizotinib-resistant NSCLC (ALK^{L1196M}) [86–88]. Additional resistance mutations have been identified in these targets outside the ATP binding region, which likely function by altering the conformational state to one that is not conducive to drug binding [86,89,90]. Resistance mutations in the drug target are not only restricted to ATP-mimetic therapies targeting oncogenic tyrosine kinases but have also been identified for drugs with an allosteric mode of action, such as the MEK1^{P124L} mutation that can confer resistance to the MEK inhibitor AZD6244 [85], as well as for nonkinase targets such as the SMO^{D473H} mutation in the G protein-like molecule Smoothened that confers resistance to the Hh pathway inhibitor, vismodegib [80]. Genetic amplification and subsequent overexpression of the drug target can represent another mechanism of resistance that operates through a classical increased gene dosage effect. As an example, amplification of BCR-ABL oncogene has been identified in cases of imatinib-resistant CML [87]. Finally, drug resistance could occur through the selection of an alternatively spliced variant of the drug target, as has been recently observed in melanoma wherein an alternatively spliced form of mutant BRAF^{V600E} that lacks the RAS binding domain (p61BRAF^{V600E}) had emerged following vemurafenib therapy [91].

Drug resistance could also occur through the activation of alternative pathways that maintain oncogenic signaling, bypassing the requirement for the targeted oncogenic protein. This mechanism was first identified as another mode of resistance of EGFR^{mut} NSCLCs to EGFR inhibitors, in which oncogenic signaling was maintained due to the activation of the c-Met receptor through *MET* gene amplification or upregulation of its ligand, HGF [97]. Resistance to EGFR-targeted therapy has also been linked to upregulation of additional kinase activities,

Table 2.3 Examples of drug resistance mechanisms and markers for targeted agents.

Type of mechanism	Therapy	Disease	Driver mutation	Mechanism/marker of resistance	Clinical evidence	References
Genetic alteration in drug target “Gatekeeper” mutations	Imatinib	CML	BCR-ABL translocation	ABL ^{T315I}	Y	[87]
	Imatinib	GIST	KIT mutation	KIT ^{T670I}	Y	[88]
	Gefitinib, erlotinib	NSCLC	EGFR mutation	EGFR ^{T790M}	Y	[89]
	Crizotinib	NSCLC	ALK translocation	ALK ^{L1196M}	Y	[86]
Additional mutations	Imatinib	CML	BCR-ABL translocation	ABL ^{mut} -multiple	Y	[92a]
	Vismodegib	MB	PTCH1 mutation	SMO ^{D473H} , other	Y	[80,93]
	AZD6244	Melanoma	BRAF mutation	MEK ^{P124L}	Y	[85]
	Crizotinib	NSCLC	ALK translocation	ALK ^{E1174L} , ALK ^{C1156Y} , ALK ^{L1152R}	Y	[86,90,94]
Gene amplification	Quizartinib	AML	FIT3-ITD mutations	FIT3 ^{mut} multiple	Y	[95]
	Imatinib	CML	BCR-ABL translocation	BCR-ABL	Y	[87]
	Dacomitinib	NSCLC	EGFR mutations	EGFR ^{T790M}	N	[92b]
Alternative spliced form	Vemurafenib	Melanoma	BRAF mutation	p61BRAF ^{V600E}	Y	[91]
Alternative pathway/bypass mechanism	Gefitinib, erlotinib	NSCLC	EGFR mutation	MET amplification, HGF upregulation	Y	[97,98]
	Cetuximab	CRC	None	ERBB2 amplification, NRG1 upregulation	Y	[99]
	Erlotinib	NSCLC	EGFR mutation	AXL upregulation, GAS6 expression	Y	[100]
	Vemurafenib	Melanoma	BRAF mutation	COT, PDGRA, IGF1R overexpression, NRAS mutation	Y	[101–103]

(continued)

Table 2.3 (Continued)

Type of mechanism	Therapy	Disease	Driver mutation	Mechanism/marker of resistance	Clinical evidence	References
Alteration in downstream effector	Vemurafenib	CRC	BRAF mutation	EGFR activation	Y	[83,104]
	Ruxolitinib	MPN	JAK2 mutation	JAK1, TYK2 heterodimerization	Y	[105]
	Crizotinib	NSCLC	MET amplification	EGFR activation	N	[106]
	Gefitinib	NSCLC	EGFR mutation	Activating PIK3CA mutation	Y	[107]
	Vemurafenib	Melanoma	BRAF mutation	Activating MEK mutation	Y	[85]
	Vismodegib	MB	PTCH1 mutation	GLI2, CCND1 amplification	N	[93,108]
Nongenetic and/or pathway independent	Trastuzumab	Breast	ERBB2 amplification	PTEN inactivation	Y	[81]
	Imatinib, erlotinib	CML	BCR-ABL, EGFR mutation	BIM polymorphism	Y	[109]
	Erlotinib	NSCLC	EGFR mutation	Histological transformation	Y	[107]
	Erlotinib	NSCLC	EGFR mutation	Epithelial-to-mesenchymal transition	Y	[110]
	Erlotinib	NSCLC	EGFR mutation	Altered chromatin state involving KDM5A	N	[111]
	Vemurafenib	Melanoma	BRAF mutation	Stromal-derived HGF production	Y	[112,113]

including ERBB2 and AXL [99,100]. Several mechanisms of resistance to the BRAF^{V600E}-selective molecule, vemurafenib, have been described that function by maintaining active ERK signaling through activation of CRAF, bypassing the need for oncogenic BRAF^{V600E}. This could occur through activating mutations in *NRAS* or via activation of growth factor receptor signaling through PDGFRA or IGF1R overexpression or through feedback activation of EGFR [83,101,102,104]. Alternatively, upregulation of the COT kinase could sustain ERK signaling in a RAF-independent manner, also bypassing the requirement for the oncogenic BRAF^{V600E} mutation [103]. Bypass mechanisms could also occur directly at the level of the drug target. In a recent example involving resistance to JAK2 inhibitors in myeloproliferative neoplasms, downstream JAK-STAT pathway activity was maintained in the presence of JAK2 inhibitors, due to the activation of JAK2 *in trans* via heterodimerization with JAK1 or TYK2, thus bypassing JAK2 kinase inhibition [105].

Cancer cells can also evade targeted therapy through the selection of genetic variants in signaling proteins that are downstream of the drug target. In this setting, oncogenic pathway signaling is maintained, despite suppression of the upstream target. Several examples supporting this mode of resistance have been described, including the identification of activating PIK3CA mutations in the context of gefitinib resistance [107], activating MEK mutations in the context of vemurafenib resistance [85], activated PI3K signaling through PTEN loss in the context of trastuzumab resistance [81], and *GLI2* or *CCND1* activation through gene amplification in the context of vismodegib resistance [93,108]. The germ line genetics of an individual can also impact the functional outcome of target inhibition, as was recently highlighted by the identification of a common deletion polymorphism in the pro-apoptotic protein BIM that leads to an intrinsic resistance to therapy in CML and EGFR^{mut} NSCLC [109]. The downstream upregulation of BIM following TKI treatment is normally required to induce apoptosis to these targeted therapies [114,115]. Finally, evidence exists for nongenetic mechanisms that may be independent of pathway activity, including drug efflux [116], induction of drug-tolerant states involving chromatin modification [111], transdifferentiation [107,110], and contributions of microenvironmental factors [112,113].

The discovery of such drug resistance mechanisms through these modeling efforts has facilitated the development of second-generation therapies and/or rational combination approaches to target resistance in the clinic. One of the first examples was the identification and clinical development of second-generation SMIs, such as dasatinib or nilotinib, that target the imatinib-resistance mutations causing conformational changes in ABL [117]. Both of these therapies have proven to be effective in treating imatinib-resistant CML characterized by these resistance mutations, as well as have shown improved efficacy over imatinib in treatment-naïve patients [118,119]. Newer therapies that target the gatekeeper mutation in Abl (ABL^{T315I}) are currently under clinical investigation and have shown to be effective in preclinical models driven by this variant [120]. Similarly, irreversible inhibitors that selectively target EGFR^{T790M} have been identified [121]. Given that this resistance mutation has been identified in approximately 50% of the EGFR mutant patients that progress on EGFR-targeted SMIs, a predictive marker to identify this

population of patients at relapse would be critical. Resistance modeling efforts have also led to rational therapeutic combination strategies that are being tested clinically (see Section 2.5). The ability of vemurafenib-resistant tumors to bypass oncogenic BRAF^{V600E} signaling through CRAF activation has led to clinical trials evaluating combinations with MEK inhibitors. Combining EGFR pathway inhibitors with agents that block alternative RTK pathways, such as Met, represents another rational therapeutic strategy to extend survival in patients in EGFR-mutant NSCLC patients. Overall, the full potential of drug resistance modeling efforts to ultimately impact patient outcome on targeted therapies has only begun to be realized. However, it is already becoming abundantly clear that the identification of specific resistance mechanisms is playing a critical role in the identification of predictive biomarkers for a variety of anticancer drugs.

2.5

Discovery of Predictive Biomarkers in the Context of Treatment Combinations

Agents with approved companion diagnostics are frequently used in combination therapy. For example, Herceptin (trastuzumab), which is indicated for HER2 positive breast cancer, is primarily used in combination with a taxane-based regimen. However, the diagnostic was developed to predict response to trastuzumab, not the combination. In the case of the currently approved usage, this does not represent a problem, as there are no companion diagnostics for the chemotherapy regimens, and trastuzumab is expected to contribute to efficacy only in HER2-positive cancers. However, the next set of targeted agents coming up for regulatory approval includes agents that are designed to be effective as combinations based upon the biology of the drug target(s). These “rational combinations” may require a new approach to biomarker identification that accounts for combination activity that is distinct from simple additive effects. Rational combinations include combinations that simultaneously target two redundant pathways (synthetic lethality), combinations that simultaneously target a primary pathway and a known resistance mechanism, and combinations where one drug activates the pathway that is targeted by the other drug (e.g., DNA damage repair).

Traditionally, combination therapy is defined by an empirical combination in which there is hope of gaining advantage by being effective on cancers with diverse genetic lesions or combating innate and acquired resistance. However, this is done without knowledge of the determinants of response or the molecular characteristics of the tumor. Rational combinations are based on a mechanistic understanding of the drugs as single agents and in combination. They also require an understanding of the molecular determinants of response to the agents alone or in combination. In these mechanism-based combinations, there may be distinct markers that predict response to combination treatment compared to a single agent.

As already described, preclinical discovery of predictive biomarkers has been enabled by the use of screening platforms that include cell line panels that represent the diversity of human tumors. In order to correlate a biomarker with response, a

quantitative assay is required in order to rank-order responsiveness. Likewise, preclinical discovery of predictive biomarkers for treatment combinations requires a quantitative measure of response to the combination of drugs. Here the problem becomes more complex, as there are a variety of ways to measure combination effects. It is useful to review the more common approaches to measuring combination effects and discuss factors that determine which method is the most appropriate. There is no single best method, and the choice of analysis method usually depends upon the mechanism of the agents and the combination effect. In order to select the best model, it is helpful to understand the assumptions of each model.

There are several excellent published reviews describing the various methods of combination analysis [122–125]. Briefly, the two most common models for measuring combination effects are Loewe additivity [126] and Bliss independence [127]. The Loewe additivity model assumes that the expected additive effect of two agents that act independently is identical to a combination in which each single agent is added to itself. These data are generally represented as an isobologram with agents combined at a fixed ratio that represents the ratio of their IC_{50} values. For example, two agents with IC_{50} values of 10 and 1 would be at a ratio of 10:1 across a range of doses (Figure 2.3a). The employment of this method requires calculation of single agent IC_{50} values, which can represent a problem in examples

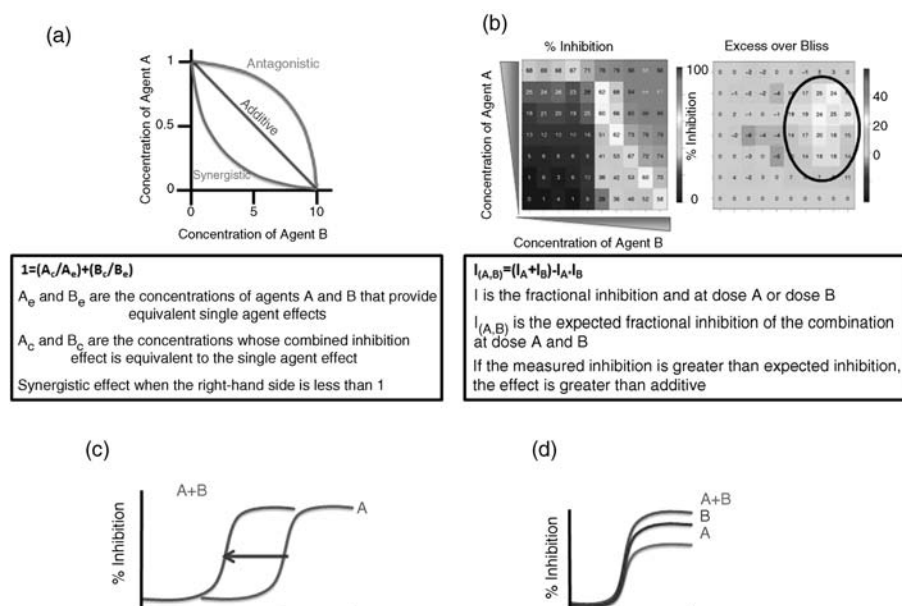


Figure 2.3 Models for measuring combination effects. (a) Loewe additivity, which is usually represented with an isobologram. (b) Bliss independence: the heat maps show the relationship between inhibition and “excess over Bliss,” the

difference in the Bliss expectation and the measured effect. Positive values identify dose combinations that are greater than additive, shown here as the increase in percent inhibition. (c) Potentiation factor. (d) Excess over highest single agent.

where one or both agents exhibit only measurable dose responses in the combination setting. The Bliss independence model assumes that the expected effect of two independent agents is the mathematical product of the effect of each single agent. For example, two independent cytotoxic agents combined at their respective IC_{50} doses should result in 75% cell killing, as the first agent would kill 50% of the cells and the second agent would kill half of the remaining 50%. This works well when the effect is binary, as described in this example (killing versus no effect), but it does not account for partial effects that can influence the response to a second agent. The model does have a practical advantage in that it does not require agent to exhibit a single agent IC_{50} and it can be carried out with a variety of dose ratios or sampled sparsely. Thus, it is possible to carry out a dense dose matrix in culture, choose relevant doses for an animal study, and use the same synergy metric to evaluate a sparse sampling *in vivo* (Figure 2.3b).

Two additional models that have practical applications in measuring drug combination effects on cells are “potentiation factor” and the excess over highest single agent (HSA). To measure a potentiation factor, “agent A” is added across an appropriate dose range on the presence or absence of a single efficacious dose of “agent B”; the IC_{50} values are measured with or without agent B and represented as a ratio (Figure 2.3c). This is a useful model in cases where one agent has a rather binary effect, as is often the case with targeted antibodies, or when the desired effect is to determine if addition of agent B can lower the efficacious dose of agent A to reduce toxicity. The final model is “excess over highest single agent”. This is considered a “low bar” model, as it does not require synergy or even additivity; rather, it only requires that the combined effect at any dose combination is better than the best response of either single agent (Figure 2.3d). In practical terms, this is the effect that is evaluated in the clinical setting. It can also be practical in a large-scale screen, when the goal is to seek signals, and it may not be possible to test at the best dose combinations or sample a dense matrix of conditions. “Positives” can be followed up in detail to select the best combinations for follow-up.

Several examples of rational combinations are currently under evaluation in the clinic. One combination that has received considerable attention is the combination of inhibitors of PI3K and MAPK pathway components [128,129]. In this example, PI3K and MAPK function in parallel pathways that signal from EGFR to the pro-apoptotic protein BAD. Pathway activation, for example, through activating mutations in RTKs, PI3K, or KRAS, is considered to be a predictive biomarker for efficacy of the individual agents. However, cancer cells can evade one inhibitor by signaling through the alternative pathway. In addition, the two pathways exert feedback inhibition on the signaling cascade, such that blocking one pathway can activate upstream of the other pathway. Thus, the combination is expected to enhance efficacy by both preventing drug resistance that occurs by changing signaling dependency and suppressing the effects due to lost feedback inhibition. The predictive biomarkers are the activating mutations that sensitize cells to either single agent (Figure 2.4a). Patients that harbor activating mutations in both pathways could require combination treatment and there are hints from emerging clinical data of combination efficacy in this population [130].

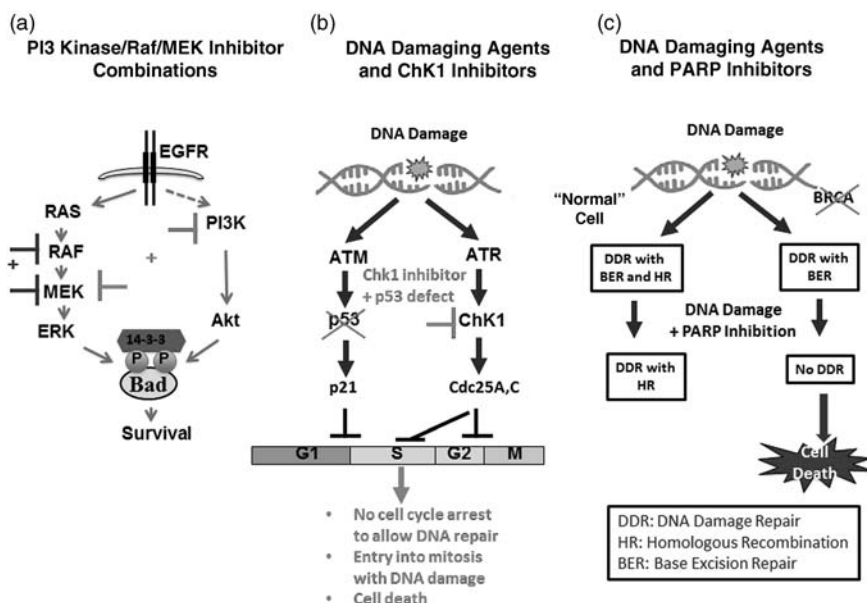


Figure 2.4 Examples of rational combinations that include targeted agents with predictive biomarkers. (a) Examples of targeting the PI3K pathway in combination with the Ras/MEK/ERK pathway or cotargeting RAF and MEK. (b) Loss of the G1 checkpoint is predicted to sensitize cells to the combination of a DNA damaging agent and a Chk1

inhibitor, p53 mutation serves as a biomarker for sensitivity to the combination. (c) The combination of a DNA damaging agent and a PARP inhibitor is predicted to be more efficacious when there is additional defect in DNA damage repair by homologous recombination due to loss of BRCA1 or BRCA2 activity.

Another rational combination approach is the cotargeting of BRAF^{V600E} and MEK [131]. The rationale is to combat acquired resistance to the BRAF inhibitor that arises by alternative activation of the downstream kinase, MEK. The companion diagnostic for the BRAF inhibitor (BRAF^{V600E}) is used to select responsive patients, and the MEK inhibitor prevents resistance via MEK/ERK-dependent mechanisms (Figure 2.4a). Mechanisms of resistance to BRAF inhibitors that are not MEK/ERK dependent have been observed preclinically, and as mutational screening of tumor biopsies becomes more routine, it may be feasible to evaluate cancers for a variety of genetic lesions in order to tailor the combination therapy to the individual cancer [132].

Additional rational combination strategies that are under evaluation in the clinic include the combination of DNA damaging agents with inhibitors of Chk1 or PARP. DNA damage causes normal cells to arrest their cell cycle and repair DNA before committing to DNA synthesis and cell division. In these examples, DNA is damaged and one of these pathways is inhibited pharmacologically, causing G2 arrest in the case of Chk1 and base excision repair in the case of PARP. Thus, cells treated with a Chk1 inhibitor and a DNA damaging agent may be entirely dependent upon the G1

checkpoint, rendering cancer cells with defects in G1 arrest (e.g., p53 mutants) more sensitive (Figure 2.4b) [133]. Likewise, cells treated with a DNA damaging agent and PARP inhibitor are now dependent upon other pathways for DNA damage repair (Figure 2.4c) [134,135]. A lot of attention has been paid to the evaluation of this combination in patients with germ line mutations in BRCA1 or BRCA2. However, because these are germ line mutations, there may be no increase in therapeutic window. New data emerging from large-scale cell line profiling efforts have demonstrated that rare somatic events such as the translocation of EWS-FLI1 can sensitize tumor cells to PARP inhibitors [4]. Whether these lesions turn out to be predictive biomarkers, and whether combination with a DNA damaging agent will be required for clinical efficacy remain open questions.

The candidate biomarkers in these examples were all discovered based on detailed knowledge of the relevant biological pathways. The mathematical analyses of synergy described above were largely confirmatory and focused on a relevant subset of cell lines. However, recent advances in high-throughput screening and the increase in scale with which genetic and epigenetic profiles are becoming available for large cell line panels open the possibility of empirical biomarker discovery for targeted drug combinations. This “hypothesis generating” approach would entail systematic screening of combinations across genetically characterized cell panels. In this manner, unexpected synergies could be identified and the wealth of correlative data could facilitate the mechanistic dissection as well as identification of treatment-responsive subsets. Efforts of this type are already underway and we expect to see significantly more in the near future [136,137].

2.6

Discovery of Predictive Biomarkers for Antiangiogenic Agents

Angiogenesis, the growth of blood vessels from preexisting ones, is required for tissue maintenance and growth, and hence plays a pivotal role in the development of malignant lesions [138]. Preclinical studies have defined critical drivers required for the outgrowth of tumors from indolent lesions, many of which are initiators or direct drivers of angiogenesis (e.g., Kras). Inhibition of angiogenesis has been established as an important therapeutic strategy against solid tumors, including, but not limited to, metastatic colorectal carcinoma, NSCLC, breast cancer, glioblastoma, and renal cell carcinoma. The VEGF pathway has emerged as the dominant driver of tumor angiogenesis, and the majority of targeted agents aim to inhibit the ligand VEGFA or its receptors directly. The first antiangiogenesis therapeutic, bevacizumab, a monoclonal antibody directed against VEGFA, was approved in 2004, with subsequent approval of several VEGF receptor tyrosine kinase inhibitors. Based on scientific rationale and preclinical data, broad clinical efficacy could be expected for this type of therapy, which targets the basic mechanism of supplying nutrients and oxygen for sustained tumor growth. However, clinical data have highlighted the complexity of human tumors, prompting many groups to determine why only subsets of patients derive survival benefit from treatment with these therapies.

Table 2.4 Candidate biomarkers that might determine or modulate antiangiogenic drug activity for agents targeting the VEGF axis.

Parameter	Example	Modality	References
Target	VEGF, VEGFR	Tumor IHC, qPCR, ISH; plasma ELISA	[139–141]
Target pathway activity	Phospho-VEGFR, Dll4, VEGF signaling RNA signatures	Tumor IHC, qPCR	[142]
Degree of vascularization	VEGFR, NRP, CD31, CDH5, PLVAP, perfusion, permeability	Tumor IHC, DCE-MRI imaging	[139,143]
Active angiogenesis	Circulating endothelial (progenitor) cells, Dll4, tip cell markers, and RNA signatures	Tumor IHC, qPCR; blood sample FACS	[142]
Angiogenic phenotype	Kras, p53 mutation, hypoxia (CA9)	Tumor DNA analysis, IHC, PET imaging	[144,145]
Compensatory ligands	Additional VEGFR ligands (for therapeutics blocking only specific ligand or receptor)	Tumor IHC	[142]
Compensatory growth factors and cytokines	FGF2, Bv8	Tumor IHC	[142]
Angiogenesis inhibitors	THBS2	Tumor IHC	[139]
VEGF pathway-independent vessels	High pericyte coverage (SMA, desmin, NG2)	Tumor IHC, qPCR	
Drug activity	Permeability changes, VEGFA, soluble VEGFR, changes in circulating endothelial cells	DCE-MRI imaging, plasma ELISA, blood sample FACS	[10,146,147]
Adverse events	Hypertension	Sphygmomanometer	[148]
Systemic modifiers of drug activity	SNPs in VEGFA, VEGFR	DNA isolated from peripheral blood	[143,149]

IHC: immunohistochemistry, ISH: *in situ* hybridization, ELISA: enzyme-linked immunosorbent assay, FACS: fluorescence-activated cell sorting.

Summarized below is the high-level status of current knowledge relating to predictive biomarkers for antiangiogenic agents from larger studies with control arms (Table 2.4). Comprehensive summaries of all efforts in this field have recently been reviewed in several high-quality publications where references for most of the primary studies discussed here can be found [10,146,147].

2.6.1

Challenges

There is little precedent for the identification of biomarkers that predict efficacy of a cancer drug targeting the stromal microenvironment. Challenges in developing and implementing a useful diagnostic, faced by all oncology drugs, are exemplified

for antiangiogenics [150]. Preclinical hypothesis generation is hampered due to the inability to harness the power of large cell panels to establish differentials in sensitivity. Consequently, candidate discovery or testing relies heavily on preclinical tumor models. Subcutaneous or orthotopic mouse models, as well as genetically engineered tumor models driven by specific oncogenic transgenes, each emulate distinct aspects of tumor angiogenesis, but allow limited comparison across models to identify drivers of efficacy. In the clinic, analysis of archival material appears to be of limited value, as this class of drugs target a highly “plastic” mechanism that is bound to be associated with large temporal as well as site-specific effects, theoretically requiring analysis of pretreatment biopsies of all target lesions (primary and metastasis) for precise profiling of analytes, something that would be difficult to implement, especially in adequately sized patient cohorts. As the vasculature constitutes only a small percentage of all cells in the tumor, assays might be too insensitive or differentials too small to enable meaningful correlations with clinical endpoints. Finally, conventional response criteria initially set in place to evaluate efficacy of cytotoxic agents could be suboptimal to determine benefit from drugs that, due to their mechanism of action, maximally induce tumor stasis.

2.6.2

Pathway Activity as a Predictor of Drug Efficacy

Pioneering biomarker work was initially focused on known modulators of angiogenesis. VEGFA as the dominant driver of the pathway was measured by several groups in tumor samples [139], as well as circulating levels in plasma or serum [140]. While high VEGFA levels have been reported to pose a worse prognosis in some studies, data thus far have failed to establish a conclusive relationship with the efficacy of antiangiogenics, even for therapeutics such as bevacizumab, which targets VEGFA directly. Other VEGF ligands have thus far not been evaluated in a systematic manner, and tumor and plasma levels do not correlate, requiring further careful analysis of the relative contribution of tumor and systemic sources to the circulating VEGF ligand levels. Similar negative results were obtained for VEGF receptor and NRP1 coreceptor levels in tumor samples, activated phospho forms, and circulating soluble forms of the VEGF receptors; however, the quality of some of the detection reagents have been called into question.

Vascular density, as measured by pan-endothelial markers such as CD31, is a quantitation of target cell population and a more downstream marker for angiogenic activity. Despite analysis in several studies, findings have thus far failed to establish a link to drug sensitivity. More detailed analyses focusing on direct VEGFA target genes such as Dll4 (a putative marker for new vascular sprouts induced by VEGFA), other more VEGFA-dependent vascular phenotypes (low pericyte association), or VEGFA transcriptional signatures are promising efforts [151], but still require confirmatory analysis in larger patient cohorts.

Other approaches have focused on the enumeration of circulating endothelial cell populations, as they might be indicative of active angiogenesis in certain

contexts [152]; imaging modalities such as DCE-MRI (most established approach) or PET imaging have been used to define vascular flow and permeability, or hypoxia as an indirect readout for vascular density and dependency. However, these approaches are technically challenging, especially when implemented across multiple treatment centers, and have not been validated thus far in a larger randomized trial. Finally, several genetic alterations, such as activated KRAS or mutant p53, are associated with a more angiogenic phenotype during tumor evolution. However, several studies have clearly demonstrated that patients benefited from antiangiogenic agents independent of the mutation status of their tumor [144,145]. Attempts to link drug benefit to host characteristics such as genetic variations (SNPs) in VEGF pathway genes reported inconsistent findings between studies and require further analysis into the functional consequences of these variants.

2.6.3

Predicting Inherent Resistance

Without a clear link between VEGF pathway biomarkers and clinical efficacy from antiangiogenic agents, it is plausible that compensatory mechanisms can promote angiogenesis in certain tumors despite inhibition of VEGF signaling. Other proangiogenic (ANGPT, FGF, TGF β , IL8, and PlGF) as well as antiangiogenic cytokines (e.g., thrombospondins) were analyzed in tumor tissue and circulation, but failed to show a clear association with outcome. Preclinical studies identified subsets of immune cell infiltrates or certain cancer-associated fibroblasts as sources of cytokines (e.g., Bv8 and PDGFC) that can convey resistance to VEGF-targeted drugs. Future studies will have to determine if this biology is recapitulated in human disease.

2.6.4

On-Treatment Effects as a Surrogate of Drug Efficacy

With none of the baseline parameters analyzed thus far showing strong association with sensitivity to antiangiogenic drugs, several studies have evaluated whether the extent of posttreatment drug effects could be used as a predictor of long-term benefit. Although less desirable than a diagnostic that can be implemented pretreatment, these efforts could help further our understanding of drug mechanism of action, and if proven useful, it could be used to spare patients of drug regimens from which they are unlikely to derive lasting benefit. Most studies have focused on the extent of changes in plasma VEGFA or VEGF receptor levels, changes in number or phenotype of circulating endothelial cells, the extent of decrease in vascular permeability measured by DCE-MRI, or even the severity of adverse events such as hypertension that can be linked to target inhibition [148]. Several studies have provided encouraging data showing greater benefit in patients with more pronounced changes induced by target inhibition. However, the data have been inconsistent between studies and indications, and the predictive value of these biomarkers will certainly require further investigation.

2.6.5

Summary

Why do we not have a predictive biomarker for antiangiogenic treatments thus far? As described above, the complex biology associated with tumor angiogenesis is certainly a contributing factor. In addition, there have only been a limited number of large randomized clinical trials with adequate sample collection, and even in these studies samples are typically only available from a subset of patients. It is therefore difficult to delineate prognostic from predictive associations in much of the published data, and most analyses might not be powered sufficiently to identify more subtle associations with outcome. Furthermore, biomarkers related to antiangiogenics can be difficult to delineate in the context of standard of care, for example, chemotherapy combinations.

There is also the issue of the plastic nature of tumors. Based on studies of on-treatment pharmacodynamic biomarkers, most patients experience an initial drug effect on the tumor vasculature (e.g., reduction in tumor vascular permeability). However, some tumors might be able to engage a mechanism that allows tumors to grow despite continuous VEGF pathway blockage. While drivers of this ability might exist at baseline, these markers might be too distal and varied to enable preselection of patients with sufficient precision. Another major issue might be the underestimation of true benefit by using classical clinical endpoints from the pivotal studies. New data indicate that patients benefit from addition of antiangiogenics beyond first line progression [153,154], indicating that the progression is largely driven by loss of activity of the chemotherapeutic treatment component, and not by lack of antiangiogenic drug activity.

It is difficult to understand why target expression or pathway activity would not be tightly linked to efficacy, and recent new data have indicated that focusing on the most proximal analytes might provide the most promising avenue for defining a biomarker for antiangiogenics. Utilizing a new assay platform that detects smaller isoforms of VEGFA, elevated plasma levels of VEGFA at baseline were found to be associated with increased benefit from bevacizumab-containing therapy [141], albeit only in a subset of indications (positive association in breast, gastric and pancreatic cancer, no correlation in colon, lung or renal cancer). These differences are puzzling and further research into the basis for these discrepancies is warranted, but the findings have prompted the proposal of a phase 3 clinical study with prospective patient stratification by plasma VEGFA levels, which hopefully will provide conclusive data defining the utility of this biomarker [155]. While proof is still outstanding, this will be the first diagnostic-driven clinical trial for any antiangiogenic to date, and hopefully is an indicator of the increased awareness of the critical need for biomarker discovery and implementation of comprehensive sample collection, which will further enable research in this important area, ultimately benefiting patients by matching them with drugs most likely to be efficacious.

2.7

Gene Expression Signatures as Predictive Biomarkers

As detailed above, cancer is a highly heterogeneous disease whose treatment is complicated by differences in tumors' innate molecular features – even among tumors that are clinically and pathologically indistinguishable. The most important consequence of this heterogeneity, from the point of view of patient health, is variability in response to therapy and chance of relapse; and the identification of an optimal, personalized course of therapy for each patient is one of the main challenges in oncology today.

Gene expression profiling – by qPCR, gene expression microarrays, or now with increasing frequency, RNA sequencing – provides one way of characterizing tumor heterogeneity. In two seminal papers published in 2000, the identification of expression “signatures” in lymphomas [156] and intrinsic gene expression subtypes in breast cancer [157] touched off an explosion of interest in the discovery of gene expression patterns that are associated with important aspects of tumor biology and pathology.

The term “signature” is evocative and familiar, but wholly unspecific in the context of molecular biology. As a consequence, it is now often used loosely to refer to a variety of distinct concepts and approaches that build on these early studies. In what follows, we address this by clarifying the difference between (i) the discovery of gene signatures and molecular substructure within nominally unitary diseases, and (ii) the process of converting this knowledge into expression-based diagnostics. Signature-based strategies have been pursued in many cancer types, but to focus discussion, we will restrict attention from this point forward to signatures and diagnostics developed in the context of breast cancer, where the field has advanced the furthest.

2.7.1

Signature Discovery: Unsupervised Clustering

Alizadeh *et al.* first coined the term “gene expression signature” to refer to a set of coexpressed genes whose expression pattern picks out a specific subset of the samples under study [156]. Shortly thereafter, Perou *et al.* identified multiple signatures in a collection of breast tissues and cell lines [157]. According to this definition, two genes in the same signature should provide largely redundant information, whereas two genes in different signatures should provide orthogonal information. To achieve this, a signature must necessarily contain only a small, coherent subset drawn from the full collection of genes assayed. The choice of genes to include in a signature, more generally referred to as the *feature selection problem*, may be made in a supervised or unsupervised fashion – that is, with or without reference to a nonexpression variable of interest. Perou *et al.* constructed their signatures in an unsupervised manner. First, the full set of features interrogated by their two-color microarrays was filtered on the basis of dynamic range considerations only: Did signal differ significantly for at least a subset of

samples or show substantially greater variation across tumors from different subjects than between repeated biopsies from the same tumor? Next, as is common practice for unsupervised analyses, they applied hierarchical clustering to the relative expression data for their selected genes – clustering both samples and, separately, genes. The resulting heat map is shown in Figure 2.5.

Several patterns are immediately apparent in Figure 2.5. At a very coarse level, tumors split into two large groups that corresponded to estrogen receptor- α (ER) status as assessed by traditional IHC. Also, ER-negative tumors that scored highly for *ErbB2* IHC (Figure 2.5, pink samples) show clear overexpression of the *ERBB2* transcript as well as transcripts from a small group of genes often found to be coamplified with *ERBB2* (pink genes). Other aspects of these data, however, are much more ambiguous. The ER-positive half of the tree appears to have further subgroups, but how many? What do we make of the signature identified by Perou *et al.* as being relevant for tumors derived from a basal epithelial lineage (red bar in Figure 2.5), but which in fact appears to be part of a larger signature that tags normal-like tumors rather than basal tumors?

Importantly, heat maps of this type may suggest natural divisions of the data, but the question of how many gene signatures or sample clusters are actually “there” is often ill posed: genes and samples need not fall into discrete and mutually exclusive subsets, nor respect just one mechanism of grouping. Numerous algorithms exist to suggest a number of clusters k , and using simulated data in which a discrete cluster structure is enforced, several algorithms have been shown to perform well [158–160]. The use of such algorithms permits automation and consistency across different data sets. However, when presented with data that do not satisfy their basic assumptions, such algorithms will offer a suggestion for k and go on to partition the samples, but there is no guarantee that an objectively true k has been found or that the resulting clusters are interpretable.

Perou and coworkers avoided this trap by making a conservative preliminary characterization of their results. Specifically, they used the heat map in Figure 2.5 as a guide, but manually based breast cancer subtype labels on (i) the behavior of sets of genes whose expression had been previously associated with a specific lineage (basal versus luminal epithelial), and (ii) similarity of expression patterns between tumors and well-characterized cultured cell lines. Although the existence of a true normal-like subtype has since been questioned [161,162], the basic divisions suggested by Perou and coworkers are still well supported, albeit with two notable refinements: the division of the luminal subtype into a good prognosis A and poor prognosis B [163], and the division of the triple-negative basal subtype into claudin-low versus true basal-like [164,165].

2.7.2

Diagnostic Development: Supervised Classification

Soon after the discovery of expression signature-based subtypes in breast cancer, Sørlie *et al.* showed that subtype classification may have prognostic value [163]. Clearly, the ultimate objective of such research must be the identification of new

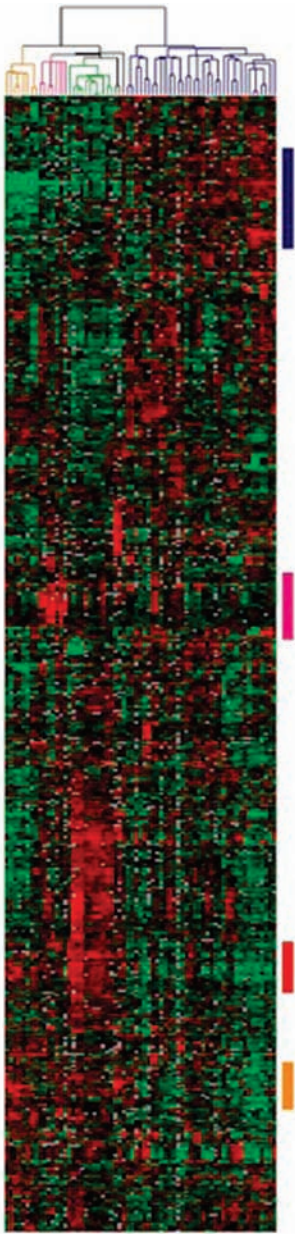


Figure 2.5 Reproduction of Figure 3b from Ref. [157]. Cluster analysis using their “intrinsic” gene subset, with genes in rows and tumor samples in columns. Sample dendrogram colored by assigned tumor subtype: basal-like, orange; Erbb2+, pink; normal breast-like, green; and luminal

epithelial/ER+, dark blue. Gene sets particularly associated with a given assigned tumor subtype are indicated with colored bars: basal epithelial, red and orange; Erbb2 overexpression cluster, pink; and luminal epithelial/ER+, dark blue.

therapeutic strategies or, through diagnostic development, improved utilization of existing ones.

Do gene signatures provide a logical basis for improved diagnostics? Combining expression measurements from multiple functionally related genes into a single signature is conceptually appealing for a variety of reasons. First, a signature has built-in redundancy of measurement, providing robustness with respect to both technical error and some degree of true biological variability. Furthermore, it is conceivable that distinct causal events in different tumors – which could not simultaneously be detected by any single assay – have a convergent impact on the downstream process (e.g., proliferation) driving the expression signature. Finally, a well-designed gene expression assay may be more reproducible and less prone to technical issues and subjective interpretation than, say, histological grading or IHC [156,166–168].

Although there have been some spectacular failures [169,170], much of the subsequent diagnostic development work that built on this subtype foundation – work that has focused on recurrence risk and potential benefit from adjuvant chemotherapy in ER-positive subjects – has, in fact, been successful. The PAM50 multigene expression classifier for predicting tumor subtype has been shown (retrospectively) to have significant prognostic and predictive values [171], other distinct but related multigene diagnostic classifiers have performed similarly in a number of independent breast cancer data sets [165,172,173], and two major, multicenter clinical trials (MINDACT and TAILORx) are currently in progress to prospectively evaluate the MammaPrint (Agendia) and Oncotype Dx (Genomic Health) expression-based diagnostics – both of which have already received regulatory approval in the United States [174–177].

Notably, the gene signatures identified during the original discovery of subtypes were *not* directly used for diagnostic development. Instead, the feature selection process was revisited in a supervised fashion. Specifically, a supervised approach requires a training data set with gene expression measurements *and* an explicit, nonexpression response variable: patient outcome [178–180], ER or BRCA1 status [179], tumor histological grade [181], or even molecular subtype labels manually assigned to a small collection of “prototype samples” [171,182]. A wide range of statistical tools are available for supervised classifier construction, but in all cases, the size and composition of the final feature set are wholly based on estimated predictive power.

2.7.3

Summary

We have reviewed two complementary but distinct phases in the construction of multigene expression-based diagnostics: the unsupervised discovery of tumor heterogeneity and of gene signatures that reflect this heterogeneity and the supervised construction of a final diagnostic classifier. While multiple signatures encompassing hundreds or thousands of genes may be found in the first phase, only a small fraction of these genes are typically utilized in the second. This is expected: supervised classifier construction methods typically extract all benefit

from relevant signatures with just a few, key member genes. This is optimal from a prediction accuracy point of view, although it has two consequences that frequently cause concern. First, the selected features are often not the most well-characterized genes in the signature. Second, competing diagnostic classifiers constructed from different training data, and typically using different statistical approaches, often share few if any features. Concern here, however, is not necessary. Signature interpretability in the first discovery phase gives us confidence that our data and analyses are sound; final diagnostic validity, on the other hand, should not be assessed on the basis of interpretability, but rather on prediction consistency and accuracy. Thus far, most of the superficially distinct multigene breast cancer classifiers discussed above have performed well in this regard [165,173]. Whether these diagnostics ultimately go on to routine use in clinical practice is, therefore, less a question of their analytical validity – which seems sound – and more a question of eventual cost, ease of use, and the degree to which they are perceived as truly improving decision-making for adjuvant chemotherapy.

2.8

Current Challenges in Discovering Predictive Biomarkers

As described earlier in this chapter, human cancer is a complex disease associated with alterations in gene expression, dysregulated activation of cellular signal transduction pathways, enhanced or accelerated proliferation, and defective cell differentiation or cell death. New drugs are being developed to target each aspect of this complex biology, with a rapidly growing appreciation by pharmaceutical companies for the importance of precise companion diagnostic strategies that have the potential to significantly improve the likelihood of clinical success. As described in previous sections, with better characterization of the molecular mechanisms of tumorigenesis, completion of human genome sequencing, as well as the advances in DNA technologies such as microarray, next-generation sequencing, combined with more powerful bioinformatics tools, tremendous progress has been made in our ability to identify new biomarkers, especially in the scope of activating oncogenic mutations. However, there are still many challenges faced by physicians and scientists in their efforts to provide a truly predictive diagnosis for each individual cancer patient. Unfortunately, in most cases, the simple concept of “oncogene addiction” may not adequately define a patient’s tumor and its associated vulnerability (Figure 2.6). In this section, we will review examples describing the complexity of cancer biology we face in our efforts to discover better diagnostics and potential approaches to address these challenges.

2.8.1

Access to Tumor Cells Is Limited during Treatment

For hematopoietic tumors, or leukemias, a large number of tumor cells can be easily obtained for biomarker assessment from peripheral blood through

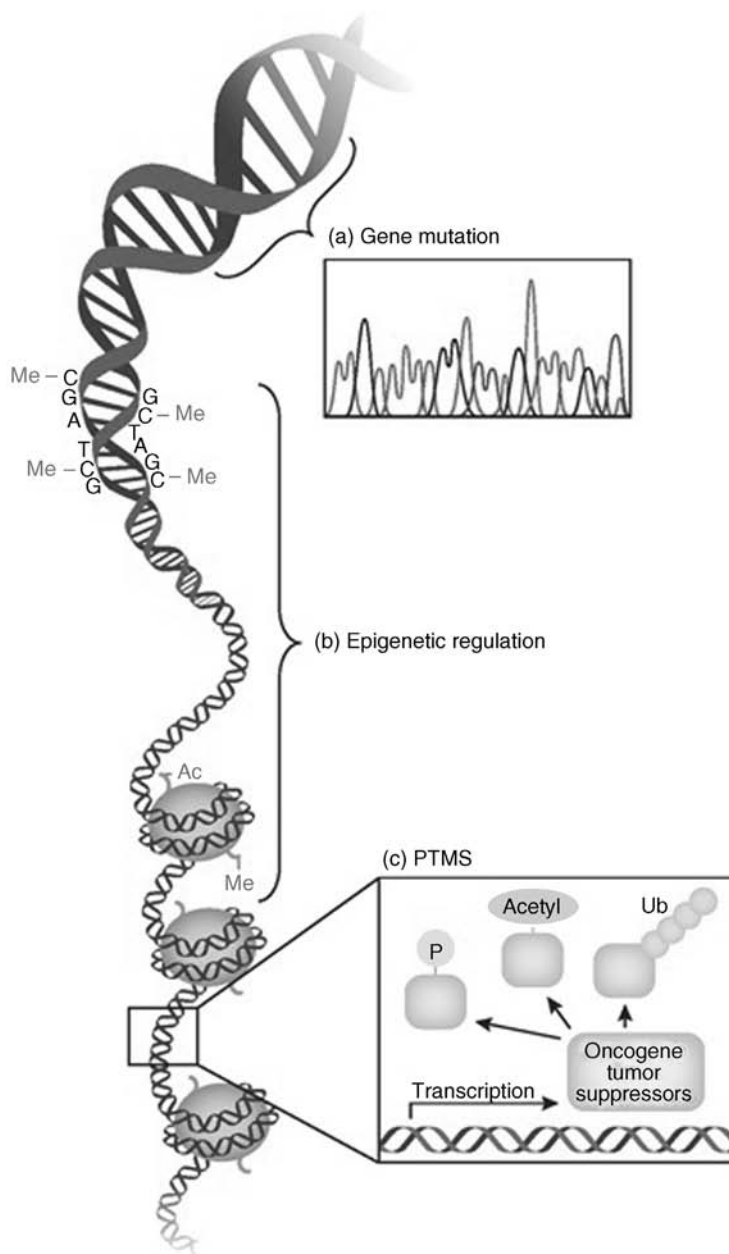


Figure 2.6 Challenges in discovering predictive biomarkers. The evolution and progression of the malignant phenotype is governed by complex biological processes. These may include some or all the following processes: (a) “Driver” or “passenger”

mutations due to unstable cancer genomes.

(b) Epigenetic modification of the genetic code. (c) Posttranslational modifications regulating activities of oncoproteins and tumor suppressors.

technologies such as immunoaffinity sorting. On the other hand, for most solid tumors, access to a patient's tumor tissue is limited, and it is consequently challenging to evaluate predictive or pharmacodynamic biomarkers during treatment. Substantial effort has been initiated to develop alternative noninvasive approaches to gain access to tumor cells from patients, including the isolation of circulating tumor cells (CTCs) from a patient's blood. CTCs can potentially be used to monitor disease progression and response to treatment. Such cells can also be used to interrogate biomarkers, assuming that they reflect the properties of the tumor from which they arose.

During the initial steps of metastasis, CTCs detach from primary tumors, breach the basement membrane, intravasate into either blood or lymphatic vessels, and eventually migrate to distant organs. CTCs are extremely rare, as evidenced by a study of metastatic breast cancers (MBC) in which less than 10 CTCs were typically detected in 7.5 ml of peripheral blood [183]. The number of detected CTCs can potentially serve as a prognostic biomarker. This was best elucidated by a clinical trial involving 177 MBC patients, in which CTCs were collected at different time points after treatment and enriched through an immunomagnetic approach. This study concluded that progression-free survival (PFS) was well correlated with the number of CTCs and patients with ≥ 5 CTCs had a significantly shorter PFS [184]. Furthermore, molecular characterization of these rare CTCs also provides additional biomarkers to assess tumor origins, prognosis, and treatment. For example, a FISH analysis of CTCs from 31 patients with different cancer indications suggested that the majority of CTCs exhibited aneuploidy, which was consistent with their malignant origin [185]. A different study confirmed that *EGFR* mutations in lung CTCs from NSCLC patients were consistent with the corresponding original tumors in 12/13 cases [186]. Furthermore, the follow-up of these patients after prolonged *EGFR* inhibitor therapy demonstrated the acquisition of additional *EGFR* mutations in CTCs, which could not be detected in the primary tumor biopsy, suggesting the evolution of drug resistance [186].

Although CTCs can potentially revolutionize our understanding of tumorigenesis, as well as provide a platform to identify and assess new biomarkers, current application of this system is still largely limited due to technique hurdles, especially the inability to robustly and consistently isolate relatively rare CTCs. With the advancement of new technologies, such as microscopic scanning, and improvement of microfluidic isolation approaches, it may become more feasible to recover CTCs from the vast number of surrounding normal leukocytes [187].

2.8.2

Drivers and Passengers

Cancer is largely a genetic disease. Normally, many layers of protective mechanisms maintain the stability of DNA molecules to ensure a robust defense system against tumor initiation. However, with the breach of one or many of these defense lines, tumors inevitably will acquire many mutations during clonal expansion, including point mutations, deletions, frame shifts, copy number gains and losses,

and chromosomal rearrangements. Those alterations that cause or promote cancers are often referred to as “drivers.” A good example of this category of genomic alteration is CML, which is caused by a reciprocal translocation between chromosome 9 and chromosome 22 to form the constitutively activated BCR-ABL kinase [188]. On the contrary, those mutations present in the cancer genome but without obvious advantage to the tumor cells when they occurred are referred to as “passengers.” For example, most melanoma, colorectal, and lung cancers have a mutation rate that is close to 10–100 mutations per mega base of DNA [189].

The ability to distinguish “driver” and “passenger” events in tumors has been a significant challenge to cancer researchers, and presents an additional challenge to the use of tumor genome profiles to identify potentially informative biomarkers. In 2005, NIH initiated the Cancer Genome Atlas (TCGA) project, using high-throughput sequencing technology and sophisticated bioinformatics tools to identify somatically acquired mutations. Since then, many genes that appear to play a critical role in tumorigenesis have been identified, providing new insights into both the targeted therapy strategies and the discovery of predictive biomarkers. For example, by applying next-generation sequencing, Seshagiri *et al.* have performed a systematic comparison of more than 70 normal-tumor matched pairs of primary human colorectal cancer. They found that in 10% of colon tumors, there are multiple fusion transcripts, including recurrent gene fusions involving R-spondin family members RSPO2 and RSPO3. Interestingly, these RSPO fusions exist exclusively with mutations of adenomatous polyposis coli gene (*APC*) [190]. This discovery not only indicates that fusion of R-spondin genes probably has a role in the initiation of tumorigenesis but also provides a potential new biomarker for *APC* wild-type colon cancer patients.

Although the value of cancer genome sequencing in tumor patients is now generally accepted, there are formidable challenges associated with the clinical implementation of costly genotyping to guide treatment decisions. Recent advances in genomic analysis, including exome sequencing, may pave the way toward practical approaches to achieving a sufficiently comprehensive, yet economical assessment of each patient’s tumor. Currently, a hypothesis-driven approach, such as genotyping patients for known cancer-associated mutations, has provided a more practical interim solution. For example, a microsatellite instable form of a human *HSP110* gene mutation was identified in almost all primary CRC tumors examined. This mutant *HSP110* is then translated to an aberrantly spliced protein, which sensitizes CRC cells to anticancer agents, providing a candidate biomarker for both prognosis and treatment response [191]. It would be impossible, however, to discover such a biomarker by either exome sequencing or targeted genotyping.

2.8.3

Epigenetic Regulation Adds Another Layer of Complexity

Modern cancer biology has invested much effort in the identification of genetic mutations in cancers, which has been invaluable in advancing our understanding of human tumorigenesis. This is especially true for “driver” mutations since these

are likely to be associated with tumor initiation. In contrast, few specific genetic mutations have been specifically linked to tumor progression, leading to the suspicion that epigenetic changes may also play a substantial role in tumor progression. Indeed, the development and maintenance of a tumor is often orchestrated by cellular programs that switch cancer genes on and off in a dysregulated manner by modifying both DNA directly and the chromatin-associated histone molecules [192].

Some chromosomal regions contain elevated numbers of CpG islands, which generally localize near transcriptional initiation sites. Methylation of the 5-position of cytosine (5-^mC) in these CpG sites usually results in the transcriptional silencing of the downstream gene by preventing the interaction of transcriptional factors with promoters [193]. In the genome of cancer cells, tumor suppressor genes are rarely inactivated by mutations in the exon sequences. Rather, these genes are often repressed by hypermethylation of the CpG sites in their promoter regions – such as *p14^{ARF}*, *p57^{Kip2}*, and *RARβ2* [194]. Aberrant methylation of these tumor suppressor genes can potentially be used as a prognostic biomarker to predict drug response or drug resistance. Furthermore, the methylated cytosine in CpG sites can be converted to hydroxymethylcytosine (5-^{hm}C) by ten-eleven translocation (TET) hydroxylases. A recent genome-wide mapping study of 5-^{hm}C has shown loss of the 5-^{hm}C landscape in the melanoma epigenome, which is an epigenetic hallmark of these tumors and can be used as diagnostic and prognostic biomarker for melanoma patients [195].

Many diagnostics-focused companies are actively analyzing DNA methylation profiles in tumor cells to facilitate the development of accurate prognostic and predictive cancer biomarkers. The best example thus far of the application of DNA methylation to predict drug response is the use of hypermethylation of the *O*-(6)-methylguanine-DNA methyltransferase (*MGMT*) gene promoter for glioblastoma patients in clinical trials to predict response to alkylating agents. While *MGMT* promoter hypermethylation is associated with loss of function, deficiency of the modification leads to the activation of *MGMT* in tumor cells, which can directly remove *O*-(6)-alkyl adducts, leading to diminished response to therapies using alkylating agents [196]. The aberrantly methylated Septin9 (*SEPT9*) gene similarly constitutes a predictive epigenetic biomarker for early detection of colorectal cancer in blood specimens [197]. This biomarker has been evaluated in a study called PRESEPT involving a cohort of almost 8000 CRC patients with an overall sensitivity of 67 and 88%, respectively [198].

2.8.4

Many Oncoproteins and Tumor Suppressors Undergo Regulatory Posttranslational Modifications

The function of many proteins that contribute to tumor cell biology is subject to critical posttranslational modifications (PTMs), such as phosphorylation, methylation, ubiquitination, and many others. Consequently, PTMs can play pivotal roles in tumor cell biology that impact the response to treatment. For example,

the p53 tumor suppressor is normally maintained at low levels by ubiquitination by the ubiquitin E3 ligase MDM2 and subsequent degradation [199]. Upon DNA damage, ATR or DNA-PK kinases phosphorylate p53 at Ser15 [200,201], which impairs the ability of MDM2 to bind p53, promoting the accumulation and activation of p53. In sharp contrast, phosphorylation of p53 at Ser392 negatively affects the DNA binding and transcriptional activities of p53 [202]. It has been reported that phosphorylation of p53 at Ser15 is decreased, whereas phosphorylation at Ser392 is increased in human tumors [202], making them potentially useful diagnostic biomarkers, particularly in the context of treatment with DNA damaging agents.

Although many protein-based diagnostic assays have been developed to detect protein in tumors, such as IHC, they largely detect only protein expression levels. Assessment of the specifically modified forms of proteins requires antibodies that detect particular PTMs. Given the complexity and low abundance of PTMs in tumor samples, most PTM studies have employed *in vitro* cancer cell lines as a model system for biomarker discovery. Proteins that are differentially modified under different treatment conditions can be further explored as potential biomarkers. For example, in a phosphorylation dynamics study, Olsen *et al.* detected 6600 phosphorylation sites on 2244 proteins. Furthermore, by treating HeLa cells with EGF, they found that 14% of the phosphorylation events are regulated greater than twofold after EGF stimulation [203]. Moving forward, these phosphorylation events could be further analyzed to identify potentially informative biomarkers in cancer patients with constitutively active EGF signaling.

2.9

Future Perspective

A rapidly accelerating broad effort to personalize cancer drug therapy is now well underway. Clinical proof of concept for the successful implementation of biomarker-guided treatment decision-making for several antitumor agents has recently been achieved, prompting a new paradigm for medical oncology in which patients are matched with drug treatments deemed most likely to be efficacious based on the detection of specific and measurable molecular features of cancer cells that vary substantially across patient populations. Improvements in our ability to rapidly profile cancer cells for gene expression, mutation, and proteomic features, together with software tools that facilitate large-scale data analysis, have given rise to powerful platforms for the discovery of candidate predictive biomarkers. However, as described above, there is no shortage of challenges associated with the discovery and development of useful biomarkers for the vast array of putative anticancer agents currently undergoing development. Future efforts to address these challenges will undoubtedly require advances on several fronts: (i) improved preclinical models that faithfully recapitulate the biology of tumor cells and the diversity of human cancers and their response to treatment; (ii) improved technologies for “omic” profiling of tumors and tumor-derived cell lines; (iii) a

better understanding of the role of tumor–host interactions in response to treatment; and (iv) more sophisticated tools to enable a meaningful analysis of increasingly large and complex data sets. Considering that the recent experience with “rationally targeted” therapeutics suggest that the activity of these agents, and almost certainly many of the agents currently being developed, is invariably limited to relatively small patient subsets, it will certainly be essential to continue the vigorous pursuit of predictive biomarkers.

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3

Crizotinib

Jean Cui, Robert S. Kania, and Martin P. Edwards

3.1

Introduction

The human body is composed of trillions of living cells with a tightly regulated program for growth, division, and death. Abnormal cell growth and invasion of tissues, resulting from a breakdown in this program that is caused by DNA damage, are the characteristics of cancers. Chemotherapy has been used to treat cancer since the beginning of the twentieth century, became the dominant cancer treatment paradigm in the 1970s and 1980s, and is used as first-line treatment against many different forms of cancer today [1]. Chemotherapy kills both normal rapidly dividing cells and cancer cells, often resulting in severe side effects. Research aimed toward targeted cancer therapies, a paradigm shift in cancer treatment that takes advantage of tumor-specific biology for anticancer activity while sparing healthy cells, has become a major focus. Increased understanding of the complex signaling systems used by both normal cells and cancer cells, especially the discovery of cancer-specific abnormalities, has underpinned the success of targeted therapies.

Receptor tyrosine kinase (RTK) activity is tightly controlled in normal cells because the 58 known human RTKs play fundamental roles in cellular processes, including cell proliferation, migration, metabolism, differentiation, and survival [2]. All the RTKs share a similar molecular architecture, including a ligand binding extracellular region, a single transmembrane helix, an intracellular regulatory domain, and a cytoplasmic tyrosine kinase domain. Constitutively enhanced RTK activity arising from point mutation, amplification or rearrangement of the corresponding genes, and aberrant RTK activation through enhanced autocrine or paracrine ligand activation have been implicated in the development and progression of many types of cancer [3]. A number of RTK inhibitors have been developed and approved to produce therapeutic benefit by blocking aberrant RTK signaling in various cancers, including axitinib for targeting the VHL-dependent VEGF pathway in renal cell carcinoma, and erlotinib and gefitinib for targeting mutant EGFR in non-small cell lung cancer (NSCLC).

MET, also called hepatocyte growth factor receptor (HGFR), belongs to a unique subfamily of RTKs and is normally expressed by epithelial and endothelial cells. Hepatocyte growth factor (HGF), a member of the plasminogen-related growth factor family, also known as scatter factor (SF), is a high-affinity natural ligand of MET that is mainly produced by mesenchymal cells [4,5]. The HGF/MET signaling pathway is critical during embryo development and postnatal organ regeneration, but under normal physiological conditions, the HGF/MET signaling pathway is only fully active in adults for wound healing and tissue regeneration processes [6]. The HGF/MET axis is frequently hijacked by cancer cells for tumorigenesis, invasive growth, and metastasis [7]. The expression of HGF and/or MET at abnormally high levels in a wide range of solid tumors is associated with a metastatic phenotype and poor prognosis [8,9]. MET mutations have been identified in many tumors, including hereditary and sporadic human papillary renal carcinomas, ovarian cancer, childhood hepatocellular carcinomas, gastric cancer, and lung cancer [10]. Experiments have demonstrated that MET is required for both tumor and metastasis growth and maintenance, suggesting MET targeting as a possible therapeutic approach to treat even late-stage cancer [11]. HGF/MET signaling is emerging as a player in cancers resistant to EGFR and BRAF kinase inhibitors. MET amplification has been detected in up to 20% of NSCLC patients with tumorigenic EGFR mutations and acquired resistance to gefitinib or erlotinib treatment. The resistance mechanism is associated with activation of ERBB3/PI3K/AKT signaling [12]. HGF mediates EGFR TKI resistance in a distinct mechanism by rescuing both PI3K/AKT and ERK signaling through signaling adaptor GAB1 [13]. Growth factor-driven resistance from the tumor micro-environment represents another potential mechanism for anticancer kinase inhibitors [14,15]. HGF is present in patient stromal cells of melanoma and correlates with a poor response to the BRAF inhibitor vemurafenib treatment. In summary, because of the role of aberrant HGF/MET signaling in human oncogenesis, invasion/metastasis, and acquired drug resistance, the inhibition of the HGF/MET signaling pathway has great potential in cancer therapy [16].

Anaplastic lymphoma kinase (ALK) is an RTK grouped in a subfamily with leukocyte tyrosine kinase (LTK) within the insulin receptor (IR) superfamily. ALK is mainly expressed in the central and peripheral nervous systems suggesting a possible role in normal development and function of the nervous system. Pleiotrophin (PTN) and midkine (MK) have been proposed as ALK ligands in mammals. The phosphorylation of ALK in the absence of the direct interaction with PTN in several cell systems indicates that ALK may be a dependent receptor tyrosine kinase, and PTN and MK may not be the only ALK activators [17]. Although the normal physiological roles of ALK are not completely elucidated, cancer-related interest in ALK has drawn attention due to its oncogenic roles in hematopoietic, solid, and mesenchymal tumors. ALK was first discovered as a fusion protein NPM (nucleophosmin)-ALK encoded by a fusion gene arising from the t(2;5)(p23;q35) chromosomal translocation in anaplastic large cell lymphoma (ALCL) cell lines in 1994 [18]. More than 20

distinct *ALK* translocation partners have been discovered in many cancers, including ALCL (60–90% incidence), inflammatory myofibroblastic tumors (IMT, 50–60%), NSCLC (3–7%), colorectal cancers (CRC, 0–2.4%), breast cancers (0–2.4%), and other carcinomas with rare incidence [19]. The fusion partners with *ALK* play a role in dimerization or oligomerization of the fusion proteins to generate constitutive activation of *ALK* kinase function [20]. The *EML4-ALK* fusion gene, comprising portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the *ALK* gene, was first discovered in non-small cell lung cancer archived clinical specimens and cell lines [21,22]. *EML4-ALK* fusion variants are highly oncogenic and caused lung adenocarcinoma in transgenic mice [23]. *ALK* has been reported to be highly expressed in breast cancer [24]. The oncogenic mutations of *ALK* in both familial and sporadic cases of neuroblastoma and in anaplastic thyroid cancer have also been reported [25–27]. Therefore, *ALK* is an attractive molecular target for cancer therapeutic intervention.

Proto-oncogene tyrosine-protein kinase *ROS* is one of the last two remaining orphan receptor tyrosine kinases with an unidentified ligand and was discovered in 1981 as the oncogene product of the avian sarcoma RNA tumor virus UR2 [28]. There has been very limited data reported on *ROS* expression in humans. *ROS* is present throughout the human epididymis, but the highest levels of *ROS* expression in adults are in lung tissues [29,30]. Although the normal physiological functions of *ROS* are not fully understood, both abnormal expression and variable mutant forms of *ROS* kinase have been reported in a number of cancers [31]. *ROS* is aberrantly expressed in 33–56% of glioblastoma tumors [32] and up to 55% of meningeal tumors [33]. *FIG-ROS* fusion protein was the first fusion protein of *ROS* discovered in 2003 in a human glioblastoma multiforme [34,35]. The *FIG-ROS* fusion kinase activates an SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice [36]. Dysregulated expression of *ROS* kinase may contribute to pathogenesis of human lung cancer, given that elevated *ROS* expression levels were observed in 20–30% of patients with NSCLC [37]. Several fusion proteins with *ROS* kinase have been found in human lung cancers, suggesting an oncogenic role for *ROS* kinase in lung cancers [22,38,39]. In summary, *ROS* kinase is a promising molecular based target candidate for cancers with aberrant *ROS* kinase activities.

Crizotinib, 3-[(1*R*)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridin-2-amine, is an ATP competitive and multitargeted protein kinase inhibitor of *MET/ALK/ROS*. Crizotinib has demonstrated marked human clinical efficacies for cancer patients with abnormal *ALK*, *ROS*, and *MET* protein kinase activities, respectively. On August 26, 2011, the US Food and Drug Administration approved Xalkori® (crizotinib) to treat certain patients with late-stage (locally advanced or metastatic) NSCLC who express the abnormal *ALK* gene. Xalkori was approved with a companion diagnostic test, the Vysis *ALK* Break Apart FISH Probe Kit that would help determine if a patient has the abnormal *ALK* gene.

3.2

Discovery of Crizotinib (PF-02341066) [40]

Crizotinib is the product of a drug discovery program originally aimed at inhibiting the MET RTK. (R,Z)-5-((2,6-Dichlorobenzyl)sulfonyl)-3-((3,5-dimethyl-4-(2-(pyrrolidin-1-ylmethyl)pyrrolidine-1-carbonyl)-1*H*-pyrrol-2-yl)methylene)indolin-2-one (PHA-665752) was the first reported MET inhibitor with potent cellular activity against MET autophosphorylation ($IC_{50} = 9 \text{ nM}$ in GTL-16 cell line) and selectivity (>50-fold for MET compared with a panel of diverse tyrosine and serine-threonine kinases) [41]. PHA-665752 was used in preclinical studies to build confidence in MET as a target for cancer therapy and to identify potential patient populations. In a variety of tumor cells, PHA-665752 potently inhibited HGF-stimulated and constitutive MET phosphorylation, downstream signal transduction of MET, and HGF/MET-driven phenotypes, for example, cell growth, cell motility, invasion, and morphology. *In vivo*, PHA-665752 inhibited MET phosphorylation in tumor xenografts and tumor growth in a dose-dependent manner [41]. However, the poor pharmaceutical properties of PHA-665752 (low solubility, high metabolic clearance, and low permeability) limited its further development as a clinical candidate. The cocrystal structure of PHA-665752 revealed a binding environment of unphosphorylated MET kinase domain (Figure 3.1), in an autoinhibited conformation observed previously in crystal structures of the apoenzyme and a complex with the staurosporine analog K252a [42]. In these MET crystal structures, the beginning of the kinase activation loop (residues 1222–1227) forms a turn that wedges between the β -sheet and the α C-helix. Consequently, the activation loop significantly displaces the α C-helix from a catalytically competent position and the downstream activation loop residues (1228–1245) to a position that interferes with ATP and substrate binding. This unusual kinase activation loop conformation creates a unique inhibitor binding pocket that presents an opportunity for the design of selective inhibitors.

The 2-amino-5-aryl-3-benzyloxy pyridine series was created as a mimic of PHA-665752 with 2-aminopyridine replacing oxindole as a hinge binder. The 3-benzyloxy

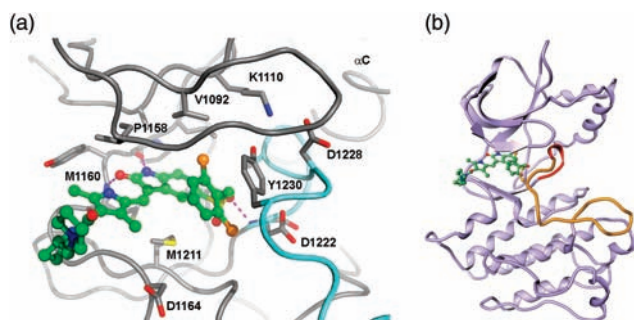


Figure 3.1 Cocrystal structure of PHA-665752 with unphosphorylated MET kinase domain.

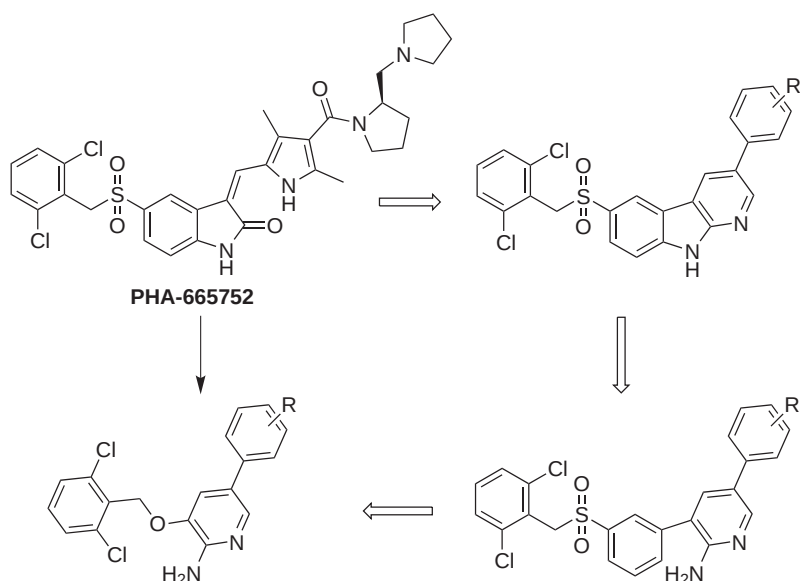


Figure 3.2 Design of 2-amino-5-aryl-3-benzyloxy pyridine scaffold to replace oxindole.

group was designed to more efficiently access the hydrophobic pocket occupied by the 2,6-dichlorophenyl group of PHA-665752 in a manner that permitted less molecular weight and conformational strain (Figure 3.2). The design was validated first with compound **1** (Figure 3.3), which showed moderate enzymatic and cell potencies against MET. Medicinal chemistry optimization led to the identification of the 2,6-dichloro-3-fluoro- α -methyl-benzyl group as an important component for MET cell potency, exemplified with compound **2**. Compound **2** demonstrated potent inhibition against MET *in vitro* and *in vivo* leading to the complete tumor growth inhibition in nude mice in the human U87 glioblastoma xenograph tumor model. However, compound **2** was a potent CYP3A4 inhibitor with an IC_{50} of $0.6\ \mu\text{M}$ [43].

Further lead optimization at the aminopyridine 5-position, targeting improved lipophilic efficiency (LipE) to reduce potential drug–drug interactions and achieve acceptable physical and ADME properties, led to the discovery of the 5-pyrazol-4-yl group as a more efficient substituent than the 5-phenyl group. *N*-Substituents on the 5-pyrazol-4-yl group maintained or enhanced MET inhibition potency while lowering cLogD by up to almost three full units in some cases, thus improving LipE and pharmaceutical properties dramatically [43]. The MET cell potency versus cLogD data from this and earlier subseries, all 5-aryl derivatives of the 3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine core, are plotted in Figure 3.4 to graphically illustrate different efficiency zones. The data points colored blue represent the 5-pyrazol-4-yl subseries that generally occupied higher LipE space compared to the 5-phenyl subseries (red color). Structural features that resulted in efficiencies crossing constant LipE lines by moving up and to the left, a design goal

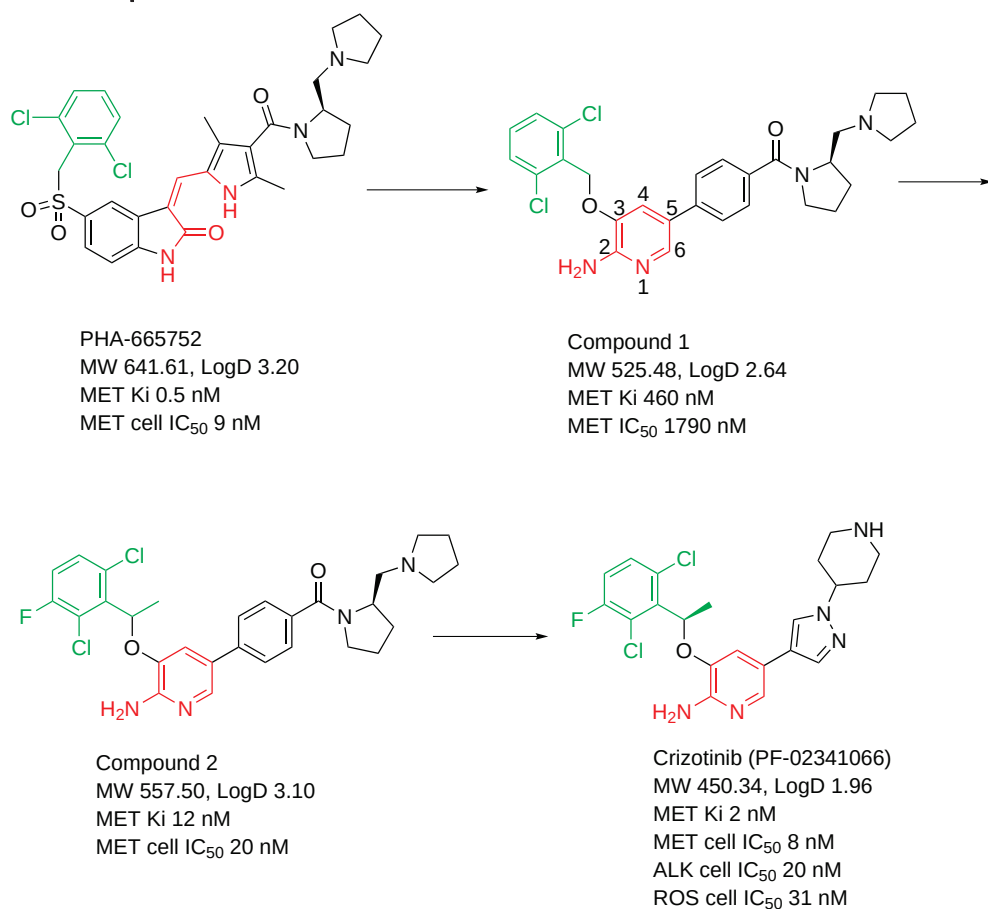


Figure 3.3 Discovery of crizotinib (PF-02341066).

during optimization, were analyzed for trends. Optimization of the *N*-substituent on the 5-pyrazol-4-yl group in this series generated a clinical candidate PF-02341066, later named as crizotinib with a MET cell IC₅₀ of 0.008 μ M and much reduced inhibition of CYP3A4 (IC₅₀ = 5 μ M) relative to compound 2 (CYP3A4 IC₅₀ = 0.6 μ M).

The cocrystal structure of crizotinib bound to the unphosphorylated state of MET kinase domain shows that, as with PHA-665752, the compound binds to an autoinhibitory kinase conformation in which a portion of the kinase activation loop makes direct interactions with the inhibitor. The similar binding modes revealed by an overlay of cocrystal structures of crizotinib and PHA-665752 with MET confirmed the original design hypotheses (Figure 3.5). However, crizotinib binds the MET kinase domain more efficiently than PHA-665752, resulting in much improved cell-based ligand efficiency (LE) and LipE (LE = 0.38 and LipE = 6.1 for crizotinib, and LE = 0.26 and LipE = 4.8 for PHA-665752).

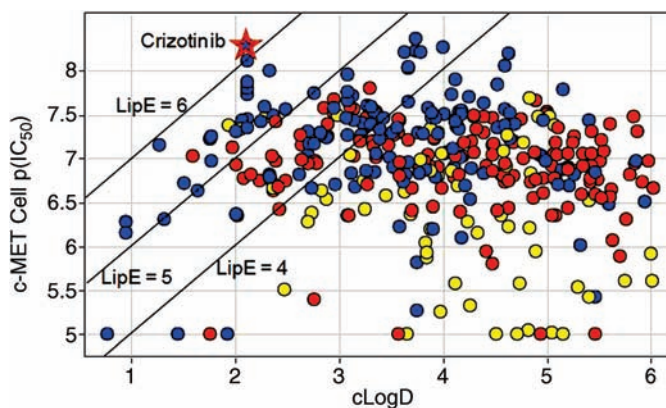


Figure 3.4 MET cell $p(IC_{50})$ versus $cLogD$ (blue for 5-pyrazol-4-yl, red for 5-phenyl, and yellow for others).

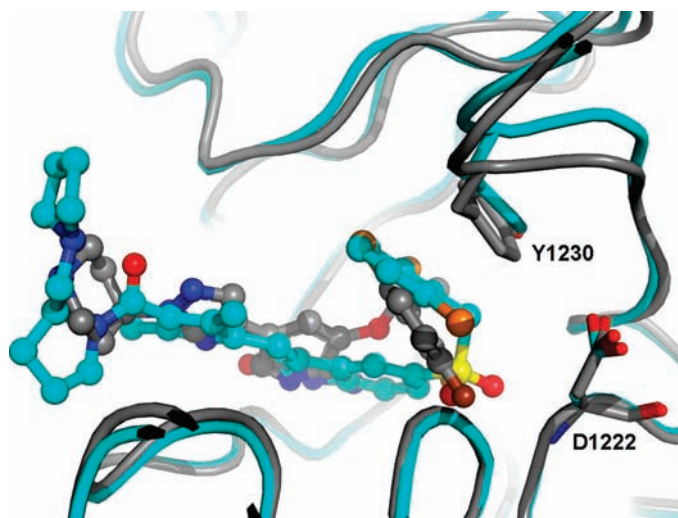


Figure 3.5 Overlay of crizotinib (gray color) and PHA-665752 (cyan color) bound to MET.

3.3

Kinase Selectivity of Crizotinib

Crizotinib, when evaluated against a panel of more than 120 human kinases from Upstate Inc., inhibited only 13 kinases with enzymatic potency within 100-fold of crizotinib enzymatic MET potency. Cell-based autophosphorylation assays were employed to determine a more accurate picture of kinase selectivity in the whole cell context (Table 3.1). Crizotinib demonstrated a potent cell IC_{50} of 20 nM against an oncogenic ALK kinase fusion protein, NPM-ALK, in a human lymphoma cell line and inhibited ROS autophosphorylation ($IC_{50} = 31$ nM) in the HCC78 cell line.

Table 3.1 Kinase selectivity of crizotinib.

Kinase	MET	ALK	ROS	RON	AXL	TIE2	TRKA	TRKB	ABL	IR	LCK
Enzyme IC ₅₀ (nM)	<1.0	<1.0	<1.0	NA	<1.0	5.0	<1.0	2.0	24	102	<1.0
Cell IC ₅₀ (nM)	8.0	24	31	80	294	448	580	399	1159	2887	2741

Ron in the transfected NIH 3T3 cell line (IC₅₀ = 80 nM) was the only other kinase tested that was inhibited in cells at less than almost 300 nM. In summary, crizotinib was determined to be a selective and potent inhibitor of only MET/ALK/ROS in cells.

The unique autoinhibitory conformation of MET and the stabilizing interactions of crizotinib to the Tyr-1230 residue of the A-loop in this inactive conformation confer good potency and selectivity against a broad panel of kinases in cells. Crizotinib is 10-fold less potent against RON even though RON and MET have a high sequence similarity, likely resulting from the RON Ile residue in the corresponding MET Tyr-1230 position.

As with MET, the unphosphorylated apo-ALK crystal structure revealed a unique autoinhibited conformation. The ALK protein adopted a conformation with the A-loop in an inhibitory pose, where a short proximal A-loop helix (α AL) packs against the α C-helix and a novel N-terminal β -turn motif, with the distal portion obstructing part of the predicted peptide-binding region [44]. The cocrystal structure of ALK with crizotinib (PF-02341066) (PDB ID 2xp2) and the apo-ALK crystal structure (PDB ID 3L9P) align closely (Figure 3.6), suggesting that potent whole cell inhibition of ALK by crizotinib resulted from the stabilization of the basal inhibited ALK conformation.

3.4

Pharmacology of Crizotinib [45,46]

The MET-derived pharmacological effects of crizotinib were evaluated in a variety of pro-oncogenic tumor cell phenotypes, tumor models, and molecular mediators of cancer progression in MET-driven tumor cell lines and models having minimum ALK and ROS expression [45]. Crizotinib potently inhibited MET phosphorylation and MET-dependent proliferation, migration, and invasion in *MET*-amplified GTL-16 tumor cells *in vitro* (IC₅₀ = 5–20 nmol/l). Crizotinib showed marked efficacies at well-tolerated doses in MET-driven tumor models with minimum ALK and ROS expression, including GTL-16 gastric, NCI-H441 NSCLC, CAKi-1 renal, and PC-3 prostate carcinoma xenograft models. The antitumor efficacy of crizotinib was dose dependent and showed a strong correlation with the inhibition of MET phosphorylation *in vivo*. Near-maximal inhibition of MET activity for the full dosing interval was necessary to maximize the efficacy. The antitumor mechanisms *in vivo* were associated with the inhibition of MET-dependent signal transduction, tumor

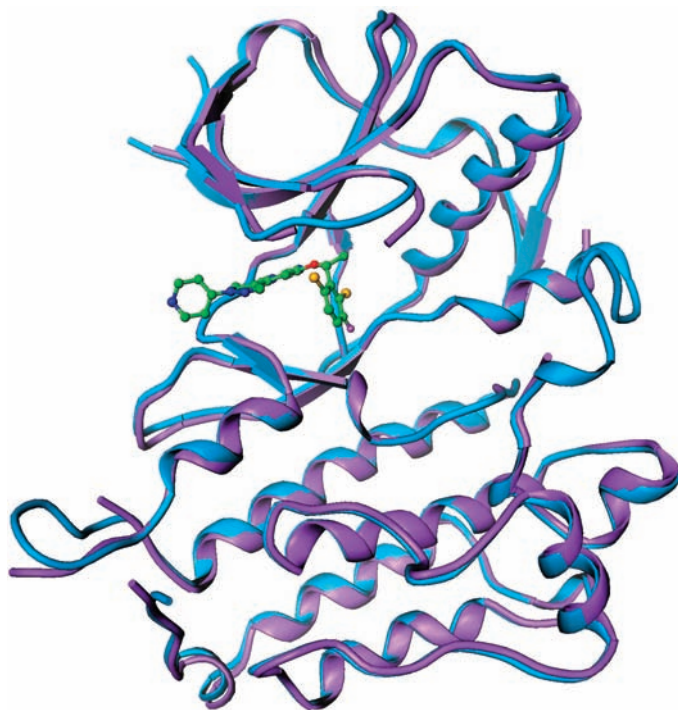


Figure 3.6 Overlay of crizotinib/ALK cocrystal (purple color) with apo-ALK crystal (blue color) structures.

cell proliferation (Ki67), induction of apoptosis (caspase-3), and reduction of microvessel density (CD31).

The ALK-derived pharmacological effects of crizotinib were evaluated in NPM-ALK-positive ALCL cell lines and related tumor models with minimum expression of c-MET [46]. To explore the potential clinical utility in treatment of patients with ALK-positive ALCL, crizotinib was evaluated *in vitro* and *in vivo* in NPM-ALK-positive ALCL cell lines and related tumor models [46]. Crizotinib potently inhibited NPM-ALK phosphorylation in Karpas299 or SU-DHL-1 ALCL cells (mean $IC_{50} = 24$ nmol/l) leading to the inhibition of NPM-ALK-dependent cell proliferation ($IC_{50} = \sim 30$ nmol/l). The observed cytoreductive activity of crizotinib was associated with the inhibition of NPM-ALK-dependent cell cycle progression at the G1–S-phase checkpoint and the induction of cell death pathways. Crizotinib demonstrated dose-dependent antitumor efficacy with complete regression of all tumors at the 100 mg/kg/day oral dose within 15 days of initial compound administration in severe combined immunodeficient-Beige mice bearing Karpas299 ALCL tumor xenografts. A strong correlation was observed between antitumor response and inhibition of NPM-ALK phosphorylation and induction of apoptosis in tumor tissue. In addition, inhibition of key NPM-ALK signaling mediators, including phospholipase C- γ , signal transducers and activators of transcription 3, extracellular signal-regulated kinases,

Table 3.2 Pharmacokinetic parameters of crizotinib in preclinical species and humans after intravenous or oral administration.^{a)}

Species	Route	Dose (mg/ kg)	CL _{plasma} (ml/min/ kg)	V _{ss} (l/kg)	C _{max} (μg/ml)	t _{max} (h)	AUC _{0–∞} (μg h/ml)	t _{1/2} (h)	F _{oral} (%)
Rat	i.v.	5	29 ± 8	13 ± 4	— ^{b)}	—	3.0 ± 0.9	7.7 ± 1.8	—
	p.o.	25	—	—	0.53 ± 0.10	4.7 ± 1.2	5.6 ± 0.8	7.0 ± 0.4	63
Dog	i.v.	5	9.0 ± 0.8	13 ± 2	—	—	9.3 ± 0.8	17 ± 4	—
	p.o.	25	—	—	0.62 ± 0.37	4.0 ± 2.0	12 ± 8	12 ± 3	65
Monkey	i.v.	5	34 ± 4	13 ± 1	—	—	2.5 ± 0.3	5.5 ± 0.2	—
	p.o.	25	—	—	0.24 ± 0.11	6 ± 0	4.1 ± 1.7	12 ± 3	42
Human	i.v.	100 ^{c)}	—	—	0.061 ± 0.03	2.5 ± 1.7	0.60 ± 0.22	10 ± 2	—
	p.o.	100 ^{c)}	—	—	0.061 ± 0.03	2.5 ± 1.7	0.60 ± 0.22	10 ± 2	—

a) Data are expressed as mean ± S.D. (n = three animals or animal patients per group).

b) Not applicable.aa

c) mg/body.

and Akt by crizotinib was observed at concentrations or dose levels that correlated with inhibition of NPM-ALK phosphorylation and function.

Crizotinib is a free base anhydrous crystalline compound with an onset melting point of 195 °C and experimentally measured pK_a values of 8.9 and 5.4. It is not surprising that crizotinib has a pH-dependent solubility: 0.034 mg/ml in pure water, 41 mg/ml in simulated gastric fluid, and 0.19 mg/ml in simulated intestinal fluid. Crizotinib showed moderate metabolic stability in human hepatocytes, moderate to high plasma protein binding (92–97%) in human and preclinical species, and low to moderate permeability in the Caco-2 cell assay. Biotransformation mediated by CYP3A4 is likely to be the major clearance mechanism. Crizotinib is not a potent inhibitor of CYP1A2, 2C8, 2C9, 2C19, and 2D6 (IC₅₀ > 30 μM) in human liver microsomes, but does inhibit CYP3A isozymes in a time-dependent manner (K_i = 3.0 μM and k_{inact} = 0.11 min^{−1}). The pharmacokinetic parameters of crizotinib in preclinical species and humans are summarized in Table 3.2 [47].

Crizotinib was negative in the BioLume Ames assay with or without rat liver S9 metabolic activation. In the CHO cell micronucleus assay, crizotinib was positive in the absence of S9 and negative in the presence of S9. Crizotinib inhibited binding of [³H]-dofetilide to hERG ion channels with a K_i value of 1.8 μM and inhibited hERG potassium currents with an IC₅₀ of 1.1 μM as measured by patch-clamp electrophysiology. A 7-day repeat-dose oral toxicity study with crizotinib in male and female rats indicated a NOAEL for crizotinib of 150 mg/kg corresponding to a plasma C_{ave} of 120 nM (free).

3.5

Human Clinical Efficacies of Crizotinib

The human clinical phase I trial of crizotinib was initiated in 2006 in patients with advanced cancer to determine the maximal tolerated dose (MTD) and the

recommended phase II dose (RP2D). Crizotinib was first dosed at 50 mg orally once daily and eventually escalated to 300 mg orally BID, at which dose two patients experienced grade 3 fatigue. The MTD and the recommended clinical dose is 250 mg BID [48]. After a single dose of crizotinib at 250 mg, C_{max} was achieved at a median T_{max} of 4.0 h and the mean apparent terminal half-life was 42 h [49]. After repeated dosing at 250 mg BID, crizotinib plasma concentrations appeared to reach steady state within 15 days with a mean steady-state trough plasma level of 256 ng/ml or 45 nM of free drug, which exceeded the target efficacy levels predicted for the inhibition of MET and ALK based on preclinical mouse models [50]. Decreased clearance following multiple crizotinib doses was observed (64.5 and 60.1 l/h following 15 and 28 days in comparison with 100 l/h after a single-day dosing), possibly resulting from crizotinib autoinhibition of CYP3A [50]. A 3.6-fold increase in the single-dose oral midazolam AUC was observed following 28 days of dosing at 250 mg BID, suggesting that crizotinib is a moderate CYP3A4 inhibitor [50]. AUC generally increased proportionally with doses over the therapeutic dose range studied, and accumulated by 4.0–5.9-fold after multiple doses with a terminal half-life of 43–51 h. A high-fat meal did not appear to change crizotinib PK [50]. The exposure level of crizotinib in cerebrospinal fluid (CSF) was reported in one patient who received crizotinib 250 mg orally BID with a median trough total plasma concentration of 256 ng/ml and CSF concentration of 0.616 ng/ml [51].

Crizotinib has demonstrated remarkable efficacy in patients suffering from non-small cell lung cancer, inflammatory myofibroblastic tumor, anaplastic large cell lymphoma, and neuroblastoma harboring fusion *ALK* or *ROS* genes or having *de novo* *MET* gene amplification. The publication of the discovery of the transforming *EML4-ALK* fusion gene in patients of NSCLC in August 2007 accelerated the clinical studies of crizotinib in patients with aberrant ALK expression, especially in NSCLC [21]. A subset of NSCLC patients with *EML4-ALK* fusion gene was enrolled in the second part of the crizotinib phase I study (A8081001) and a phase II study (A8081005) that was launched in August 2009 based on the marked efficacy results from the crizotinib phase I study. The efficacy results from both trials were summarized in Table 3.3.

Overall, the treatment-related adverse events were mostly grade 1 or 2. The most common adverse events were visual effects, nausea, diarrhea, constipation, vomiting, and peripheral edema. The most common treatment-related grade 3 or 4 adverse events were neutropenia, raised alanine aminotransferase, hypophosphatemia, and lymphopenia [52,53].

ALCL is a rare type of non-Hodgkin lymphoma (NHL) and comprises about 3% of all NHLs in adults and between 10 and 30% of all NHLs in children. Fifty to sixty percent of ALCL are ALK positive. Three out of four ALK-positive ALCL patients (age 20–26), resistant to at least three lines of chemotherapy treatment, achieved a complete response (CR) following treatment with crizotinib at 250 mg BID [54]. Remarkably, in a Children's Oncology Group (COG) phase I consortium study of crizotinib, a CR resulted for 7/8 children with ALK-positive ALCL [55]. Neuroblastoma is another rare childhood cancer and the most common cancer in infancy, with an annual incidence of about 650 cases in the United States. Activating ALK

Table 3.3 Summary of clinical activity of crizotinib in patients enrolled in phase I A8081001 [52] trial and phase II A8081005 [53] trial.

Clinical trial	Number of patients	ORR ^{a)} (95% CI ^{b)})	DCR ^{c)} (95% CI)	Estimated PFS ^{d)} (range) (months)	Median duration of response (weeks)
A8081001	143	60.8 (52.3–68.9)	82.5 at 8 weeks (75.3–88.4)	9.7 (7.7–12.8)	49.1
A8081005	255	53 (47–60)	85 at 12 weeks (80–89)	8.5 (6.2–9.9)	43

a) ORR: overall response rate.

b) CI: confidence interval.

c) DCR: disease control rate.

d) PFS: progression-free survival.

mutations were recently detected in most familial and 10% of sporadic neuroblastomas [24,25]. Clinical evaluation of crizotinib for the treatment of neuroblastomas has been initiated. From the COG phase I consortium study, two patients with neuroblastoma had CRs, one with a documented ALK mutation [55]. ALK translocations have been discovered in about 50% of IMT, a distinctive mesenchymal neoplasm. A sustained partial response to crizotinib in a patient with ALK-translocated IMT was reported [56]. In summary, inhibition of ALK in both adult and children patients with abnormal ALK kinase activity correlates with objective antitumor activity with minimal toxicity.

As seen with other TKI drugs such as imatinib and gefitinib, cancers develop resistance to crizotinib, despite the remarkable initial responses, within 1 year on average. Understanding the biology of resistance will be critical to selecting the best therapeutic strategies for treating patients with acquired resistance to crizotinib. Secondary mutation in the ALK kinase domain (22–36%) is one of the mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers [57–59]. Unlike EGFR TKI resistance in NSCLC, which mainly has gatekeeper T790M mutation, the crizotinib secondary mutation resistance is more reminiscent of imatinib and has a greater diversity of resistance mutations including gatekeeper L1196M mutation, C1156Y, G1202R, S1206Y, and G1269A mutations, and one insertion mutation 1151Tins. Also, ALK fusion gene amplification was identified in tumors from some patients resistant to crizotinib treatment [58,59]. Other crizotinib resistance mechanisms identified from a limited number of patients include aberrant activation of other kinases (c-KIT, EGFR) and KRAS mutation [58,59]. Multiple resistance mechanisms developing simultaneously were found in a subset of patients [59]. Other resistance mechanisms will surely emerge as clinical experience with crizotinib increases. Efforts to clinically evaluate second-generation ALK inhibitors that inhibit both wild and mutant ALK are underway with the hope that extended patient survival will result by addressing at least a subset of the crizotinib resistance mechanisms [60].

Approximately up to 2% of patients with NSCLC harbor *ROS1* rearrangements [61]. Crizotinib demonstrates marked antitumor activity in patients with advanced NSCLC harboring *ROS1* rearrangements as a result of ROS TKI inhibition, analogous to the more thoroughly explored ALK TKI inhibition detailed above. An ORR of 54% (7/13), with 6 PRs and 1 CR, resulted for a group of ROS+ patients treated with crizotinib at 250 mg BID. The disease control rate at 8 weeks was 85% (11/13), and the median duration of treatment was 20 weeks (range 4+–59+) [62].

The clinical role of MET TKI inhibitors in cancer patients continues to be investigated. It was reported that inhibition of MET signaling by crizotinib or by RNA interference-mediated MET depletion resulted in the induction of apoptosis accompanied by inhibition of AKT and extracellular signal-regulated kinase phosphorylation in lung cancer cells with MET amplification, but not in cells with a MET mutation or in those without amplification or mutation of MET [63]. Crizotinib also demonstrated effects on signal transduction and survival in gastric cancer cells with MET amplification [64]. Both of these examples suggest that MET gene amplification might be an appropriate patient selection biomarker for treatment with crizotinib. One patient with a *de novo* highly MET-amplified NSCLC achieved a confirmed partial response with crizotinib [65]. Two out of four patients with esophagogastric adenocarcinoma harboring amplified MET treated with crizotinib experienced tumor shrinkage (–30 and –16%), but subsequent progression after 3.7- and 3.5-month treatments [66]. A 62-year-old woman with MET-amplified glioblastoma in A8081001 phase I trial demonstrated 40% reduction of the contrast-enhancing lesion in MRI after two monthly cycles of crizotinib [67]. The patient was neurologically and radiographically stable after 4.5 months on crizotinib at the time of report.

There is an emerging concept that MET amplification and elevated levels of stromal HGF may play an important role in resistance to TKIs such as EGFR inhibitors in NSCLC and other cancers, suggesting an additional possible therapeutic utility for crizotinib [68].

3.6 Summary

Crizotinib is a multitargeted tyrosine kinase inhibitor of MET/ALK/ROS. The 2-amino-3-benzoyloxypyridine series, an essential scaffold for crizotinib, was designed from the cocrystal structure of PHA-665752 bound to the unphosphorylated MET kinase domain, which has an inactive autoinhibited conformation and aligns with the apo-MET autoinhibited conformation. The new design achieved more effective interactions with the inactive MET protein and stabilized the inactive MET protein in an autoinhibited conformation. Optimization of the lead series by focusing on ligand efficiency and lipophilic efficiency generated the clinical candidate crizotinib (PF-02341066), which demonstrated potent *in vitro* and *in vivo* MET kinase and ALK inhibition, effective tumor

growth inhibition, and good pharmaceutical and safety properties. Crizotinib was well tolerated in human phase I studies, and the treatment-related adverse events were typically gastrointestinal (grade 1/2) and visual disorders (grade 1). The discovery of EML4-ALK as an oncogenic driver in non-small cell lung cancer accelerated the clinical development of crizotinib in the ALK-driven patient population. The US Food and Drug Administration granted fast-track approval of crizotinib on August 26, 2011 based on the marked high response rates of crizotinib in patients with ALK-positive advanced NSCLC and its good safety profile in phase I and II trials. Promising antitumor activities have been observed with crizotinib in patients with ALK-positive anaplastic large cell lymphoma, inflammatory myofibroblastic tumor, and neuroblastoma; ROS-positive non-small cell lung cancer; and MET gene-amplified non-small cell lung cancer, esophagogastric adenocarcinoma, and glioblastoma. The broad antitumor activities of crizotinib based on molecular targets across many cancers indicate the importance of understanding tumor biology to identify the driver targets for the stratification of the right patient population. The swift approval of crizotinib by the Food and Drug Administration based on 255 late-stage NSCLC patients with aberrant ALK gene represents a major triumph for personalized medicine. The early use of enriched patient populations should accelerate clinical evaluation of future targeted agents and allow accelerated drug approval in molecularly targeted patient populations.

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4

Discovery and Development of Vemurafenib: First-in-Class Inhibitor of Mutant BRAF for the Treatment of Cancer

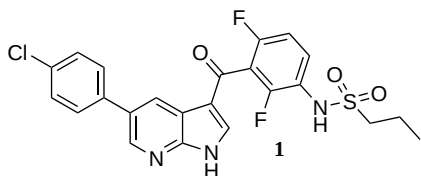
Prabha Ibrahim, Jiazhong Zhang, Chao Zhang, James Tsai, Gaston Habets, and Gideon Bollag

4.1

Background

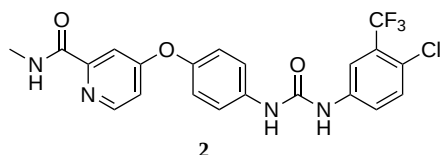
RAF kinases are a family of three (ARAF, BRAF, and CRAF) serine/threonine protein kinases that participate in the RAS–RAF–MEK–ERK signal transduction (MAPK) cascade. In 2002, BRAF was identified as a driver oncogene in many different cancers [1]. The majority of BRAF mutations consist of glutamic acid substitution for valine at position 600 (V600E). This mutation leads to constitutive kinase activity and correlates with uncontrolled cellular proliferation [2]. The identification of oncogenic BRAF mutations in about half of the patients with metastatic melanoma [3] presented the opportunity to develop oncogene-selective inhibitors that could be beneficial to melanoma patients.

Although previous generations of BRAF inhibitors possess low nanomolar activity against V600E, the therapeutic efficacy has been hindered either by lack of bioavailability or by lack of kinase selectivity [4–9]. Vemurafenib (**1**, PLX4032, RG7204, Zelboraf[®]), an orally active drug discovered by Plexxikon, is a first-in-class inhibitor of oncogenic BRAF kinase activity, approved in 2011 (USA), for the treatment of patients with unresectable or metastatic melanoma with the BRAF^{V600E} mutation. In addition, a companion diagnostic test to detect the BRAF^{V600E} mutation, in formalin-fixed paraffin-embedded human melanoma tissue (cobas[®] 4800), was developed in parallel to the development of vemurafenib. The coupling of drug and companion diagnostic development serves as a new approach to personalized medicines in the postgenomic era.

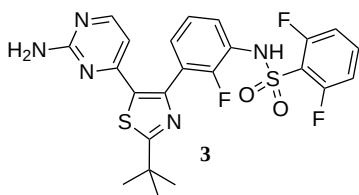


USAN: Vemurafenib (Vem)
Trade Name: Zelboraf[®]
Roche & Plexxikon Inc.
Launched: 2011

The multitargeted kinase inhibitor sorafenib (2), originally discovered as a CRAF inhibitor and approved for the treatment of renal and hepatocellular carcinoma, is a weak inhibitor of BRAF^{V600E}, and it did not reveal clinical benefits in melanoma patients [10]. Recently, dabrafenib (3, GSK2118436), inhibitor of mutant BRAF became the second compound to show clinical activity in patients with BRAF (V600)-mutated metastatic melanoma [11].



USAN: Sorafenib
Trade name: Nexavar®
Bayer
Launched: 2005



USAN: Dabrafenib
Trade name:
GSK
Status: Preregistration

4.2

Discovery and Development of Vemurafenib (PLX4032)

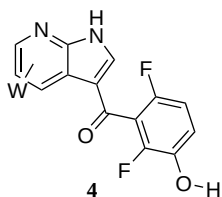
Vemurafenib, formerly known as PLX4032, was built around the 7-azaindole scaffold. This novel scaffold was identified from a target-naïve screening library, utilizing a combination of biochemical and high-throughput cocrystallography screening filters. Plexikon's screening library (scaffold library) of more than 20,000 low molecular weight (150–350 Da) compounds were identified using a novel approach described by Zhang *et al.* [12]. The process of low-affinity biochemical screening of the scaffold library at high concentrations (100–200 μM) against multiple members of target protein family yielded scaffolds for further screening by cocrystallography. This approach (scaffold-based drug discovery) has been successfully utilized in identifying small-molecule inhibitors for targets in several protein families, including phosphodiesterases [13], and nuclear receptors [14]. Recently, we have described the expansion of this strategy to discover 7-azaindole as a scaffold targeting protein kinases [15], using Pim-1 kinase as a robust system for cocrystallography [16]. This novel kinase scaffold was one among 70 different compounds bound in the ATP binding pocket and was the starting point to rationally design the selective oncogenic BRAF inhibitor vemurafenib [17], as well as a selective dual FMS–KIT kinase inhibitor PLX647 [18].

The 7-azaindole profiler library was built through diverse chemical modifications at the productive sites of the scaffold as guided by the cocrystal structures. This proprietary library was screened against recombinant BRAF^{V600E} kinase to identify potential leads. The initial submicromolar hits revealed a set of compounds, containing a difluorophenol substituent (4) connected through a ketone linker to

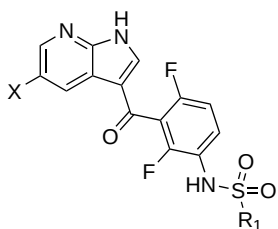
Table 4.1 Optimization of the sulfonamide functional group.

Compound number	R ₁	IC ₅₀ (BRAF ^{V600E}) μ M
4	Me	0.22
5	Et	0.11
6	<i>n</i> -Pr	0.008
7	<i>n</i> -Bu	0.02

the 7-azaindole scaffold. One of these hits was cocrystallized with a mutated p38 kinase that served as the surrogate of BRAF, while the crystallization of BRAF was being developed. The structure revealed key hydrogen bond interactions with the back-bone and side chains in the ATP binding pocket. Although these difluorophenol compounds were potent inhibitors of oncogenic BRAF, they lacked selectivity against other kinases and were poorly absorbed when dosed in rats. Further lead optimization approaches included bioisostere replacement of the —OH (phenol) in order to address metabolic liability and to improve *in vivo* profiles. Replacement of the —OH group by sulfonamides is a well-known medicinal chemistry approach [19]. The utility of this bioisosteric replacement was demonstrated in the discovery of soterenol, a potent and selective β -adrenoceptor agonist with improved potency, selectivity, and metabolic profile, resulting in longer duration of action [20].



As a next step in lead optimization, a number of sulfonamides with varying alkyl chain lengths were used to replace the —OH group with a fixed substitution on the 5-position of the azaindole ($X = \text{Br}$). The SAR revealed the *n*-propyl sulfonamide as the optimal functional group for oncogenic BRAF potency (Table 4.1).



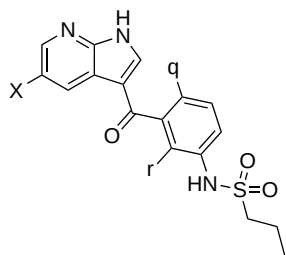
The impact of the substitutions (*q* and *r*) in the middle phenyl ring on the BRAF^{V600E} potency was then examined by maintaining $X = \text{H}$ (Table 4.2). Replacing fluorine at the *para*-position (*q*) of the sulfonamide with hydrogen (**9**) or with chlorine (**10**) had a moderate impact on the potency. On the contrary, the fluorine

Table 4.2 Optimization of the 5-position of the azaindole core.

Compound number	X	q	r	IC ₅₀ (BRAF ^{V600E}) μ M
8	H	F	F	0.108
9	H	H	F	0.679
10	H	Cl	F	0.238
11	H	F	H	>10
12 (PLX4720)	Cl	F	F	0.008
6	Br	F	F	0.008
13	Phenyl	F	F	0.016
14	3-Pyridyl	F	F	0.003
15	4-NMe2-Phenyl	F	F	0.055
16	4-OMe-Phenyl	F	F	0.02
Vemurafenib (1; PLX4032)	4-Cl-Phenyl	F	F	0.031

in between the carbonyl and the sulfonamide (r) was critical, as substitution to hydrogen (**11**) resulted in loss of activity (>1000-fold). The crystal structure of compound **8** in complex with BRAF^{V600E} showed a hydrogen bond between the nitrogen from *n*-propyl sulfonamide motif and the backbone NH of Asp594 of the protein. The electron-withdrawing fluorine helps to lower the intrinsic pK_a of the sulfonamide nitrogen and makes it a better hydrogen bond acceptor. The size and electronegativity of fluorine allow it to participate in the hydrogen bond interaction without creating steric hindrance. Finally, the fluorine may help to break the tendency of the phenyl ring to be coupled to the azaindole-linker coplanarity.

The next step of optimization was focused on substitutions at the 5-position (X) of azaindole. Halogens Cl and Br were equivalent (compounds **12** and **6**, respectively), showing ~10-fold improvement in activity compared to compound **8**. In addition, a number of aromatic and heteroaromatic substituents at the 5-position of the azaindole were synthesized and screened through the oncogenic BRAF biochemical assay, and the data for a selected set of compounds are shown in Table 4.2 (compounds **13–16** and **1**). The various 5-substitutions did not significantly affect biochemical potency; however, these substituents had impact on the *in vivo* exposure and efficacy (xenograft) studies. Due to their pharmaceutical properties, *in vitro* safety profile, and consistent rodent pharmacokinetic properties, both PLX4032 and PLX4720 (compounds **1** and **12**, respectively) were chosen for preclinical evaluation. Finally, based on the favorable pharmacokinetic properties in higher species, vemurafenib was chosen for further development.



The cocrystal structure of vemurafenib with the BRAF^{V600E} kinase domain revealed an asymmetric dimer with preferential compound binding to the protomer with active (DFG-in) conformation. The favored binding to this conformation was likely due to the unique hydrogen bonding interaction between the backbone NH of Asp594 and the nitrogen from the *n*-propyl sulfonamide substructural motif [21]. The vemurafenib class of BRAF inhibitors prefers the active conformation of the kinase, and the binding of the inhibitors caused a shift in the regulatory α C-helix involved in RAF dimerization. This distinct structural effect may underlie the different efficacy and safety profiles of vemurafenib compared to earlier generation of RAF inhibitors such as sorafenib.

4.3

Pharmacology

Vemurafenib displayed similar potency for BRAF^{V600E} (31 nM) and CRAF (48 nM) with modest selectivity (approximately threefold) over wild-type BRAF (100 nM). When screened against a panel of 200 kinases, the majority of them were minimally affected, while some non-RAF kinases were found to be sensitive to vemurafenib (Table 4.3). It is important to note that most of these kinases were assayed at a lower ATP concentration (10 μ M for the counterscreens compared to 100 μ M for RAF kinases) [21]. Vemurafenib potently inhibited ERK phosphorylation and cell proliferation in BRAF mutant but not wild BRAF wild type cell lines, thus demonstrating the translation of the biochemical selectivity to cellular selectivity. In *in vivo* studies vemurafenib demonstrated efficacy in multiple BRAF^{V600E}-bearing colon cancer (Colo205) [21] and melanoma (LOX and Colo829) [22] xenograft models.

Table 4.3 *In vitro* profiling of vemurafenib.

Biochemical assay	IC ₅₀ nM	Biochemical assay	IC ₅₀ nM	Cellular assay	IC ₅₀ nM
BRAF ^{V600E}	31	NEK11	317	Colo205	40
CRAF	48	BLK	547	Colo829	80
BRAF	100	LYNB	599	A375	300
SRMS	18	YES1	604	SW620	7828
ACK1	19	WNK3	877	SKMEL2	7107
MAP4K5 (KHS1)	51	MNK2	1717		
FGR	63	FRK (PTK5)	1884		
LCK	183	CSK	2339		
BRK	213	SRC	2389		

4.4

Clinical Efficacy and Safety

Vemurafenib was evaluated in a phase I study [23] in patients with metastatic cancer, initially with a crystalline formulation and later on with an amorphous formulation [24] microprecipitated bulk powder (MBP). Efficacy was observed in patients with tumors that carry V600E mutation. In a phase II study, vemurafenib induced clinical response in patients with BRAF^{V600E}-positive metastatic melanoma, with a median overall survival of approximately 16 months [25]. Vemurafenib produced improved rates of overall and progression-free survival in a phase III-randomized clinical trial, comparing vemurafenib with dacarbazine in patients with previously untreated, metastatic melanoma with the BRAF^{V600E} mutation [26]. Common adverse events associated with vemurafenib were arthralgia, rash, fatigue, alopecia, keratoacanthoma or squamous cell carcinoma, photosensitivity, nausea, and diarrhea. Following this rapid clinical development path that demonstrated striking tumor shrinkage, a significant survival benefit, and a manageable safety profile, vemurafenib was approved in the United States in 2011 for the treatment of patients with unresectable or metastatic melanoma with BRAF^{V600E} mutation as detected by an FDA-approved test.

4.5

Companion Diagnostic (cobas 4800) Development

A real-time PCR reaction to detect the BRAF^{V600E} mutation was developed by the scientists at Roche Molecular Systems, Inc. This assay can detect BRAF mutations directly from formalin-fixed, paraffin-embedded tissue (FFPET), allowing the testing of archived samples [27]. A prototype diagnostic assay was developed concurrent with the phase I trial, which was used as enrollment criterion for the phase II and phase III trials. The cobas 4800 diagnostic assay was approved in 2011, concurrent with the approval of vemurafenib.

4.6

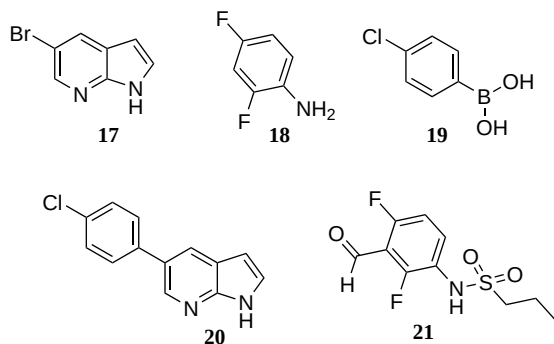
Synthesis

4.6.1

Discovery Route(s)

Multiple synthetic routes were used for the discovery phase synthesis of vemurafenib [28]. However, for preclinical needs a routine scalable route was identified, utilizing the three commercially available building blocks, 5-bromoazaindole (**17**), 2,4-difluoroaniline (**18**), and 4-chlorophenylboronic acid (**19**). Suzuki coupling of 5-bromo-7-azaindole with 4-chlorophenylboronic acid provided the intermediate 5-(4-chlorophenyl)-1*H*-pyrrolo[2,3-*b*]pyridine (**20**) in high yield. Key intermediate propane-1-sulfonic acid (2,4-difluoro-3-formyl-phenyl)-amide (**21**) was prepared from compound **18** in two steps, with moderate yields. Aldol coupling of

the intermediates **20** and **21** followed by demethylation and oxidation provided vemurafenib (overall yield ~10%).

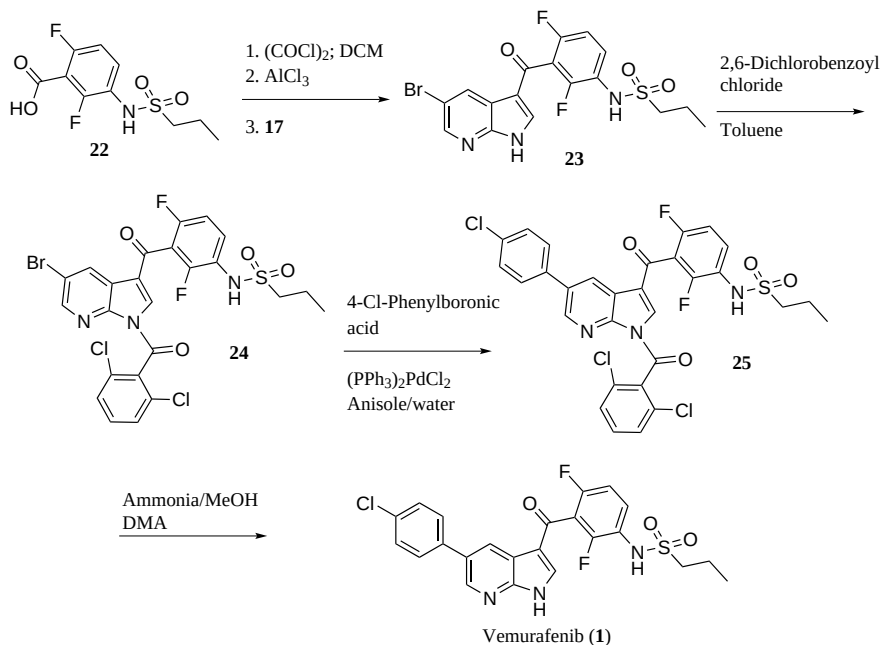


This route was further optimized to avoid chromatographic purifications and used for the synthesis of vemurafenib in kilogram quantities.

4.6.2

Process Route

A four-step process for synthesis of vemurafenib was described in patent WO2011015522 (Scheme 4.1). The synthesis began with the conversion of the acid (**22**) to the acid chloride followed by Friedel–Crafts reaction with 5-bromoazaindole



Scheme 4.1

(17), resulting in the formation of compound **23**. Subsequent protection of compound **23** using 2,6-dichlorobenzoyl chloride and Suzuki coupling with 4-chlorophenylboronic acid (**19**) resulted in the formation of compound **25**, which when reacted with a solution of ammonia in methanol provided vemurafenib. This synthesis could potentially be the industrial-scale production process of vemurafenib.

4.7

Summary

In summary, vemurafenib (**1**) is marketed as Zelboraf for the treatment of patients with unresectable or metastatic melanoma, with BRAF^{V600E} mutation, as detected by an FDA-approved test, and is currently undergoing clinical trials for a number of additional indications.

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5

Targeting Basal-Cell Carcinoma: Discovery and Development of Vismodegib (GDC-0449), a First-in-Class Inhibitor of the Hedgehog Pathway

James C. Marsters Jr. and Harvey Wong

5.1

Introduction

Innovative approaches in the treatment of cancer focus on the identification of key oncogenic proteins and the development of pharmaceutical agents that target these oncogenes. So-called “driver mutations” establish a direct linkage of oncogene activity and disease initiation and/or progression. Targeted agents that block the activity of these aberrant proteins offer an opportunity to treat cancer effectively while limiting the toxicity of more broadly used chemotherapeutics. The association of target and disease forms the foundation of a personalized approach to cancer treatment.

First identified in a genetic screen using *Drosophila* [1], the hedgehog pathway is important in embryonic development and is critical to the initiation and patterning of many organ systems, from flies to humans. In mammals, the activity of hedgehog ligands, designated Sonic, Desert, and Indian, are critical for the proper development of the nervous system, skeleton, lung, digits, and gut [2]. The key morphogenic role of these ligands is underscored in humans where loss of function mutations have been linked to several developmental disorders such as neural defects and skeletal deformities [3,4]. In normal adult tissues, however, hedgehog pathway activity is generally quiescent [5].

Two transmembrane proteins, the smoothened homolog (SMO) and the patched homolog 1 (PTCH1), control signaling at the cell surface (Figure 5.1) [6]. PTCH1, in the absence of ligand, represses SMO and blocks its activity. Upon hedgehog ligand binding to PTCH1, it is internalized and degraded within lysosomes, thus releasing its repression of SMO and allowing signaling to occur. Signal transduction leads to the transcriptional activation of hedgehog target genes such as *GLI1* and *PTCH1* [7]. The specific induction of hedgehog target gene transcription in response to ligand binding (or mutational activation) provides a convenient marker for hedgehog activity in cells and tissues [8].

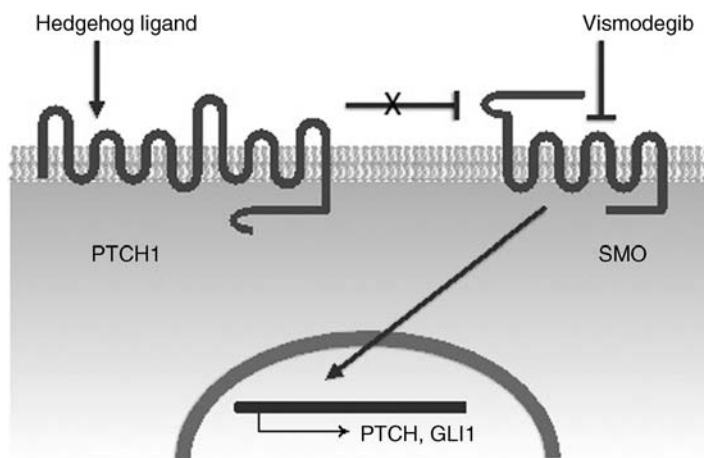


Figure 5.1 Schematic representation of the hedgehog signaling pathway. The binding of hedgehog ligand to PTCH1 relieves PTCH1 inhibition of SMO signaling, driving the

activation of GLI1 transcription factors and the expression of hedgehog target genes. Vismodegib blocks hedgehog signaling by binding and inactivating SMO.

5.2

Hedgehog and Basal-Cell Carcinoma

Aberrant activation of the hedgehog pathway has been associated with cancer in humans. Germline mutations in the repressor PTCH1 were found in patients with Gorlin syndrome [9,10], a genetic disease associated with the development of numerous basal-cell carcinomas (BCCs) in patients throughout their lifetimes. Similarly, the majority of sporadic BCCs were also shown to contain inactivating mutations in PTCH1 [10], with ~10% of cases showing direct activating mutations in SMO [11]. The risk of development of BCC is highest in fair-skinned individuals and is associated with sun exposure, most frequently on the head and neck. In most cases, sporadically occurring BCCs show hyperactivated hedgehog signaling, demonstrated by high transcript mRNA levels of hedgehog target genes (*GLI1* and *PTCH1*) [12,13]. Unregulated activity of SMO in these cells makes it a putative target for the treatment of BCC [11]. Targeting SMO with a small-molecule inhibitor forms the basis for developing an effective therapy for the treatment of BCC. Vismodegib represents the first hedgehog pathway inhibitor approved in the US for the treatment of adults with metastatic basal-cell carcinoma or with locally advanced basal-cell carcinoma that has recurred following surgery or in those patients who are not candidates for surgery or for radiation.

5.3

Cyclopamine as an SMO Antagonist

In the late 1950s, studies on the causes of craniofacial deformities in newborn sheep identified the plant *Veratrum californicum*, or California corn lily, as the likely

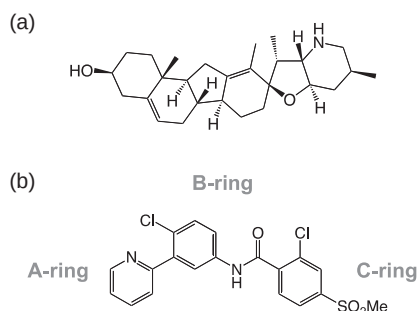


Figure 5.2 Structures of cyclopamine (a) and vismodegib (compound 15) (b).

culprit based on an association with the grazing habits of pregnant ewes [14]. Extraction of the causative agents from corn lily lead to the identification of the steroidal alkaloids cyclopamine, cycloposine, and jervine (Figure 5.2a) [15]. The teratogenic activity of cyclopamine in sheep was later linked to its ability to bind to and inhibit SMO [16]. Efforts to utilize cyclopamine as a starting point in drug development have been demonstrated in the elegant work at Infinity Pharmaceuticals on IPI-926 (saridegib), which showed greatly improved potency and pharmaceutical properties over the natural product [17].

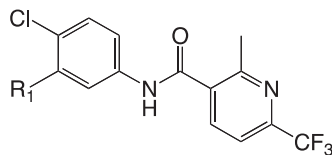
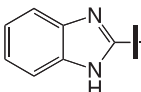
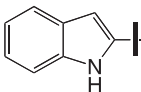
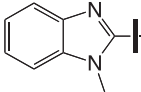
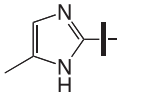
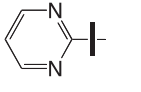
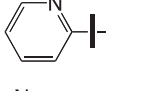
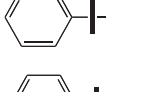
5.4

Small-Molecule Inhibitors of SMO

While studies with cyclopamine and its analogs demonstrated proof of concept in cells and in animal models, these complex steroidal compounds were not ideal lead structures. We set out to identify additional compounds with improved drug-like properties and a structural class that was more amenable to further manipulation [18]. High-throughput screening of small-molecule compound libraries was conducted in order to identify nonsteroidal SMO antagonists as lead compounds for a drug optimization effort. This cell-based screen employed murine 10T1/2 (S12) fibroblasts transfected with a plasmid encoding a luciferase reporter gene downstream of hedgehog pathway transcriptional binding sites (*GLII*) [19].

The benzimidazole 1 (Table 5.1) resulted from the hit-to-lead optimization effort utilizing the structure–activity data generated in the high-throughput screen [18]. This compound was potent, with an IC_{50} of $0.012\mu\text{M}$ in the Gli-luciferase assay, and proved to be useful in animal models as a tool compound. However, its poor metabolic stability in human microsomes and low aqueous solubility made it a poor choice for clinical studies. A preferable candidate profile would entail a molecule with sufficient bioavailability and metabolic stability to bind and inhibit SMO continuously over the treatment period. Our optimization program focused on modifications of the A-ring benzimidazole and C-ring nicotinic amide functionalities of the molecule (Tables 5.1 and 5.2 and Figure 5.2b).

Table 5.1 Structure–activity relationship examining heterocyclic replacements of the A-ring benzimidazole.

		
Entry	R1	IC ₅₀ (μM)
1		0.012
2		0.500
3		0.009
4		0.256
5		8.00
6		0.042
7		10.0
8		2.40

The SMO binding potencies following systematic structural replacement of the benzimidazole are shown in Table 5.1 [18]. The 40-fold loss of potency observed in compound **2** illustrates the preference for a hydrogen bond acceptor at a position adjacent to the ring junction, while the *N*-methylated analog (**3**) showed little change in affinity. Replacing the fused ring benzimidazole with the imidazole (**4**) or pyrimidine (**5**) led to a 20- and 600-fold drop in potency. However, the 2-pyridyl

Table 5.2 Structure–activity relationship examining phenyl- and pyridyl-based substituents at the C-ring amide.

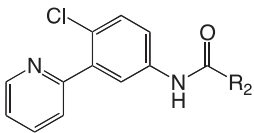
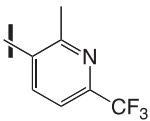
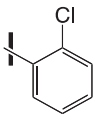
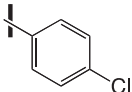
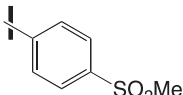
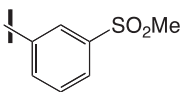
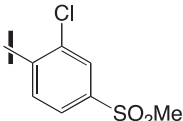
		
Entry	R2	IC ₅₀ (μM)
6		0.042
9		0.600
10		0.800
11		0.110
12		1.5
13		0.040
14		0.120
15		0.013

Table 5.3 Comparison of the potencies and screening pharmacological properties of candidate inhibitors of the hedgehog pathway.

Entry	IC ₅₀ (μM)	CLR predicted from microsomes (ml/(min kg))			CLR measured (ml/(min kg))		clog P	Solubility (μg/ml)	
		Rat	Dog	Human	Rat	Dog		pH 1	pH 6.5
1	0.012	18.5	22	9.4	3.0	124	3.9	300	0.3
6	0.042	5.9	10	0.6	4.5	1.9	3.7	1000	1.8
13	0.040	2.8	11	5.9	1.2	1.4	3.2	420	0.5
15	0.013	3.7	11	4.5	3.7	0.4	4.0	>3000	9.5

replacement, compound **6**, retained much of the affinity of the benzimidazole lead. The preference for a basic nitrogen adjacent to the ring junction was maintained in this series, as the 3- and 4-pyridines (**7** and **8**) showed a 200- and 50-fold drop in potency relative to compound **6**.

Next, we performed an extensive evaluation of substituents at the amide linked C-ring of the molecule, based on compound **6** [18]. The nitrogen atom of the nicotinamide was found to be relatively unimportant, as the phenyl and nicotinic amides (**9** and **10**) were equipotent. Substitution at the 2-position of the ring, however, gave improved potency as evidenced by compound **11**, possibly due to additional hydrophobic interactions and/or conformational effects that move the aromatic ring out of plane with the amide bond. Sulfonyl substitution at the 4-position (**13**) improved potency 20-fold relative to the phenyl analog (**10**). Combining the best substituents (**11** and **13**) provided the lead candidate, compound **15**, with good potency (IC₅₀ = 0.013 μM). The results of candidate screening studies provided the predicted and observed clearance rates (CLRs) in rat and dog shown in Table 5.3, along with calculated log P (clog P) and measured aqueous solubilities (pH 1–6.5). Compound **15** demonstrated improved pharmaceutical properties compared with **1** – particularly, lower observed clearance in dog (~300× slower) and better aqueous solubility at pH 6.5 (~30× higher) even though the clog P values were very similar.

Head-to-head evaluation of these compounds was performed *in vivo* using CALU-6 tumor xenografts in mice. Nude mice bearing CALU-6 tumors were treated orally with compounds **6**, **13**, and **15** at 100 mg/kg BID for 3 days. The extent of hedgehog pathway inhibition in tumors was measured using quantitative RT-PCR of GLI1 transcription relative to tumors treated with a vehicle control (Figure 5.3). Compound **15** inhibited >90% of hedgehog target gene expression in tumor, and maintained high exposure in plasma. Tumor growth studies using a medulloblastoma allograft mouse model showed that doses of compound **15** as low as 12.5 mg/kg BID led to tumor regression [18]. Compound **15** (GDC-0449 or vismodegib) met our criteria for candidate nomination, and was more fully characterized prior to clinical testing.

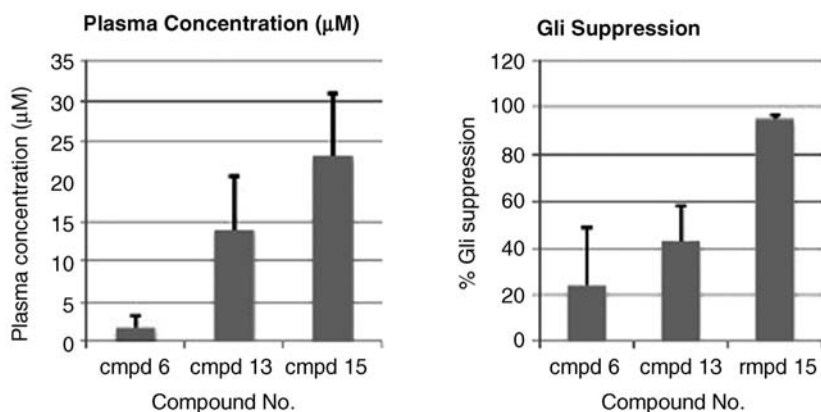


Figure 5.3 Pharmacodynamic evaluation of candidate inhibitors in CALU-6 tumors in nude mice. Plasma concentrations (a) and percentage of Gli suppression in tumors (b) following oral dosing at 100 mg/kg BID for compounds 6, 13, and 15 (sampled at 4 h following the fifth dose).

5.5

Preclinical Characterization of Vismodegib

To support the selection of vismodegib as a potential drug candidate, a battery of *in vitro* studies as well as *in vivo* pharmacokinetic studies were performed in order to characterize its absorption, distribution, metabolism, and excretion (ADME) profile. Vismodegib was assessed in hepatocyte metabolic stability studies to both confirm and augment information gathered from similar screening assays performed in liver microsomes [18]. The metabolic stability of vismodegib (1 μM) was assessed in mouse, rat, dog, monkey, and human cryopreserved hepatocytes following a 3 h incubation [20]. In mouse, rat, dog, and human hepatocytes, metabolic stability was high with the percent vismodegib remaining at the end of the incubation being 94, 88, 100, and 96%, respectively. The molecule proved to be less stable in monkey hepatocytes with only 41% vismodegib remaining at the end of the incubation period. Interestingly, the decreased stability in monkey hepatocytes proved to be consistent with observations from *in vivo* pharmacokinetic studies (Section 5.5.4).

5.5.1

Plasma Protein Binding and Blood Plasma Partitioning

In order to better understand the distribution characteristics of vismodegib, an assessment of free concentration, plasma protein binding, and blood plasma partitioning was conducted. The plasma protein binding of vismodegib was evaluated *in vitro* using mouse, rat, rabbit, dog, cynomolgus monkey, and human-pooled plasma [20]. Vismodegib is highly protein bound with the percentage bound being ≥94% in all the six species tested. Blood plasma partitioning of vismodegib was evaluated in mouse, rat, dog, cynomolgus monkey, and human-pooled whole

blood. Mean blood–plasma partition ratios ranged from 0.608 to 0.881 in all species tested, suggesting that vismodegib does not preferentially distribute into red blood cells.

5.5.2

In Vitro and Exploratory *In Vivo* Metabolism of Vismodegib

Shortly following the identification of vismodegib as a drug candidate, exploratory metabolite identification studies were performed *in vitro* using rat, dog, and human liver microsomes, and *in vivo* following oral dosing in rat and dog from collected urine [20]. Vismodegib metabolites were characterized using mass spectrometry in positive ion mode and are shown in Figure 5.4. Two oxidative metabolites (M1 and M3) were generated in rat, dog, and human microsomes. These metabolites were formed likely by oxidation of the pyridine or central 2-chlorophenyl ring. Metabolites of vismodegib were also identified in urine samples from rats and dogs. In addition to the two oxidative metabolites detected *in vitro*, another oxidative metabolite, M2, was detected. Three glucuronide conjugates of the vismodegib oxidative metabolites (M4–M6) were also detected. In general, the limited number of metabolites identified from these studies is consistent with the low turnover observed in metabolic stability studies with hepatocytes.

In order to identify the P450 enzymes responsible for the generation of M1 and M3 in human liver microsomes, vismodegib was incubated with recombinant human P450 isoforms, 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5 [20]. P450 3A4, followed by P450 3A5, was found to produce the greatest quantity of M1. P450 2C9 produced the greatest quantity of M3. Both metabolites were produced by several other P450s but to a lesser extent.

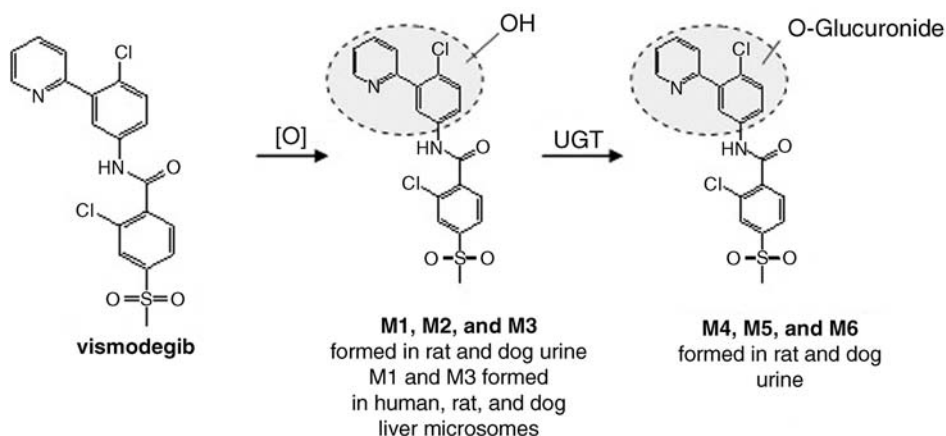


Figure 5.4 Biotransformation of vismodegib in human, rat, and dog liver microsomes and in rat and dog urine (UGT–UDP-glucuronosyltransferase).

5.5.3

Drug–Drug Interaction Potential

The drug–drug interaction potential of vismodegib against P450s was assessed in human liver microsomes using selective probe substrates [20]. Vismodegib was not a potent inhibitor of P450 isoforms 1A2, 2B6, 2D6, and 3A4/5 with IC_{50} estimates $>20\text{ }\mu\text{M}$. For P450s 2C8, 2C9, and 2C19, the inhibition constant (K_i) was determined using human liver microsomes, and paclitaxel (2C8), warfarin (2C9), and mephenytoin (2C19) as probe substrates. Estimates of K_i for vismodegib were 6.0, 5.4, and $24\text{ }\mu\text{M}$ for 2C8, 2C9, and 2C19, respectively, suggesting a moderate potential of inhibiting P450s 2C8 and 2C9. A physiologically based pharmacokinetic model (Simcyp) was used to further assess drug–drug interaction potential in order to context the *in vitro* K_i values with the anticipated *in vivo* properties of vismodegib and the targeted efficacious concentrations. Simulations were performed to assess the effect of coadministration of vismodegib with rosiglitazone, a probe substrate for P450 2C8, and S-warfarin, a probe substrate for P450 2C9. Results of the simulation indicate that coadministration of vismodegib would not substantially alter the oral exposure of these two probe substrates, and suggest that the drug–drug interaction potential of vismodegib on P450 2C8 and 2C9 is low [20]. These simulations were consistent with results of a recent drug–drug interaction study in cancer patients where coadministration of vismodegib was shown to have no effect on the oral exposure of rosiglitazone, a 2C8 substrate [21].

An additional investigation was performed to examine vismodegib's ability to inhibit P-glycoprotein using MDR1-MDCK cells and digoxin as a probe P-glycoprotein substrate [20]. The efflux ratio (B-A/A-B) using digoxin as the probe was not altered in the presence of $15\text{ }\mu\text{M}$ vismodegib, being 72 in the absence of and 81 in the presence of vismodegib, suggesting that vismodegib is not an inhibitor of P-glycoprotein.

5.5.4

Preclinical Pharmacokinetics

In order to characterize the *in vivo* pharmacokinetics of vismodegib, PK studies were performed in mouse, rat, dog, and monkeys. Table 5.4 is a summary of the single-dose pharmacokinetics in these preclinical species. Vismodegib shows good preclinical pharmacokinetic properties having low plasma clearance in all species with the exception of the cynomolgus monkey that exhibited moderate clearance. In particular, the dog had a very low plasma clearance, approximately 1% of hepatic blood flow [22]. The *in vivo* clearance estimates were consistent with results of the *in vitro* hepatocyte metabolic stability studies with vismodegib being stable in all species except for the cynomolgus monkey. The terminal half-life ($t_{1/2}$) ranged from 0.976 h in the mouse to 41.8 h in the dog. Vismodegib's volume of distribution at steady state (V_{ss}) was low to moderate in all species evaluated, being approximately total body water [22]. Following oral dosing in 0.5% methylcellulose with 0.2% Tween-80 (MCT) suspension, the

Table 5.4 Pharmacokinetics (mean $\pm$ SD) of vismodegib in the mouse ($n = 27$; three animals per time point), rat ($n = 3$ per route of administration), dog ($n = 3$ per route of administration), and monkey ($n = 3$ per route of administration).

Parameters	Mouse (IV, $n = 27$; PO, $n = 27$)	Rat (IV, $n = 3$; PO, $n = 3$)	Dog (IV, $n = 3$; PO, $n = 3$)	Monkey (IV, $n = 3$; PO, $n = 3$)
<i>Intravenous</i>				
Dose (mg/kg)	1	1	1	1
Cl (ml/(min kg))	23.0	4.65 $\pm$ 1.81	0.338 $\pm$ 0.203	19.3 $\pm$ 6.93
AUC _{inf} ((ng $\cdot$ h)/ml)	725	3980 $\pm$ 1540	60 000 $\pm$ 26 800	957 $\pm$ 387
$t_{1/2}$ (h)	0.976	1.32 $\pm$ 0.258	41.8 $\pm$ 19.8	0.581 $\pm$ 0.0922
MRT (h)	1.22	1.89 $\pm$ 0.508	62.3 $\pm$ 30.0	0.855 $\pm$ 0.101
V_{ss} (l/kg)	1.68	0.490 $\pm$ 0.0653	1.03 $\pm$ 0.119	0.984 $\pm$ 0.342
<i>Oral</i>				
Dose (mg/kg)	5	5	2	2
C_{max} (ng/ml)	311	2760 $\pm$ 1020	591 $\pm$ 97.7	162 $\pm$ 121
t_{max} (h)	1.00	0.667 $\pm$ 0.289	9.33 $\pm$ 12.7	2.00 $\pm$ 0.00
AUC _{inf} (ng $\cdot$ h/ml)	696	10 500 $\pm$ 3150	39 400 $\pm$ 5800	256 $\pm$ 112
F (%)	19.2	52.9	32.9	13.4 $\pm$ 2.07
Renal clearance (ml/(min kg))	NA ^{a)}	0.00149 $\pm$ 0.00101 ^{b)}	0.000464 $\pm$ 0.000435 ^{c)}	NA ^{d)}

AUC_{inf} = area under the concentration–time curve from zero to infinity; Cl = plasma clearance; C_{max} = highest observed plasma concentration; F = bioavailability; IV = intravenous; MRT = mean residence time; NA = not available; $t_{1/2}$ = half-life; t_{max} = time at which C_{max} occurred; V_{ss} = volume of distribution at steady state.

a) No urine was collected from mice.

b) Renal clearance was assessed for IV and PO groups.

c) Renal clearance was assessed for the IV group only.

d) GDC-0449 was not detected in urine.

bioavailability ranged from 13 to 53%. The renal clearance of vismodegib was negligible in the rat and dog, accounting for <1% of the plasma clearance. Similarly, vismodegib was not detected in monkey urine, suggestive of negligible renal excretion of the compound in this species.

5.5.5

Predicted Human Pharmacokinetics

As a final exercise in the candidate selection process, a prediction of human pharmacokinetic parameters, clearance, and volume of distribution was performed for vismodegib using simple allometry. Allometry is based on

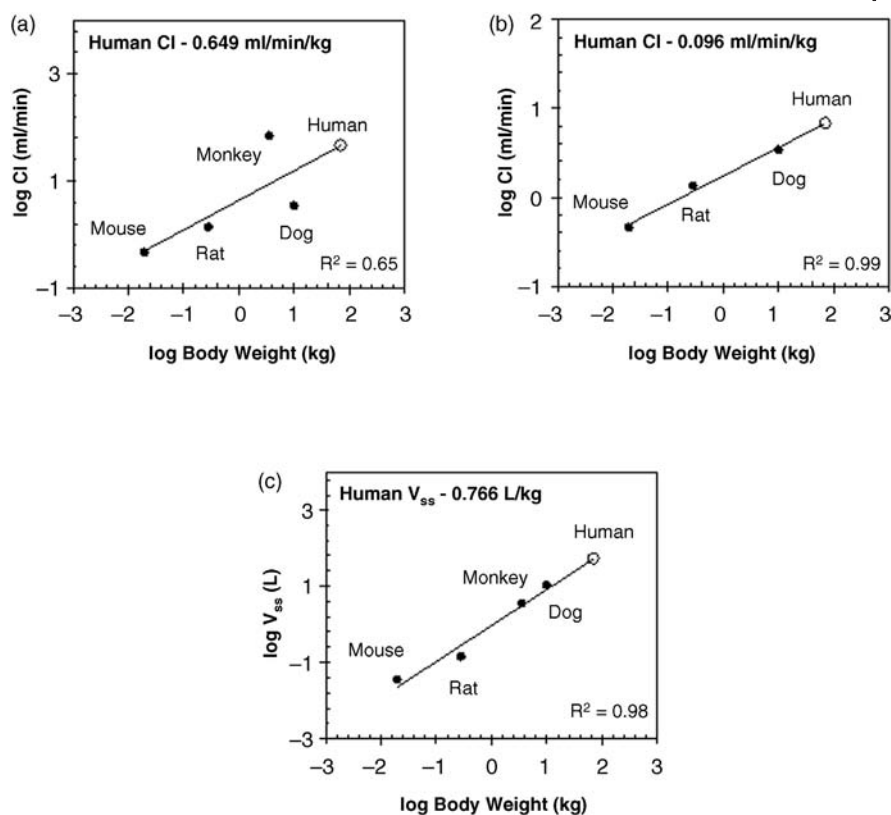


Figure 5.5 Predicted vismodegib human clearance (CI) (a and b) and volume of distribution (V_{ss}) (c) using allometric scaling.

empirical relationships between physiological parameters and body weight. Volume of distribution was similar in all preclinical species being approximately total body water and, accordingly, the strong empirical correlation yielded a V_{ss} prediction in humans that approximated total body water (0.766 l/kg) (Figure 5.5c). Using allometry to predict the rate of clearance is more challenging due to known species differences in protein binding and metabolism [23]. The approach that was taken for vismodegib was to generate two clearance predictions, one that included all species and a second that excluded the cynomolgus monkey, since that species appeared to be an outlier in hepatocyte metabolic stability studies. The allometry plot including the monkey (Figure 5.5a) showed a clear deviation of this species. Regardless of the approach, the clearance of vismodegib was predicted to be low ($\leq 3\%$ of human hepatic blood flow [22]) in humans, being 0.096 ml/(min kg) (Figure 5.5b) excluding the cynomolgus monkey data, and 0.649 ml/(min kg) (Figure 5.5a) when data from all preclinical species were included. Human hepatocyte data

were not used to make clearance predictions as the certainty in the slope of the percentage of vismodegib remaining versus time plot required for calculation of predicted hepatic clearance was difficult to ascertain due to the high stability of the compound in human hepatocytes. Based on clearance and volume of distribution predictions derived from allometry, vismodegib was predicted to have a human half-life between 14 and 92 h.

5.5.6

Summary

Overall, the preclinical properties of vismodegib were favorable and suggested that the clearance in humans would be low. Good oral bioavailability estimates from mouse, rat, and dog PK studies suggested that the compound would be well absorbed and would have good exposure in humans provided that the hepatic clearance was low. Based on the *in vitro* P450 data and the simulations performed using Simcyp, the potential for vismodegib to cause a significant drug–drug interaction was considered to be low. Finally, vismodegib was not a potent inhibitor of P-glycoprotein. Based on the favorable preclinical profile, vismodegib was advanced to phase I clinical development.

5.6

Vismodegib Clinical Experience in Phase I

During phase I clinical studies, vismodegib was administered to 66 cancer patients, including 33 patients with metastatic or locally advanced basal-cell carcinoma, at daily oral doses of 150, 270, and 540 mg [24,25]. There appeared to be no dose dependency in the oral exposure of vismodegib at steady state based on a comparison made between the 150 and 270 mg dose groups. A median steady-state concentration of 19.8 μM was observed for the 150 mg treatment group versus 15.9 μM observed for the 270 mg treatment group. The median maximal plasma level in all patients was 23.0 μM and the median steady-state concentration was 16.1 μM . The reported median time to steady state was 14 days. A very consistent steady-state plasma concentration of vismodegib was maintained throughout the treatment period with no apparent decline. This observation is in line with the long reported half-life in humans ranging from 6.5 to 14 days [26]. The excellent oral exposure and observed pharmacokinetics in patients was consistent with what was anticipated based on our preclinical characterization.

No dose-limiting toxicities or drug-related grade 5 adverse events (AEs) were observed during this study, and only one grade 4 AE of asymptomatic hyponatremia occurred during the study period in which some patients were treated for up to 19 months. Tumor responses were observed in 18 of 33 patients, which included nine metastatic patients and nine patients with locally advanced tumors (Figure 5.6). Tumor response was measured by both radiological evaluation and physical examination. Of



Figure 5.6 Vismodegib treatment in patients with locally advanced basal-cell carcinoma. Panel (a) is a patient with basal-cell nevus syndrome, at baseline (left) and after 5 months of treatment (right). Panel (b) is a

patient with lesions on the face, at baseline (left) and following 2 months of treatment (right) [24]. Copyright 2009 Massachusetts Medical Society. Reprinted with permission.

the remaining 15 patients, disease stabilization was observed in 11 patients that lasted up to 10.8 months, and 4 patients had progressive disease as the best response.

Pretreatment tissue specimens from 25 of 26 patient tumors showed high *GLI1* mRNA expression levels as measured by quantitative RT-PCR [24]. These levels are higher than those seen in normal skin or lung samples and are consistent with *GLI1* expression associated with cutaneous BCCs. This observation offers additional support to the proposal that hedgehog pathway activity is the molecular mechanism responsible for sporadic BCC.

Following the completion of a registrational study, in January 2012, vismodegib (ErivedgeTM) became the first drug to be approved by the FDA for the treatment of locally advanced or metastatic BCC [27]. A recent phase II study demonstrated that vismodegib can also reduce the tumor burden and slow the growth of new lesions in patients with Gorlin syndrome [28].

These results demonstrate the power of new therapeutic approaches to cancer treatment, in which specific agents block the aberrant protein target responsible for disease progression. Vismodegib, a potent, selective inhibitor of the hedgehog pathway, has shown promise in metastatic and advanced basal-cell carcinomas.

Efforts are ongoing to examine the mechanisms by which tumors might develop resistance to this therapy, and the possible utility of this new drug in treating earlier, less severe forms of BCC.

Acknowledgments

The authors would like to thank the patients and their families who participated in clinical studies of vismodegib, and the research and development teams for their effort guiding the discovery and clinical development of this molecule. Vismodegib was discovered by Genentech and was jointly validated through a series of preclinical studies performed under a collaborative agreement between Genentech, Inc. (South San Francisco, CA) and Curis, Inc. (Lexington, MA).

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6

G-Quadruplexes as Therapeutic Targets in Cancer

Stephen Neidle

6.1

Introduction

The concept of directly targeting individual disease-related genes as a therapeutic strategy is not new. The underlying challenges for all DNA-interacting molecules are selectivity and the need to minimize or even remove the possibility of generalized DNA-mediated toxicity. There is now an extensive literature on small molecules capable of directly “reading” DNA sequences and thus being able to discriminate between different genes. Much effort has been focused on the DNA minor groove [1,2], with the development of polyamides being especially noteworthy. These molecules have now advanced to the point where the recognition rules for many desired sequences are established and monomeric units can be assembled together in order to read a target sequence with high selectivity and affinity (see Ref. [3] for a recent example). Polyamides are inherently large molecules and this, together with the particular pharmacological challenges of the polyamide backbone, has meant that few such compounds have progressed to *in vivo* evaluation, although ways of overcoming some of the delivery problems are now becoming more successful [4].

This chapter discusses an alternative strategy for nucleic acid targeting that is based on the ability of certain guanine-rich sequences to form higher order arrangements that are fundamentally distinct from the double helix architecture.

6.2

Quadruplex Fundamentals

It has long been known by nucleic acid physical chemists that guanine-rich DNA and RNA, and even simple guanine-containing mononucleotides, have an innate tendency to aggregate and form gel-like substances. In 1962, Gellert *et al.* used fiber diffraction methods (akin to those used 10 years earlier to

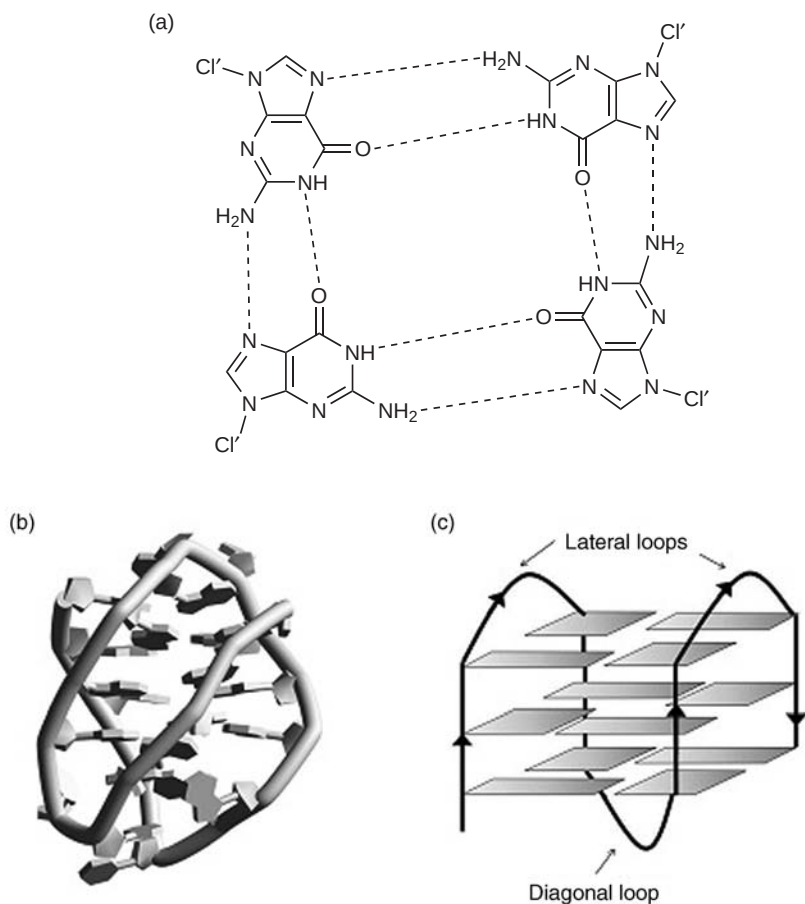


Figure 6.1 (a) The G-quartet, the core hydrogen-bonding motif of quadruplex nucleic acids, showing the hydrogen bonding between the four in-plane guanine bases. (b) Cartoon view of the NMR-derived structure [10] of the intramolecular human telomeric DNA quadruplex structure, determined in Na⁺

solution (PDB ID 143D). The backbone is shown as a smooth coil and bases are shown as solids. (c) A schematic view of the structure, with guanine bases shown as shaded parallelograms. The two types of loops present in this structure are shown.

determine the structure of the DNA double helix) to show that 5'-guanosine monophosphate forms a four-stranded helix [5], and suggested that the strands are held together by hydrogen bonding between guanine bases (Figure 6.1a), forming the so-called G-quartet (also termed the G-tetrad). Subsequent studies on poly(G) and other polymers confirmed this fourfold helical arrangement [6,7], and a range of biophysical studies (see, for example, Refs [8,9]) have also shown that the presence of an alkali metal ion is essential for structure formation and stability.

The DNA sequences at the ends of eukaryotic chromosomes, termed telomeres, comprise tandem repeats of simple guanine-rich sequences – d(TTAGGG) in humans [11,12]. These can also self-associate to form higher order arrangements comprising the G-quartet motif [13,14]. Oligonucleotides containing such sequences can fold into discrete structures, termed quadruplexes [15] (Figure 6.1b and c), which also have a metal ion requirement. X-ray crystallographic and NMR studies have established the three-dimensional structures for a number of quadruplexes (reviewed in Ref. [16]), which are outlined in Section 6.7.

6.3

Genomic Quadruplexes

The realization that quadruplex-forming sequences could exist elsewhere than in telomeric DNA, predates by some way the determination of the sequences of the human and other genomes. Such quadruplex-forming sequences had been previously identified, for example, in the promoter region of the *c-myc* oncogene [17–19] and in a region of the retinoblastoma susceptibility gene [20]. However, the knowledge of the human genome sequence enabled a systematic search for such sequences to be made. Two independent informatics studies [21,22] employed distinct algorithms but with the same generalized quadruplex search sequence

$$G_m X_n G_m X_o G_m X_p G_m,$$

where m is the number of G residues in each short G-tract and X_n , X_o , and X_p can be any combination of residues, including guanines. This general sequence does not assume that all the G-tracts are of equal length since guanines in the intervening sequences can be immediately adjacent to a G-tract. In both studies, limits were set for the length of the G-tracts and the intervening sequences (loops) such that $3 \leq m \leq 5$ and the number of nucleotides in any intervening sequence X were between 1 and 7. Over 350 000 occurrences were found – it must be emphasized that these are sequence identifications and it is not known how many of these correspond to thermodynamically stable quadruplex arrangements. Of this number, some 151 000 are within genes and almost all the rest are intergenic. Unsurprisingly, only some 14 000 are within exons. Subsequent informatics studies have revealed that there is a significant overrepresentation of putative quadruplex-forming sequences (see, for example, Refs [23–26]). Other informatics studies have extended quadruplex location to nonhuman species [27]. Overrepresentation occurs, in particular,

- i) in promoter sequences, especially in oncogenes and in a number of genes involved in cellular proliferation [28]. Examples reported to date include the *c-myc* [17–19], *c-kit* [29,30], *K-ras* [31,32], and *RET* [33] oncogenes, the HIF [34,35], androgen receptor [36], and HSP90 [37] genes, and the platelet-derived growth factor (PDGF) [38] and vascular endothelial growth factor (VEGF) [39], and

- ii) in 5'-untranslated RNA sequences [40]. Examples of genes containing such sequences include the estrogen receptor [41], the MT3 matrix metalloprotease gene [42], and the *bcl2* gene [43].

The underlying therapeutic concept that has been developed [44] is that stabilizing these quadruplexes with a small-molecule ligand will lead to inhibition of transcription (promoter quadruplexes) or translation (5'-UTR quadruplexes) of the gene involved, and that this would, by implication, confer therapeutic advantage if the gene product is critically involved in maintaining the malignant phenotype, or if it is involved in metastatic disease. The topological challenge [45] of unwinding duplex DNA at and around a quadruplex sequence may be counterbalanced by the additional stabilization produced by quadruplex formation – these highly stable tertiary structures are further stabilized by a bound ligand. A number of studies have reported the effects of ligand targeting of promoter quadruplex *in vitro* and in cells, with significant correlations being found with quadruplex binding in some instances, although the evidence that the inhibitory effects are directly related to quadruplex binding has been much more challenging to obtain. Thus, several studies have shown that the *c-kit* oncogene can be effectively downregulated in GIST (gastrointestinal stromal tumor) cells by quadruplex-binding small molecules [46–48], including the cells that are resistant to the standard treatment, Gleevec, which targets the c-kit kinase domain. A recent noteworthy report on the quadruplex-binding natural product telomestatin [49] indicates that it has activity against patient-derived glioma stem cells, and cDNA microarray data indicate that it down-regulates the *c-myc* oncogene – the assumption is that telomestatin acts by targeting the *c-myc* promoter quadruplex. A cautionary note struck by a recent study [50] using a pair of *c-myc* promoter +/- cell lines has demonstrated that the mode of action of some quinoline derivatives does not actually involve direct targeting of this quadruplex, but is the result of a (as yet undetermined) secondary effect even though these compounds were previously indicated to be *c-myc* quadruplex binders and *c-myc* transcriptional inhibitors. This suggests that observations of *in vitro* quadruplex stabilization and transcriptional inhibition may need to be augmented by more detailed molecular studies before firm mechanistic conclusions involving quadruplexes should be drawn.

6.4

Quadruplexes in Human Telomeres

The role of the telomeric DNA–protein complex in eukaryotic cells is to protect chromosomal ends from unwanted degradation and chromosomal fusions [51,52]. The extreme ~100–200 nucleotides at the 3'-end of human telomeric DNA are distinguished by being single stranded [53], albeit associated with telomere-specific proteins such as the single-stranded binding protein hPOT1 [54,55]. Due to the inability of the DNA replication machinery to fully replicate telomeric DNA ends in somatic cells, telomeric DNA becomes progressively shortened by ~50–100

nucleotides for each round of cell division. This telomere attrition eventually results in a response in which cells cease replication and enter the senescent state as a result of upregulation of the p21^{WAF} and p16^{INK4a} proteins and the consequent increase in p53 and pRb (retinoblastoma) phosphorylation, followed by upregulation of their pathways. Cellular senescence is frequently a prelude to apoptosis [56,57]. By contrast, cancer cells do not undergo telomere attrition, since in the majority of these cells (~85%), expression of the reverse transcriptase enzyme telomerase occurs [58,59]: this enzyme specifically catalyzes the synthesis of telomeric DNA d(TTAGGG) repeats from nucleotide triphosphates and thus maintains telomere length [52,60–62]. Those cancer cells that do not express telomerase maintain telomere length by so-called ALT (alternative) mechanisms, involving telomeric DNA strand recombination [63].

Telomerase is not significantly expressed in normal somatic cells, suggesting that there is a large therapeutic window for telomerase inhibitors. Furthermore, inhibition of telomerase by, for example, antisense RNA, a small-molecule inhibitor (2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid: BIBR1532), or siRNA, results in selective cancer cell death both in cells and *in vivo*, strongly supportive of the concept of telomerase as a therapeutic target [64–66], with potential wide applicability across a number of major solid and hematological cancers. Telomerase expression is now well established as a key hallmark of cancer [67], and especially of initial tumorigenic events. However, the important caveat to this is that senescence is initiated only once mean telomere length becomes critically short, requiring a considerable time lag for this to occur. For MCF7 human breast carcinoma cells with a mean telomere length of ~4.3 kb, about 100 nucleotides are lost per round of replication; so about 30–40 rounds of replication are required. This time lag was considered to be a major practical obstacle to the use of telomerase inhibitors in the treatment of human cancers, and most pharmaceutical companies dropped their initial interest in the field once this became apparent.

While the behavior of more conventional telomerase inhibitors, notably the Boehringer compound BIBR1532 or those binding at the active site of the telomerase reverse transcriptase (hTERT) domain, appeared to follow this time lag model, the development of an alternative approach based on considerations of the normal substrate for telomerase, the 3'-end of human telomeric DNA, indicated that the time lag is not an inevitable consequence of inhibition, as described below for trisubstituted acridines. The initial step in the catalytic cycle of telomerase is the capture and recognition of this end by a template of complementary sequence within the endogenous RNA domain. An important observation was that folding of telomeric DNA into a quadruplex structure effectively inhibits telomerase activity and telomere elongation is halted [68]. This folding can be driven by small molecules that stabilize telomeric quadruplexes, as was first demonstrated with a series of disubstituted amidoanthraquinone derivatives [69].

A very large number of quadruplex-binding ligands have subsequently been devised (reviewed in Refs [70–73]; see also <http://www.g4ldb.org/ci2/index.php> for a comprehensive listing), based on these simple structural principles, representing a considerable diversity of chemical type since the heteroaromatic group

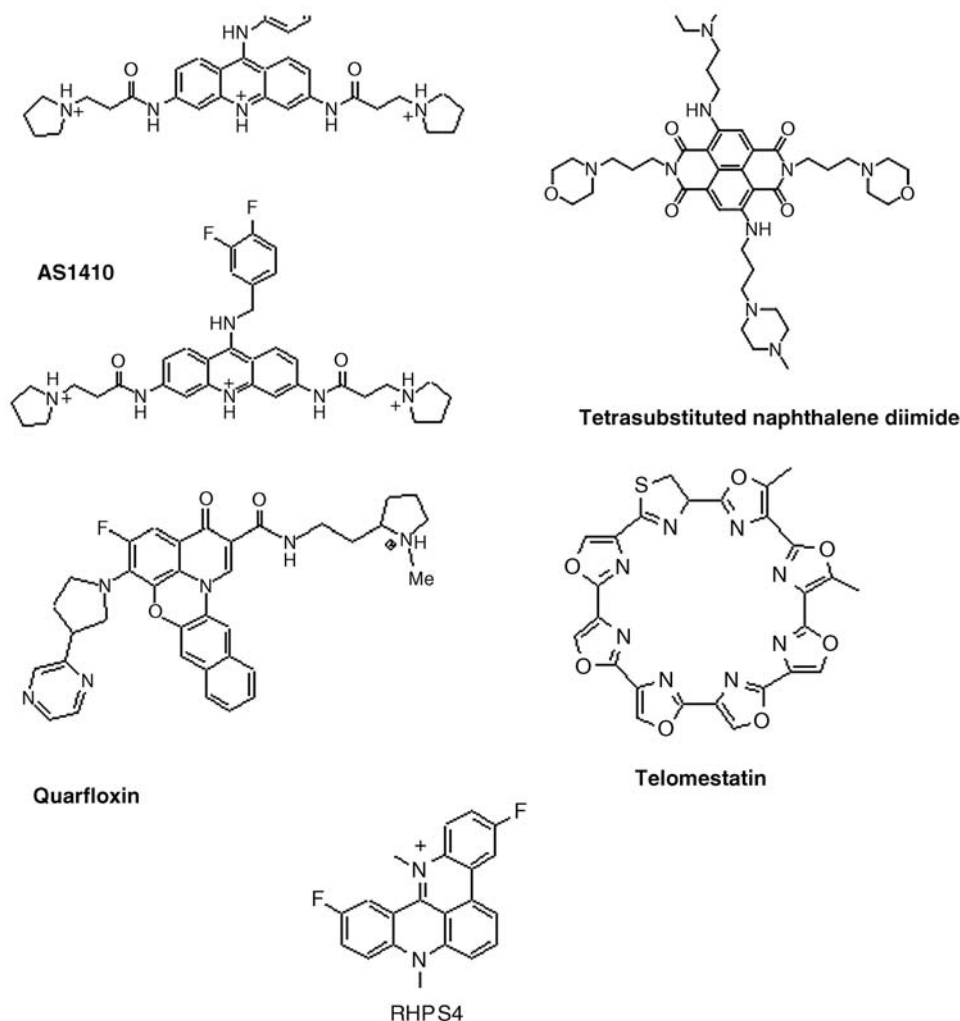


Figure 6.2 Structures of representative quadruplex-binding small molecules.

requirement can be met on the one hand by extended polycyclic moieties and on the other by conjugated yet separated ring systems. Some representative examples are shown in Figure 6.2. The overall structural features for small-molecule quadruplex binding have been defined as (i) the possession of an extended planar heteroaromatic surface, and (ii) flexible side chains terminating in cationic groups. A further design requirement has been that these compounds are able to discriminate between quadruplex and duplex DNA, so that in biological terms, any unwanted generalized cytotoxicity to normal cells would be minimized. Disubstituted acridines were an early molecular design improvement over the first-generation amidoanthraquinones, which had shown equal affinity for quadruplex

and duplex DNA, and also had suboptimal physicochemical properties [74]. The acridines have inherently superior aqueous solubility, in large part as a consequence of the cationic ring nitrogen atom, which has enabled cell-based experiments to be more readily undertaken [75]. A further step was taken with the design of trisubstituted acridines, with an anilino group at the 9-position of the acridine ring [76,77]. Here the key design concept was that the introduction of a third substituent would impart added selectivity in favor of quadruplex binding since the two alkylaminoalkyl substituents bind in one of the two grooves of duplex DNA each and the third substituent would be able to bind into one of the two additional grooves of a quadruplex. This prediction was borne out by experiment, with a 10-fold difference in affinity between the two nucleic acid types, as well as enhanced telomerase inhibitory activity and cellular selectivity in favor of telomerase (+) cell types. Cell growth inhibition studies with the lead compound, BRACO-19, showed that senescence occurred significantly more rapidly, over a few days, than had been predicted by the telomere attrition model, even though telomere shortening was still slow [78]. This finding was borne out by subsequent tumor xenograft experiments [79].

Studies with BRACO-19 [80,81] and with another quadruplex-binding acridine-based compound, RHPS4 [82–85], as well as with the acyclic G-quadruplex ligand pyridostatin [86] have shown that the observations of rapid senescence and apoptosis in many cancer cell types are due to the induction of selective DNA damage responses, as a result of the upregulation of, in particular, the p21/p16^{INK4a} kinases, p53, PARP, and ATM/ATR pathways. The natural product telomestatin, also a high-affinity quadruplex-binding compound, has a similar profile of DNA damage in cells [87]. The cause of these pathways being rapidly upregulated is generally believed to be the exposure of 3'-telomere ends, which can occur by quadruplex formation displacing bound proteins, notably hPOT1 [88] and telomerase itself, which as well as its catalytic function is believed to physically associate with the 3'-ends, in a capping function.

6.5

Quadruplexes as Anticancer Targets – Evidence from *In Vivo* Studies

In vivo activity in xenograft cancer models has not been reported for many telomeric quadruplex-binding small molecules, with most data being for BRACO-19 [79], RHSP4 [83,89,90], and telomestatin [91] (Figure 6.2 and Table 6.1). None of these compounds has progressed as yet beyond the experimental stage into clinical trial. There is little published information on their ADME, toxicological, and pharmacokinetic properties but the extended planar structures and high net positive charge of BRACO-19 and RHPS4 suggest that properties such as half-life, tumor penetration, and protein binding are likely to be suboptimal. Also, and most importantly, none of these compounds are entirely specific for quadruplex nucleic acids – they all have some affinity for duplex DNA, suggesting that nonspecific toxicity may restrict

Table 6.1 The results of *in vivo* tumor xenograft experiments to evaluate the antitumor efficacy of selected G-quadruplex small molecules.

G4 ligand	Xenograft model	Tumor response	Days for complete response
Telomestatin	U937 human lymphoma	80% tumor shrinkage	21
BRACO-19	UXF1138L human uterine carcinoma	96% tumor shrinkage + some complete remissions	28
BRACO-19	A431 human epithelial carcinoma	Not significant	—
Quarfloxin	MDA-MB-231 human breast cancer	50% tumor shrinkage	37
Quarfloxin	MIA PaCa-2 human pancreatic cancer	59% tumor shrinkage	35
RHPS4	UXF1138L human uterine carcinoma	30% tumor shrinkage	28
RHPS4	M14, LP, LM melanoma	40–51% tumor weight reduction	15
RHPS4	CG5 breast carcinoma	75% tumor shrinkage	30

Further details and primary references are given in Ref. [96].

their therapeutic windows to narrow ranges. This is the case for BRACO-19 and its more recent analog AS1410 [92]. This latter compound was ultimately abandoned as a preclinical candidate, in part for this reason. Data from the authors' laboratory on the tetrasubstituted naphthalene diimide derivative BMSG-SH-3 [93] are also included. This particular compound has high *in vitro* affinity for a number of telomeric and promoter quadruplexes and is a potent inhibitor of cell growth in several cancer cell lines. It also shows *in vivo* anticancer activity in the MIA PaCa-2 pancreatic cancer xenograft model [94]. In accordance with the broad profile of quadruplex binding shown *in vitro*, preliminary data from treated tumors suggest that BMSG-SH-3 has several cellular quadruplex targets in addition to telomeric DNA, such as those in the HSP90 and hTERT promoters, with downregulation of these genes being observed. It should be noted that such observations are by no means proof of quadruplex targeting – this will have to wait for the application of the methodology that has recently been successful in identifying quadruplex targets in individual chromosomes [95], to treated tumor material.

The available xenograft data are mostly restricted to solid carcinomas (where promising single-agent activity has been noted for several compounds) and there is little publicly available data on hematological cancers. The fluoroquinolone derivative quarfloxin (CX-3543) [97] is the sole quadruplex-binding compound to have advanced to clinical trials (www.cylene.com), which were subsequently abandoned even though the compound appeared to have an acceptable toxicological profile and a significant therapeutic window. It is likely to reenter clinical evaluation with a new license holder (www.tetragene.com). Quarfloxin was initially suggested

to be targeting a *c-myc* promoter quadruplex, but is now believed to function by selectively disrupting nucleolin/rDNA quadruplex complexes.

6.6

Native Quadruplex Structures

Early crystallographic and NMR structure determinations on quadruplex-forming sequences from telomeres of lower organisms such as *Oxytricha nova* have demonstrated that these structures are formed by the stacking of individual G-quartets, with typically three or four in an individual quadruplex [98–100]. The essential sodium or potassium ions are held in the central channel of the structures, typically forming eightfold bipyramidal prismatic coordination with O₆ oxygen substituents of the guanine bases in the quartets.

Quadruplexes can be formed from a single oligonucleotide chain (monomolecular or sometimes termed intramolecular), from two chains (bimolecular), or from four chains (tetramolecular) [101]. Under special sequence circumstances, three-chain quadruplexes can be formed [102]. The sequences that intervene between the G-tracts form loops that help to hold the core together. Three types of loops have been described: lateral, diagonal, and propeller (also termed chain reversal). A variety of topologies are possible depending on the connectivity of the loops and the polarity of the oligonucleotide chain. This is illustrated by the diverse topologies that have been found experimentally (Figures 6.1b and c and 6.3a and b) for human telomeric intramolecular quadruplex sequences formed from four repeats of the DNA sequence d(TTAGGG) [10,103–111]. This diversity is a reflection of a number of factors, in particular (i) the effects of small changes in 5'- and 3'-flanking sequences that appear to stabilize particular structures, (ii) differing metal ions, (iii) quadruplex concentration, and (iv) the method of structural investigation, that is, NMR versus crystallographic, with circular dichroism, also normally a dilute solution technique, often being used to assign topology. What is the “preferred” topology that is most relevant to a cellular environment remains a matter of continuing debate [111,112], although it is clear that simple comparisons may be misleading. For example, NMR, which is normally a dilute solution technique, has revealed several distinct, though related, folds, mostly with flanking sequences helping to stabilize them. On the other hand, crystals of quadruplexes, in common with those of other biological molecules, are heavily hydrated and have shown just one fold type. The crystal structures may be relevant to quadruplex folds in concentrated solution, and may be closer to the crowded environment in the cell nucleus. In addition, small-molecule compounds have been reported to stabilize particular topologies of the human telomeric quadruplex, although these findings have to be tempered with caution since assignments are most often made by dilute-solution circular dichroism (see the caveat above).

It has long been assumed that telomeric DNA is not transcribed. This dogma was overturned in 2009 by the surprising finding that it is, into shorter telomeric

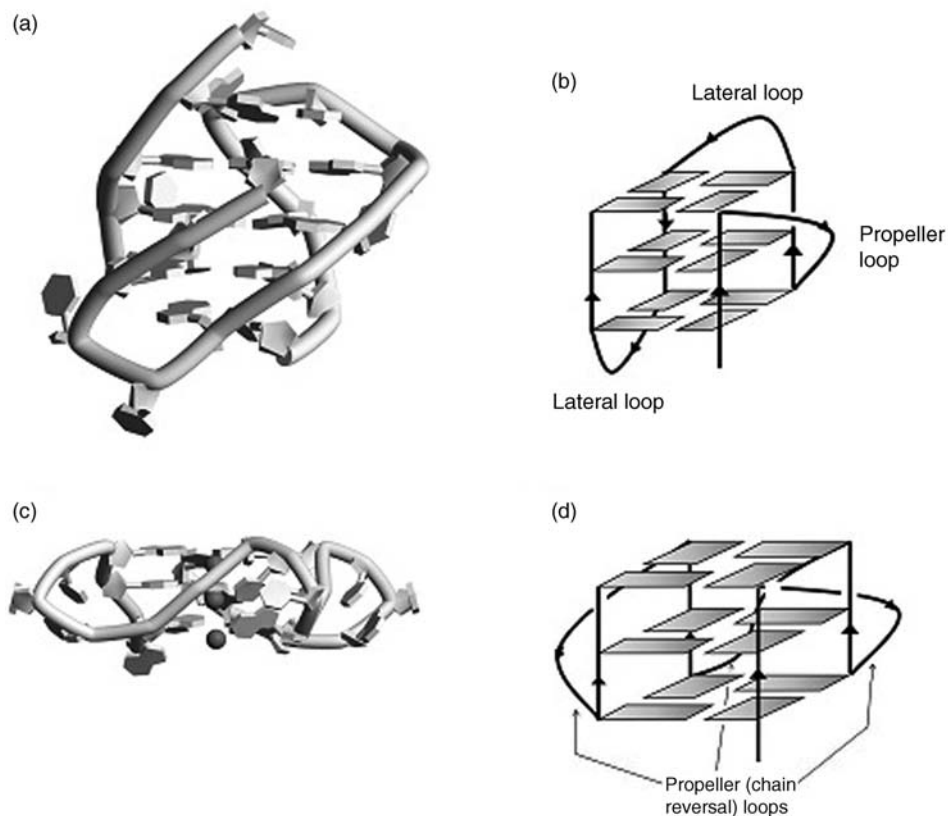


Figure 6.3 (a, b) Cartoon and schematic representations of one of the mixed parallel/antiparallel topologies formed by an intramolecular human telomeric DNA quadruplex in K^+ solution [104,107], as determined by NMR methods. (PDB ID

2GKU). (c and d) Cartoon and schematic representations of the parallel topology formed by an intramolecular human telomeric DNA quadruplex in K^+ solution [109]. (PDB ID 1KF1).

noncoding RNA fragment transcripts, termed TERRA (telomeric repeat-containing RNA) sequences [113–115]. NMR, biophysical, and crystallographic studies on TERRA sequences have found that they form highly stable parallel quadruplexes, which are not polymorphic, unlike their DNA counterparts [116–119]. The structural studies have shown that the 2'-hydroxyl groups play an essential role in stabilizing these RNA quadruplexes. The biological function of TERRA sequences has yet to be fully elucidated, although it appears likely that they play a role in regulating telomere length [120,121] and thus may be potential drug targets in their own right.

Crystal and NMR structures of single quadruplexes comprising four telomeric d(TTAGGG) repeats can only approximate some of the features of higher order structures that may be formed from, for example, eight or 12 telomeric repeats, as

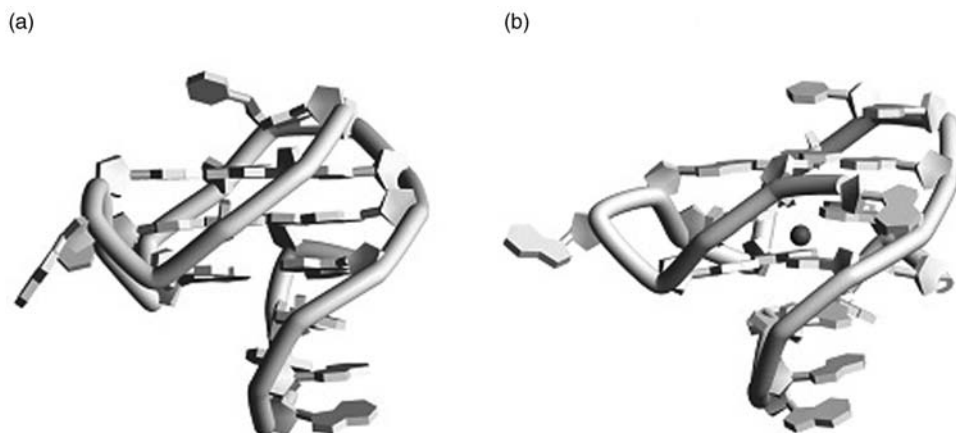


Figure 6.4 (a) NMR (PDB ID 2O3M) [126] and (b) crystal structure (PDB ID 3QXR) [127] of the *c-kit1* promoter quadruplex, viewed in the same orientation. The two sequences are identical and the differences between the structures are in the detail, not in the overall topology.

present in the telomeric DNA single-stranded overhang. There is no direct structural data on such structures, although model building has used both parallel and mixed antiparallel/parallel (3 + 1) quadruplexes as starting points [122,123]. Figure 6.4 shows a structure resulting from a molecular dynamics simulation study based on three linked individual parallel quadruplex units. Regardless of the topology of the starting point quadruplexes, it is apparent that the space between units represents new opportunities for the molecular design of small-molecule stabilizers, especially to devise those with high selectivity for this particular type of quadruplex compared with the single quadruplex of many promoter sequences. There is some biophysical data on ligand–quadruplex multimer interactions, which indicate that binding occurs at the interfaces between individual monomer quadruplex units [124,125].

The current database set of quadruplex structures (Table 6.2) represents only a very small number of the possible structural types. In view of the very large number of possible quadruplexes suggested by bioinformatics [21,22], it is important to have confidence in the biological relevance of a particular molecular structure and thus in the structural methodology being used for a given quadruplex. The crystal structures of two promoter quadruplexes have been determined by X-ray crystallography and rather more by NMR methods (Table 6.2). In the one instance [126,127] where a quadruplex has been examined by both techniques (one of the two quadruplexes encoded in the promoter sequence of the *c-kit* gene), the fold is seen to be highly conserved between crystal and dilute solution (Figure 6.5). This quadruplex also illustrates an important principle that is likely to be relevant to many other genomic quadruplexes: one of the loops in this *c-kit* quadruplex comprises the sequence CGCT, which does not behave in the same way as the TTA loops of human telomeric quadruplexes, which regardless of topology, form discrete loops between successive G-quartets. Instead, in the *c-kit*

Table 6.2 Selected crystal and NMR structures of DNA and RNA human telomeric and promoter quadruplexes (shown shaded in pink), together with selected ligand complex crystal structures (shown shaded in blue).

Sequence	Description	Resolution (Å)	PDB ID	Loops
d[U _{Br} AG ₃ U _{Br} TAG ₃ T]	Native telomeric DNA	2.40	1K8P	All P
d[AG ₃ (T ₂ AG ₃) ₃]	Native telomeric DNA	2.10	1KF1	All P
r(U _{Br} AG ₃ U ₂ AG ₃ U)	Native telomeric RNA	2.20	3IBK	All P
d[AG ₃ (T ₂ AG ₃) ₃]	Native telomeric DNA	NMR	143D	1 × D, 2 × L
d[G ₂ T ₂ AG ₃ T ₂ AG ₃ T + TAG ₃ U]	Native telomeric DNA	NMR	2AQY	1 × P, 2 × L
d[T ₂ G ₃ (T ₂ AG ₃) ₃ A]	Native telomeric DNA	NMR	2GKU	1 × P, 2 × L
d[A ₃ G ₃ (T ₂ AG ₃) ₃ AA]	Native telomeric DNA	NMR	2HY9	1 × P, 2 × L
d[TAG ₃ (T ₂ AG ₃) ₃]	Native telomeric DNA	NMR	2JSM	1 × P, 2 × L
d[(T ₂ AG ₃) ₄ TT]	Native telomeric DNA	NMR	2JPZ	2 × L, 1 × P
d[TAG ₃ (T ₂ AG ₃) ₃ TT]	Native telomeric DNA	NMR	2JSL	2 × L, 1 × P
d[TAG ₃ T ₂ AG ₃]	Porphyrin TMPyP4 complex	2.09	2HRI	All P
d[TAG ₃ T ₂ AG ₃ T]	Acridine complex	2.50	3CE5	All P
d[TAG ₃ T ₂ AG ₃ T]	ND complex	2.20	3CCO	All P
d[TAG ₃ (T ₂ AG ₃) ₃]	ND complex	2.10	3CDM	All P
r(UAG ₃ U ₂ AG ₃ U)	RNA acridine complex	2.60	3MIJ	All P
d[TAG ₃ T ₂ AG ₃ T]	Acridine complex	3.20	3QCR	All P
d[AG ₃ (T ₂ AG ₃) ₃]	ND complex	2.30	3SC8	All P
d[AG ₃ (T ₂ AG ₃) ₃]	ND complex	2.10	3T5E	All P
d[AG ₃ T ₂ AG ₃ T ₂]	Salphen complex	2.40	3QSC	All P
d[AG ₃ T ₂ AG ₃ T ₂]	Salphen complex	2.40	3QSF	All P
d[AG ₃ (T ₂ AG ₃) ₃]	ND complex	1.95	3UYH	All P
d[G ₃ (T ₂ AG ₃) ₃]	ND complex	2.40	4DA3	All P
d[G ₃ (T ₂ AG ₃) ₃]	ND complex	2.75	4DAQ	All P
d[AG ₃ (T ₂ AG ₃) ₃]	Berberine complex	2.30	3R6R	All P
d[AG ₃ (T ₂ AG ₃) ₃]	Mesoporphyrin complex	2.15	4G0F	All P
d[AG ₃ (T ₂ AG ₃) ₃]	Mesoporphyrin complex	1.65	4FXM	All P
d[T ₂ AG ₃ T] ₄	Human telomeric + RHPS4 acridine	NMR	1NMZ	All P
d(AG ₃ AG ₃ CGC ^{Br} UG ₃ AG ₂ AG ₃)	Native <i>c-kit</i> 1 promoter	1.62	3QXR	All P
d(G ₃ CG ₃ GAG ₅ A ₂ G ₃ A)	Native <i>b-raf</i> promoter	1.99	4H29	1 × L, 2 × P
d[TGAG ₃ TG ₂ TGAG ₃ TG ₄ A ₂ G ₂]	Native <i>c-myc</i> promoter	NMR	2A5P	All P
d[TGAG ₃ TG ₃ TAG ₃ TG ₃ TA ₂]	Native <i>c-myc</i> promoter	NMR	1XAV	All P
d[G ₃ CGCG ₃ AG ₂ A ₂ T ₂ G ₃ CG ₃]	Native <i>bcl-2</i> promoter	NMR	2F8U	2 × L, 1 × P

d[AG ₃ AG ₃ CGCTG ₃ AG ₂ AG ₃]	Native <i>c-kit1</i> promoter	NMR	2O3M	All P
d[CG ₃ CG ₃ CGCGAG ₃ AG ₃ T]	Native <i>c-kit2</i> promoter	NMR	2KJ2/ 2KJ0	All P
d(G ₄ CG ₄ CG ₄ CG ₄ T)	Native <i>ret</i> promoter sequence	NMR	2L88	All P
d[TGAG ₃ TG ₂ IGAG ₃ TG ₄ A ₂ G ₂ A]	<i>c-myc</i> + TMPyP4 porphyrin	NMR	2A5R	All P
d[TGAG ₃ TG ₃ TAG ₃ TG ₃ TA ₂]	<i>c-myc</i> + quindoline	NMR	2L7V	All P

P: parallel, L: lateral, D: diagonal. Further information on individual structures is available from the Protein Data Bank (<http://www.pdb.org/pdb/home/home.do>).

quadruplex, one of the guanines in this loop actively participates in a G-quartet assembly, with the effect that the overall fold is complex and not predictable on the basis of telomeric quadruplex folding. Furthermore, in the *c-kit* quadruplex, 19 out of 22 nucleotides are actively involved in stabilizing the fold, with an informatics search showing that it has a unique occurrence in the human genome [128]. Interestingly, as a result of the 19/22 requirement, it is evident that the simple sequence rule outlined above does not always guarantee that a given putative quadruplex sequence will always form a stable quadruplex, especially when it is likely that there are one or more guanines present in the loops. A picture is emerging that quadruplexes have distinct structural complexity, and that a given promoter or RNA quadruplex (for example, in a 5'-UTR sequence [40]) may have a unique sequence and hence unique structural features that make it suitable as a potential druggable target.

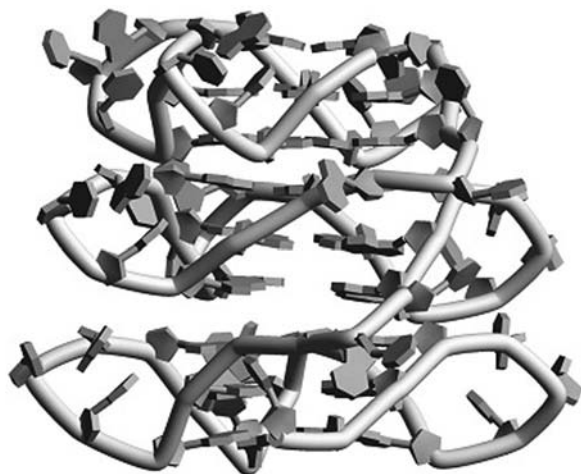


Figure 6.5 Molecular dynamics-derived structure [122] of a human telomeric DNA quadruplex multimer incorporating three parallel quadruplex units, each taken from the crystal structure [109].

To date, it is not possible to predict *ab initio* the overall topology of a given quadruplex primary sequence except when it is highly homologous to a known structure. Examination of the known quadruplex NMR and crystal structures, taken together with molecular dynamics simulations and systematic experimental studies on loop size effects [129–131], does show that the presence of single-nucleotide loops (especially when there are more than one such loops) imparts a strong preference for parallel architectures. A single nucleotide is unable to span the distance required for diagonal and lateral loops, whereas it can be accommodated by a propeller loop.

6.7

Quadruplex–Small-Molecule Structures

A number of crystal structures of small-molecule complexes with human telomeric bimolecular and intramolecular complexes are now available, together with several NMR studies using simpler quadruplexes as models for human telomeric quadruplexes. Interestingly, all these have the parallel topology found in the native crystal structures, even though there is wide diversity of ligand structure and crystal packing [16,132]. All structures also share the same type of ligand binding site, with the ligand chromophore bound externally onto a terminal G-quartet of the quadruplex by π – π stacking, together with the flexible $-(CH_2)_{2-3}-$ side chains residing in the accessible grooves and loops of the quadruplex (Figure 6.6a and b). A pronounced structure–activity requirement almost universally present in these ligands is the possession of cationic groups at the termini of the side chains; these groups tend to be close to the anionic phosphate groups lining the walls of the grooves and loops. Loop conformations are flexible and respond to the steric requirements of a particular ligand [133]. In the case of (the sole representatives to date) of an RNA quadruplex–small-molecule complex [134], with telomeric RNA, the extra 2'-hydroxyl group on the ribose sugars of the loop nucleotides is involved in stabilizing interactions that result in the formation of “adenine platforms.” These augment ligand binding, in this instance a disubstituted acridine derivative. The two available structures for promoter quadruplex–ligand complexes are both from NMR studies, and show binding to *c-myc* quadruplexes, with the native quadruplex topology being retained [135,136]. That with a quindoline derivative [136] shows that the flanking sequence actively participates in forming a binding pocket for this ligand, which may be a suitable basis for future ligand design studies (Figure 6.7).

6.8

Developing Superior Quadruplex-Binding Ligands

The search for compounds with high quadruplex affinity and selectivity, coupled with low affinity for other nucleic acids, especially duplex DNA, has also resulted in

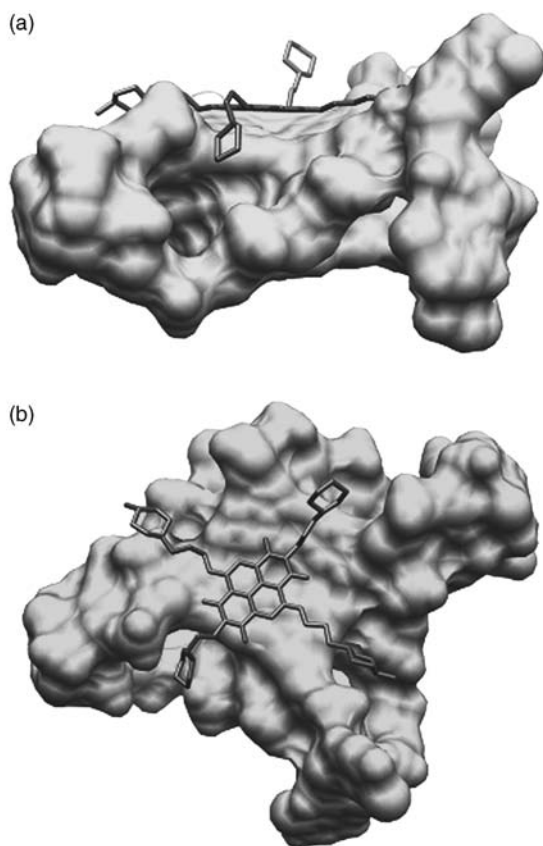


Figure 6.6 Two views of the crystal structure [137] of a tetrasubstituted naphthalene diimide compound (shown in stick representation) bound to a human intramolecular telomeric quadruplex, shown in

a solvent-accessible surface representation (PDB ID 3UYH). The morpholine and *N*-methylpiperazine end groups of the side chains are positioned in the grooves of the quadruplex.

the development of methodology for high-throughput screening of compound libraries. Two methods continue to be of particular utility:

- i) The first method is based on estimating the enhancement in quadruplex stability by changes in a quadruplex melting profile [138–140]. This can be readily monitored, on a 96- or 384-well format by means of a fluorescence resonance energy transfer (FRET) method, in which a quadruplex oligonucleotide is labeled at both ends by donor and acceptor fluorophores such as TAMRA (6-carboxytetramethylrhodamine) – acceptor fluorophore, and FAM (6-carboxyfluorescein) – donor fluorophore. These fluorophores are sensitive to change in distance between them, such as that which occurs on the melting of a quadruplex structure. On raising the temperature, usually in an RT-PCR

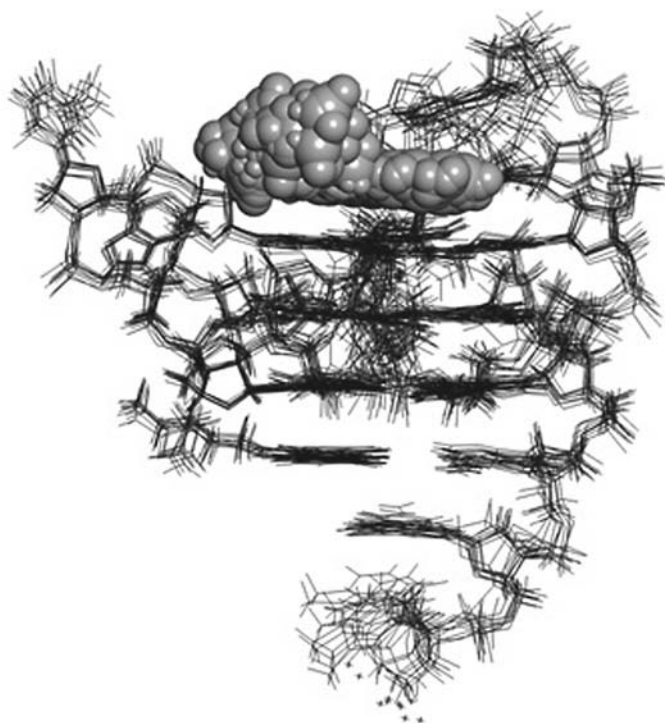


Figure 6.7 A view of the ensemble of NMR structures determined for a *c-myc* promoter complex (in stick representation) with a bound quinoline ligand, in space-filling representation), PDB ID 2L7V [138].

instrument, in which the fluorophore wavelengths can be read, a typically sharp thermal transition occurs when the quadruplex complex becomes unstructured. The midpoint of this transition is the ΔT_m value, the melting temperature difference between the oligonucleotide with ligand and the negative control native sequence in the absence of ligand. ΔT_m values are estimates of stability, not affinity, though they are often useful in selecting hits for more extended binding studies, using, for example, surface plasmon resonance techniques to determine quantitative binding constants and thermodynamics.

- ii) The Fluorescence displacement assay, especially exploiting the loss of fluorescence of thiazole orange and derivatives, is similarly amenable to a high-throughput regimen [141,142]. It has the advantage of providing readout (typically the concentration required for 50% displacement, the DC_{50} value) that does more directly relate to binding constants than ΔT_m values.

Screening libraries of small molecules for quadruplex affinity typically assay for several quadruplex types, invariably including the human telomeric quadruplex, as well as quadruplex sequences from one or several promoter quadruplexes such as *c-myc*, *c-kit*, and *k-ras*. Some though not all studies, have included a duplex DNA

sequence as a control. Few systematic library screens have been reported [142,143]; the screening of the National Cancer Institute (NCI) compound library [144] aimed at generating novel starting points or scaffolds for lead generation in an attempt to move away from the rather narrow range of quadruplex-binding small-molecule scaffolds currently in the literature [71–73,145]. The majority of published compound screens have been confined to lead optimization within small (10–30) compound libraries. A more general question of small molecule-induced selection from a large pool of possible quadruplexes has been approached using the SELEX methodology [146], though this issue has been more recently explored in a direct genome-targeted manner [95]. A number of compounds have been reported as showing high affinity for particular quadruplex targets, in particular, the *c-myc* promoter quadruplex (see for example, [147–149]). This is a desirable goal in view of the central role played by the *c-myc* protein in oncogenesis and the view that the protein is an “undruggable” target. However, such compounds have yet to be fully evaluated for their *c-myc* quadruplex selectivity compared with other potential promoter quadruplex targets. Other mechanisms of transcriptional inhibition may take place and more extensive studies using, for example, pairs of matched cell lines with validated promoter mutations are advisable [50].

The majority of quadruplex-binding compounds reported to date have been devised or discovered on the basis of the generic structure–activity requirements, of coplanarity with a terminal G-quartet and cationic charge. Thus, most of these small molecules have an extended heteroaromatic core, which may itself, as in acridine derivatives, have cationic charge, together with cationic side chains. A number of ligands have been described that contain metallo-groups see for example [150,151], almost always in the context of a heteroaromatic system that in effect mimics the characteristics of the archetypical purely organic quadruplex-binding ligands.

The natural product telomestatin is the outstanding exception to the structure–activity profile of most quadruplex-binding ligands, with a macrocyclic structure that does not contain fused rings or cationic charge [152,153]. It is notable in having exceptional quadruplex affinity combined with a very low level of duplex DNA binding. The total synthesis of telomestatin has been reported [154]. Telomestatin has inspired the design of (i) a number of libraries of macrocyclic polyoxazole compounds [155–157] and (ii) acyclic compounds comprising linked five- and six-membered rings, sometimes assembled by click chemistry [158]. Telomestatin itself has been studied in some detail in a number of cancer types, with sometimes promising results in terms of cellular and xenograft behavior [49]. However, to date there have been no clinical trials of it or related compounds.

The experimental structural data on a number of human telomeric quadruplex–small-molecule complexes provide a starting point for structure-based design approaches to compounds with superior quadruplex affinity. One important caveat (also relevant to *in silico* methods) concerns the high flexibility of the TTA loops [133,159], whose conformational features are dependent on ligand type. In part, this is a consequence of ligand side chain interactions in grooves and loops [159,160], but as yet is not predictable. Therefore, one lesson for structure-based approaches is that successful design is best attempted on closely related analogs of

a well-studied starting point. A recent example [161] bears this out, in which a tetrasubstituted naphthalene diimide compound with four cationic charges, one on each terminal *N*-methylpiperazine ring, was modified to bear two morpholine and two *N*-methylpiperazine rings, thus reducing the formal charge burden. Crystal structure analyses of complexes with both compounds showed that they were isostructural even to the extent of the fine detail of the groove interactions. However, the dimorpholine compound has improved pharmacological properties, as predicted, with, for example, low nanomolar potency against pancreatic cancer cell lines.

Several studies involving *in silico* screening of compound libraries have been reported [137,162]. In view of the loop conformational flexibility of human telomeric quadruplexes, conventional virtual screening methods are unlikely to correctly predict the ranking orders of experimental affinity, though some reports do suggest that the approach can be helpful, in particular, in finding novel nonclassical hit scaffolds, especially those that bind in quadruplex grooves [163,164]. The ZINC database [165], which currently contains over 21 million compounds (<http://zinc.docking.org/>), has been successfully screened, using both structural and physicochemical filters, a promising approach to find drug-like compounds [164]. The virtual approach has been used for *c-myc* promoter quadruplexes, where the presence of single-nucleotide loops leads to more limited conformational flexibility.

6.9 Conclusions

In terms of future quadruplex drug discovery, this chapter has highlighted the present relative paucity of structural data, which hinders current attempts to undertake structure-based design that targets non-telomeric quadruplexes. The more extensive available structural data on human telomeric quadruplexes, on the other hand, does provide some plausible starting points for rational design. It is also the case that high-throughput screening presently lacks good-quality quadruplex-focused chemical libraries. This can be addressed by first undertaking large-scale screening with current high-diversity libraries and then using the hits as starting points for further diversification. A similar situation exists for fragment-based screening since there are currently no fragment libraries that have components optimized for quadruplex targeting, although those designed for targeting riboswitches [166] would be a good starting points.

The quadruplex concept provides an alternative strategy to the more conventional approach of targeting key players in oncogenic pathways at the protein and enzyme levels. It has the advantage of circumventing the resistance mechanisms common to most kinase inhibitors (e.g., active site mutations), although as yet there is remarkably little literature on the induction and mechanism of resistance to quadruplex-binding small molecules. On the other hand, the concept of targeting a nucleic acid structure that is embedded in a sea of duplex DNA has clear challenges and small-molecule quadruplex-binding ligands need to be quite distinct from the

conventional cytotoxic agents that target duplex DNA nondiscriminately. It is apparent though that the design of quadruplex-specific small molecules that do not have any significant duplex affinity can be readily achieved (see, for example, Refs [35,167]). Now that the presence of quadruplexes in the human genome has been definitively confirmed [95], the field can move on from the point where quadruplexes have been invoked but their existence not described in cells, to them being realistic therapeutic targets, in particular at the telomere, promoter, untranslated RNA, or even open reading-frame level. Whether the current generation of small-molecule quadruplex ligands includes among them any that can be used to exploit the presence of quadruplexes in individual cancer patients, remains to be explored. Understanding the function of quadruplexes is also unclear as yet [95,168,169] and this knowledge will undoubtedly aid their exploitation.

The therapeutic potential of agents targeting specific oncogene promoters must depend on the function that an individual oncogene plays in the initiation and development of a particular cancer type. There are some advantages in the quadruplex approach that compare favorably with targeting gene products:

- i) It can target genes regardless of the “druggability” of the gene product.
- ii) There is a lower likelihood of point mutations and resistance (however, see below).
- iii) There are fewer copy numbers to target, hence low concentration of inhibitor needed.

The very recent findings [170–173] of mutations associated with disease incidence in the promoter region of the hTERT gene (the catalytic component of the telomerase complex) in melanoma, prostate, breast, and ovarian cancer patients is of considerable potential relevance since these mutations appear to be in nuclease hypersensitive regions, which are normally at or close to quadruplex-forming sequences, and at least in the case of melanoma, cause increased levels of hTERT transcription. To what extent these mutations relate to quadruplex function, formation, and stability, and how they vary with individuals, is not clear as yet, but targeting hTERT promoter quadruplexes may well provide a novel pathway to inhibiting telomerase activity in affected individuals and thus halting cancer progression.

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The quadruplex field, though young, is expanding rapidly, with an exponential increase in publications. This brief chapter can only cite a small percentage of the

literature and I have highlighted those papers and reviews that, in my opinion, may be of greatest use and interest to the wider drug discovery, chemical biology, and medicinal chemistry communities.

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7

Identifying Actionable Targets in Cancer Patients

David Uehling, Janet Dancey, Andrew M.K. Brown, John McPherson, and Rima Al-awar

7.1

Introduction and Background

The basic premise that cancer is a disease of the human genome has driven our search to fully understand how both individual and combinations of somatic genetic mutations of tumor genes can impact the prevention, diagnosis, and treatment of this disease. An appreciation for the genetic basis of human cancer has been firmly rooted for many years [1]. Indeed, discoveries that have shown a relationship between specific aberrations in the tumor genome, such as the discovery of the *BCR-ABL* fusion oncogene, to a cancer subtype, such as chronic myelogenous leukemia (CML), occurred well before the publication of the initial sequencing of the first human genome in 2001 [2]. However, due to increases in our understanding of basic tumor biology, as well as the rapid technical advances in high-throughput genomic sequencing which have occurred over the last decade, our ability to positively impact cancer management through genomic sequencing is now in the midst of a major revolution [3–8]. Not only we are able to sequence specific genes, but also to sequence entire human cancer genomes at ever-increasing rates. In parallel, our ability to use bioinformatic analyses to interpret how such data can be used to impact the prevention, diagnosis, and treatment of cancer has made major gains. A benefit of these advances is our ability to implement strategies toward personalized cancer medicine, “a form of medicine that uses information about a person’s genes, proteins and environment to prevent, diagnose, and treat disease” [9].

In order to lay the framework to describe the role of genomics for the identification of actionable mutations in cancer, we will begin with an overview of the functional and molecular classification of mutations that are associated with tumor development. In any given tumor, a large number of mutations are normally present, though the number greatly varies among tumor types. As an extreme example, in lung cancer, the presence of nearly 50 000 nonsilent mutations was determined through genetic sequencing of 178 squamous cell carcinoma lung cancer specimens [10], while the collective mutational landscape of blood tumors contains far fewer total mutations. Such genetic aberrations that are found in cancer tumor genomes can be classified as

being either “driver” or “passenger” mutations. As the name implies, driver mutations are defined as those that, either alone or in combination with other genes, play a significant role in the growth advantage of individual tumor cells and/or the tumor as a whole. Some driver mutations, termed “actionable” mutations, are defined as those that are relevant to the diagnosis or treatment of cancer. The most well-known actionable mutation is the *BCR-ABL* fusion gene (Ph+ chromosome) prevalent in the large majority of CML patients. This mutation, which arises from the fusion of the *ABL* gene with the *BCR* gene to cause a hyperactivated tyrosine kinase, is the primary target of the kinase inhibitor imatinib (GleevecTM, Novartis) as well as the second-generation kinase inhibitors such as dasatinib and nilotinib.

Other chapters in this book have highlighted more recently discovered examples of drugs that target actionable mutations. The B-Raf inhibitor vemurafenib has shown remarkable clinical efficacy in melanoma patients who have the activating driver mutation in the *BRAF* gene, coding the V600E amino acid change. Similarly, the ALK kinase inhibitor crizotinib has afforded robust responses in the subset of lung cancer patients with the driver fusion gene *EML-ALK*. These treatment advances represent the combined impact of identifying the association of a mutation with particular cancer type, understanding the basis of its biological role in driving the tumor, and the power of translational research in the discovery of a potent drug inhibitor for its protein target.

In contrast to driver mutations, passenger mutations are not believed to play a major role in tumor development or maintenance. Passenger mutations arise as a result of the inherent genomic instability of the tumor or through relatively inconsequential mutations that arise as a result of environmental factors, but do not play a role in conferring growth advantage to a tumor. Because the large majority of mutations in a given tumor are of the passenger mutations, with only a small set consisting of driver mutations, in cancer genomics discerning driver from passenger mutations in large-scale genomic analyses can represent a significant challenge.

At the molecular level, driver mutations detected by genomic sequencing can be divided into four main groups: exon mutations resulting in a single amino acid change, insertions or deletions of short coding regions (indels), copy number variations (CNVs) resulting in gain or loss of oncogenic or tumor repressor proteins, respectively, and fusion mutations resulting from chromosomal rearrangement (Figure 7.1) [4].

Table 7.1 highlights some of the marketed drugs that target actionable mutations, and the type of mutation as categorized in Figure 7.1. As can be seen in Table 7.1, small-molecule drugs or protein therapeutics have been identified that modulate the mutant protein target or a protein target in its biological pathway. Hence, these are considered druggable genes. In order for a protein to be druggable, it requires a pocket or surface that renders it amenable to modulation by a small molecule or protein therapeutic. By definition, a druggable gene is also an actionable gene. However, many aberrant proteins that arise through actionable mutations cannot be directly targeted by an existing medication. The most compelling examples are oncogenic mutations in *RAS*, which are mutations prevalent in many cancer types, including more than 90% of pancreatic cancer tumors. Although for these reasons *RAS* is a highly desirable target

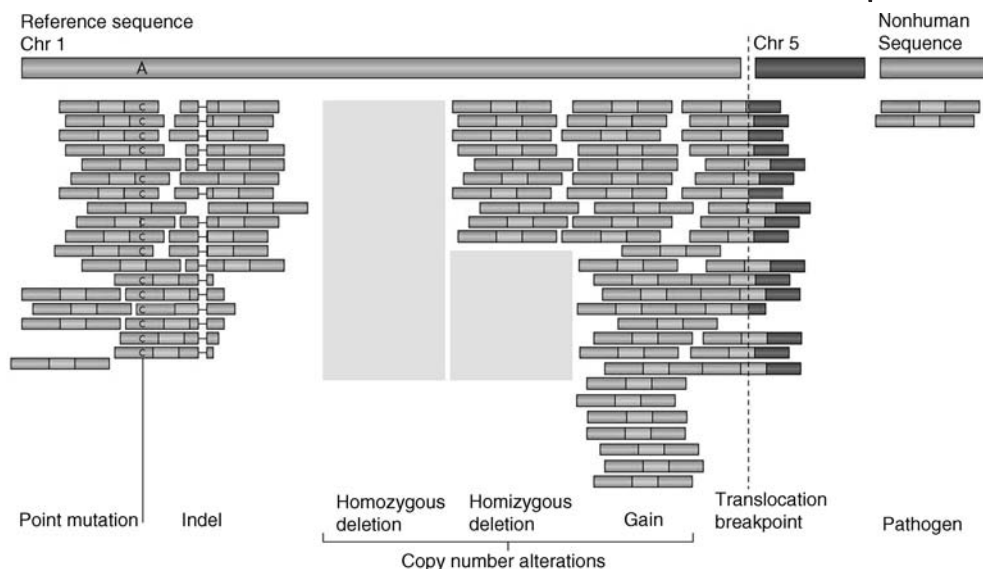


Figure 7.1 Types of driver genome alterations in human cancers. Reproduced from Ref. [4] with permission from Nature Publishing Group.

to inhibit through a small molecule, no drug has been identified to date despite it being one of the most highly validated and prevalent oncology targets. On the other hand, while not druggable, *RAS* mutations are deemed actionable because knowledge of their presence can impact treatment decisions in many cancer types, as will be discussed later [11]. Similarly, *IDH* mutations in glioblastoma are currently not targeted by an existing medication and are, at least from a current perspective, considered undruggable. However, they are actionable due to their value as a prognostic biomarker [12]. Here, it is worth pointing out that the notion of what defines druggability is constantly changing due to advances in drug discovery. For example, at one time kinases were considered by many to be “undruggable” both because their adenosine triphosphate (ATP) pockets were presumed to be highly similar to one another (making therapeutically acceptable selectivity a challenge) and because high concentrations of ATP would prohibit the ability to find sufficiently potent drugs. Indeed, the known successes in targeting kinases with therapeutic benefit in cancer provide a striking example of how dynamic the concept of druggability actually is.

7.2

Overview of Genomic Sequencing and Its Impact on the Identification of Actionable Mutations

Since the introduction of Sanger’s landmark method over 30 years ago, DNA sequencing technology has undergone a remarkable transformation that has led to

Table 7.1 Cancer therapeutics and the driver mutations they target.

Gene	Target class	Type of genetic alteration	Tumor type	Representative therapeutic agent(s)
<i>EGFR</i>	RTK	Mutation and amplification	Lung and glioblastoma	Gefitinib, erlotinib, cetuximab, and panitumumab
<i>ERBB2</i>	RTK	Amplification	Breast cancer	Lapatinib and trastuzumab
<i>FGFR1</i>	RTK	Translocation	CML	AZD-4547, ENMD-2076, and danusertib
<i>FGFR2</i>	RTK	Amplification and mutation	Gastric, breast, and endometrial cancer	AZD-4547
<i>FGFR3</i>	RTK	Translocation and mutation	Multiple myeloma	AZD-4547 and ENMD-2076
<i>PDGFRA</i>	RTK	Mutation	Glioblastoma and GIST	Imatinib, sorafenib, and sunitinib
<i>PDGFRB</i>	RTK	Translocation	Chronic myelomonocytic leukemia	Imatinib, sorafenib, and sunitinib
<i>ALK</i>	RTK	Mutation and amplification	Lung cancer, neuroblastoma, anaplastic large-cell lymphoma	Crizotinib
<i>c-KIT</i>	RTK	Mutation	GIST	Imatinib, and sunitinib
<i>FLT3</i>	RTK	Internal tandem duplication	Acute myeloid leukemia	Fedratinib, midostaurin, and quizartinib (lestaurinib, AC220)
<i>RET</i>	RTK	Mutation and translocation	Thyroid medullary carcinoma	XL184
<i>ABL</i>	NRTK	Fusion (<i>BCR-ABL</i>)	CML	Imatinib, dasatinib, nilotinib, and ponatinib
<i>JAK2</i>	NRTK	Mutation and translocation	CML and myeloproliferative disorders	INCB018424
<i>BRAF</i>	STK	Mutation	Melanoma, colon, thyroid, and lung	Vemurafenib and dabrafenib
<i>AKT</i>	STK	Activation (PTEN)	Breast and ovarian	MK-2206 and GDC-0068
<i>MTOR</i>	Lipid kinase	Activation (PTEN)	Renal cell carcinoma	Temsirolimus, everolimus, BEZ235, and others
<i>PI3K</i>	Lipid kinase	Mutation	Colorectal, breast, gastric cancer, and glioblastoma	Buparlisib, PF-4691502, and GSK-2636771
<i>PTCH1/SMO</i>	GPCR	Mutation	Medulloblastoma	Vismodegib, erismodegib, and saridegib
<i>BRCA1</i>	DNA damage/repair	Mutation	Breast and ovarian	Olaparib and MK-4827
<i>BRCA2</i>	DNA damage/repair	Mutation	Breast and ovarian	Olaparib and MK-4827

RTK: receptor tyrosine kinase, NRTK: nonreceptor tyrosine kinase, STK: serine–threonine kinase.

capabilities that would have been unthinkable only a few years ago. In a span of little more than a decade from the time the first human genome was sequenced, the length of time and cost of sequencing an entire human genome has now dropped to less than 2 weeks with costs of only a few thousands of dollars. These advances have taken place through a combination of improved technology platforms and the bioinformatic capacity to store, process, and interpret the vast amounts of sequencing data. Because a variety of genomic sequencing platform technologies have been developed, which have overlapping but distinct capabilities, a key challenge now lies in choosing the most appropriate platform for the specific application.

In the mid-2000s, the first “next-generation” sequencers (NGS) were developed. The foundation of NGS technology is based on its ability to sequence relatively short DNA reads in a “massively parallel” approach, followed by a bioinformatics intensive analysis of these reads to reconstruct the genome. The “depth” of the sequencing refers to determining and comparing redundant segments from many different DNA molecules. To analyze the genetic material obtained from biopsies, the data-intensive output of this technology requires computational and bioinformatic capabilities that are primarily found at major research institutions. Most NGS platforms can be used for either targeted sequencing of specific sets of genes (as will be discussed later) or whole genome sequencing or whole exome sequencing (WES).

A summary of some of the technology platforms and relevant applications in cancer research is provided in Table 7.2. For further information, the reader is referred to some of the excellent reviews that have been published in the past few years [13]. Although comprehensive discussion is beyond the scope of this chapter, here we wish to highlight a few technologies that have been especially important to our efforts at the Ontario Institute for Cancer Research (OICR). The NGS platform we have relied upon for much of our deep sequencing efforts is the Illumina HiSeq 2000. This technology employs a sequencing by synthesis (SBS) approach that uses the following steps: (a) A fragmented DNA library is constructed by ligating short oligonucleotide adapters onto each end of DNA fragments. (b) The library fragments are immobilized onto the surface of an eight-lane glass flow cell via hybridization to oligos complementary to the adapter sequences to form u-shaped loops. (c) PCR amplification (Amp) of fragments results in clusters of up to 1000 copies. (d) The flow cell is then loaded onto the instrument and sequencing proceeds in cycles in which DNA polymerase and four labeled, 3'-OH chemically inactivated fluorescent-labeled nucleotides are pushed through the cell, incorporating a single base and imaged according to the fluorescent group. After each cycle the 3'-OH group is deblocked to remove the fluorescent group, liberating the 3'-OH for further ligation. This cycle is repeated approximately every hour up to 200 times to produce on average 400 million sequences per lane. Our capacity using 10 Illumina HiSeq 2000 instruments, for sequencing at OICR, is at least 15 trillion bases/month and growing. Currently, an entire human genome can be routinely sequenced with 50-fold redundancy in approximately 14 days, with new upgrades enabling sequencing of a genome in 1 day (Figure 7.2).

Table 7.2 Sequencing platforms and their applications [8].

Gene sequencing platform	Method	Application	Comment
First-generation Sanger	Gel readout of fragmented DNA with end base labeled	Targeted sequencing, genotyping, and whole genome sequencing	High accuracy and long read length, but low throughput
Next generation	Strands of fragmented DNA are amplified and sequenced by imaging of individual bases incorporated by PCR	Targeted sequencing and whole genome sequencing	Significantly higher throughput than Sanger sequencing, lower material required, run time approximately 5 h
• 454 (Roche) • HiSeq (Illumina) • SOLiD (Life Technologies)			
Third generation			
• PacBio RS (Pacific Biosciences) • Ion Torrent PGM (Life Technologies) • Oxford Nanopore GridION (Oxford Nanopore Technologies Ltd.)	• Single-molecule real-time sequencing of single DNA polymerase • Semiconductor senses hydrogen ions produced, when nucleotides are incorporated in DNA • Single strand of DNA sequenced, as it moves through a pore	• Targeted sequencing and whole genome sequencing • Targeted sequencing and whole genome sequencing • Targeted sequencing and whole genome sequencing	• Long read lengths and short run time with simple sample preparation • Low technology cost and short run time • Not yet launched; promises very rapid run times at low cost

Genotyping platforms	Use allele-specific PCR	Somatic mutation analysis; SNP genotyping	Effective and accurate high-throughput genotyping platforms; suitable for clinical applications
• TaqMan OpenArray (Applied Biosystems) • MassARRAY (Sequenom) • ABI PRISM 3100 (Applied Biosystems) • iScan (Illumina) • GeneTitan (Affymetrix) • aCGH platform	• Dye-labeled probes with fluorescent readout • MALDI-TOF mass spectrometry readout • Capillary electrophoresis and optical imaging • Hybridization onto BeadChip; imaging fluorescent signals • Microarray technology and GeneChip arrays • Comparative genomic hybridization		

Adapted from Table 1 of Ref. [8] with permission from American Society of Clinical Oncology.



Figure 7.2 Illumina HiSeq 2000 and MiSeq instruments.

Despite steady improvements, the time frame for carrying out whole genome sequencing or WES and analysis is not yet tractable for many clinical applications. Rather than conducting whole genome sequencing that requires the processing of vast amounts of data, selecting a subset of predefined genes that can be quickly sequenced can be a more suitable and economical approach. For some of our clinical molecular profiling (MP) studies, we have relied on the Sequenom MassARRAY and the Pacific Biosciences RS instruments (Table 7.2). Although these two platforms are quite different, they have both proven useful to interrogate highly overlapping sets of genes in a similar short time frame. The Sequenom OncoCarta v1.0 is a commercially available assay consisting of 238 prevalidated mutations, which relies on MassARRAY multiplex PCR-based primer extension process. It is specifically designed to detect sequence differences at the single-nucleotide level, which facilitates a rapid evaluation of single-nucleotide mutations. The major limitation of the OncoCarta and other genotyping platforms is that they are only suited to evaluate single-nucleotide polymorphisms (SNPs) and short insertions or deletions that are already known, whereas sequencing can also detect novel variants. The PacBio RS is a DNA sequencing system that incorporates novel, single-molecule sequencing techniques using SMRT[®] (Single Molecule, Real-Time) technology. In contrast to other NGS DNA platforms, which rely on massive parallel sequencing of short DNA segments, this system conducts, monitors, and analyzes biochemical reactions at the individual molecule level. The instrument is capable of monitoring ~150 000 individual DNA polymerase enzymes simultaneously as they read a single DNA template molecule and incorporate fluorescent-labeled nucleotides. A schematic of this technology is shown in Figure 7.3.

Other instruments currently in use at OICR are the MiSeq and HiSeq 2500, both of which are NGS instruments from Illumina. Although based on the

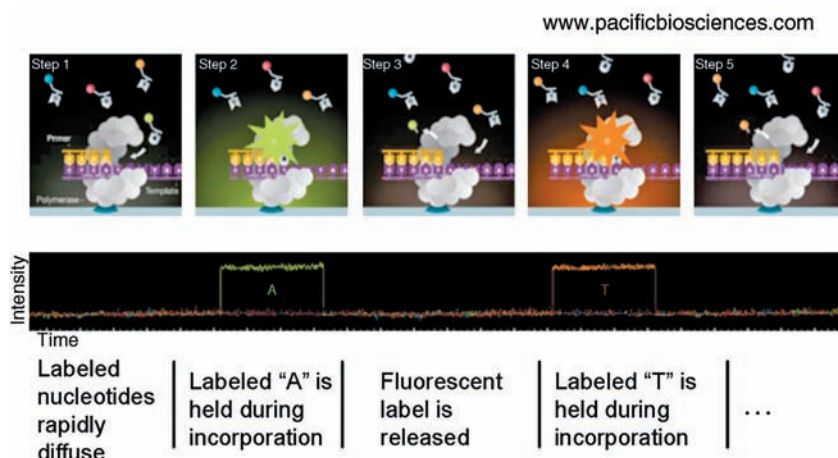


Figure 7.3 PacBio RS single-molecule sequencing platform <http://pacificbiosciences.com>.

Source: © Pacific Biosciences.

same technology platform as the Illumina HiSeq 2000, the MiSeq is a smaller version that is capable of generating clusters and sequencing the equivalent of about 1/4 lane in approximately 24 h (in comparison to eight lanes in 14 days on a HiSeq 2000), making it more amenable to certain clinical applications, where genetic analysis might impact clinical decision making. The HiSeq 2500 has somewhat enhanced sequencing capabilities relative to the HiSeq 2000, including the capability to generate clusters on instrument and the flexibility to run in a rapid mode that allows rapid run times at the expense of total data yield.

To provide some context for the many types of genomic profiling technologies and their potential clinical applications, it is useful to think of the process of identifying actionable mutations in cancer by a clinician or a scientist as falling along a continuum. At one extreme is the clinical setting wherein extensive prior knowledge leads one to evaluate mutations of a single gene with critical diagnostic implications, the results of which must be relayed back to the patient in as short a time frame as possible. Examples of this situation can be seen in the use of many personalized cancer therapies such as those targeting *ALK* and *BRAF* mutations (Table 7.1). The clinical use of these drugs is linked to an FDA-approved “companion diagnostic” test designed specifically for *one particular gene*. The clinical considerations in this setting require that such a single-gene-directed test must be fast, reliable, inexpensive, and reimbursed by the relevant health care provider [14]. The other extreme of the continuum is typified by investigative scientists working in a setting where there is limited or no prior understanding of the driving mutations that may be present in a tumor sample. In this setting, whole genome sequencing or whole exome (the DNA that code for proteins) sequencing is undertaken with the goal of discovering new mutations or novel combinations of known mutations that

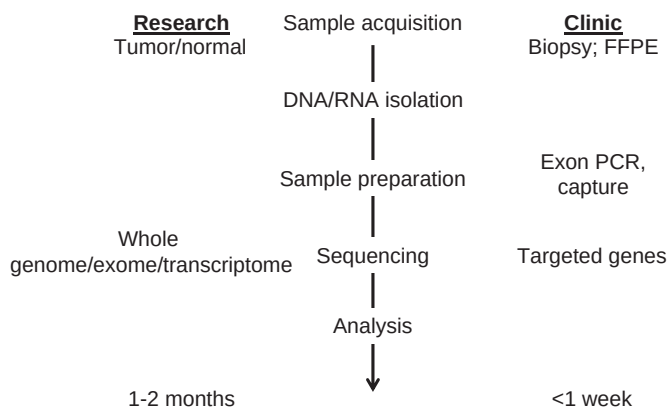


Figure 7.4 Genomic analysis workflow for sample collection, data generation, and analysis.

may have little or no clinical validation. The large amount of data produced is unlikely to provide benefit to the patient whose sample has been analyzed, although the knowledge gained over time may lead to new insights into cancer biology, determinants of clinical outcome, and new cancer treatments for patients [12]. Between these two extremes are studies in which multiple, predefined genes are profiled in a clinical setting to provide information in a timeframe which may benefit the cancer patient and further our understanding of the determinants of treatment responsiveness. Here, a compromise must be found between being able to characterize as many genes of interest as is feasible, while also being able to provide results to a physician within an acceptable time frame to impact treatment decisions. In this situation, making the appropriate choice in the trade-off between speed of analysis and comprehensiveness of sequencing information is a key facet of modern cancer genomic research.

Figure 7.4 highlights the typical workflow and timelines that are in place at OICR that demonstrate the balance between the requirements of basic research and clinical research applications in cancer genomics. A key difference in these two settings is the time frame needed to obtain data and the breadth of sequencing data required. Additional considerations for choosing a particular technology include the sample quality, type of samples (such as formalin-fixed paraffin-embedded, flash frozen, or fresh samples), the quantity (resulting in the amount of DNA obtainable from the sample), the cellularity (i.e., percentage of tumor cells versus normal cells), and the range in types of mutations of interest, for example, SNV, CNV, and so on. For applications that require the delivery of genetic sequencing information on a predefined set of actionable mutations in a time frame suitable for impacting clinical treatment decisions, the platform should be robust, versatile, rapid, and reliable. One final consideration worth mentioning is that there are important advantages of implementing multiple technology platforms, with overlapping roles that rely on distinct technological foundations. These benefits

include not only having the capacity for cross-validation but also serving a larger role of determining the optimal use of these technologies in a practical setting.

7.3

Actionable Targets by Clinical Molecular Profiling: the OICR/PMH Experience

Other chapters in this book have provided some detailed insights into the medicinal chemistry approaches that have resulted in major clinical benefit that oncology drugs such as vemurafenib, vismodegib, and crizotinib have had when administered in the appropriate settings. In these examples of targeted medicines, there is a strong link between the presence of the actionable mutation and particular tumor histology. While the merits of developing a targeted drug against a single tumor type are evident in the success of these therapies, the power of high-throughput genomic sequencing has led teams at OICR and other institutions to take alternative approach to personalized medicine. This alternative paradigm has emerged based on two major considerations. On the technical side, as we have described earlier, the capabilities of both NGS and genotyping technology platforms now enable the analysis of large numbers of tumor-associated genes simultaneously and quickly. Equally important, on the scientific front we have a growing appreciation of the presence of actionable mutations across many tumor histologies. This facet is illustrated in Figure 7.5, which shows a representation of how various validated actionable mutations are distributed across different tumor types [15]. A particularly good illustration of this phenomenon is the *BRAF* mutation. Although it is best known to be mutated in melanoma and colon, and is present in approximately 50 and 10% of cases, respectively, it has also been identified in varying frequencies in many other tumor types. Moreover, it is likely that the distribution of mutations shown in Figure 7.5 underrepresents the total presence of a given mutation if rarer tumor histologies were included.

Some of the first evidence for the value of interrogating the role of a particular mutation in multiple tumor types was seen in a clinical study carried out at MD Anderson. In that investigation, a group of 80 patients with mutated *BRAF* were evaluated alongside a group of 149 *BRAF*-wild type matched by cancer type. This study included multiple tumor histologies, including melanoma, lung, and papillary thyroid carcinoma. The study showed an increase in median overall survival from time of referral to beginning of treatment in phase I in those patients receiving RAF/MEK targeting agents versus other phase I treatments. For those patients receiving standard of care, it was found that progression-free survival was similar for melanoma patients, but shorter in colorectal cancer patients when comparing *mutBRAF* versus *wtBRAF*, while in papillary thyroid cancer there was no difference between the two groups. Interesting patterns were also found looking at other genes in combination with *BRAF*. While patients were identified with coexisting *mutBRAF* and *PTEN* loss or *PIK3CA* mutations, no patients were found

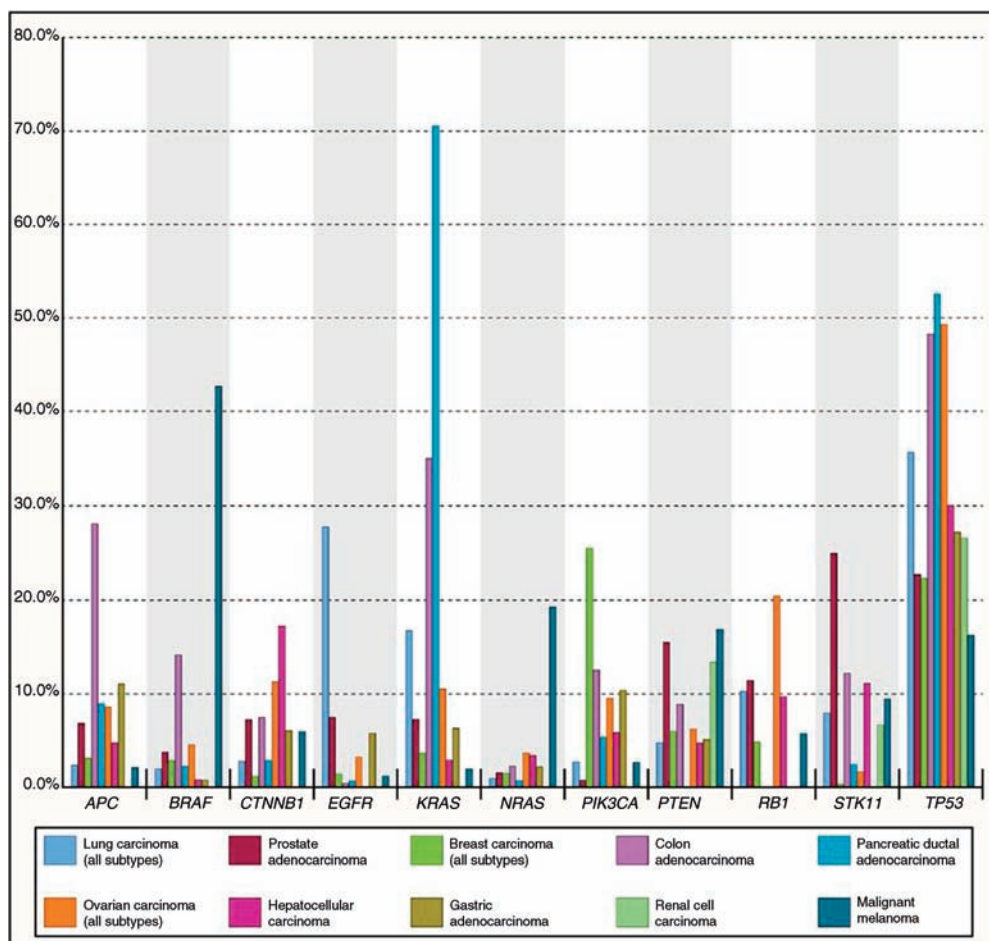


Figure 7.5 Actionable mutations in different tumor types. Reproduced from Ref. [15] with permission from Elsevier.

to have coexisting *mutBRAF* and *KRAS* mutations. Interestingly, because one patient was found to harbor both *mutBRAF* and *NRAS* mutations, it was speculated that because these mutations are considered mutually exclusive the presence of both in the tumor sample might have been the result of different cancer cell clones within the same tumor. In fact, the presence of coexisting *NRAS* and *BRAF* mutations in distinct cell populations within a given tumor has been observed in another study [16]. Given the deep sequencing capabilities of NGS technology, these observations may become more prevalent.

The above-mentioned considerations have provided an impetus to test the hypothesis that if a particular mutation observed in one tumor type acts as a “driver,” it may also act as an actionable driver mutation in other tumor types. By systematically testing a range of targeted agents in patients with different cancer

histologies and a range of mutations, we have the opportunity to assess the impact of targeted therapies and potentially expand their therapeutic applications. This approach ultimately may provide the opportunity to match mutations to their relevant targeted agents in a manner not exploited through current clinical practice. Two added benefits to this approach are that it offers the potential to gain new insights on secondary mutations and genetic backgrounds that lead to resistance to targeted agents, as will be discussed later. The primary goals of this type of study are both to further our knowledge in the identification of actionable mutations in cancer patients and to positively impact the treatment of the patients involved in the study.

To design and execute such a molecular profiling study, Princess Margaret Hospital (PMH), OICR, and four additional cancer centers collaborated on a trial to evaluate the clinical application of genotyping using the OncoCarta Panel and NGS using the PacBio [8,15]. The purpose of that study was to evaluate the feasibility and preliminarily assess potential clinical impact of genomic sequencing in patients with advanced cancers for whom there were no standard treatment options. Key criteria for patients' enrollment included that (a) the patients are a potential candidate for a clinical trial, (b) their lesions are suitable for biopsy, (c) they possessed adequate organ function, (d) archival sample as well as blood samples were available for comparative analysis, and (e) informed consent was given. A tumor biopsy and blood sample were collected on all patients. Genotyping was performed using the Sequenom and OncoCarta v1.0 panel of 238 actionable mutations in 19 oncogenes (Table 7.3) [8,15]. We chose the PacBio sequencer for several reasons, including its ability to sequence multiple genes very quickly (30–45 min), limited sample preparation requirements, relatively low cost, and flexibility in the choice of genes to sequence.

For the OICR/PMH study, an expert panel that met on a regular basis in order to deliberate on the data generated and the inclusion of multiple perspectives was critical to its success [8,15]. A clear challenge that faces such a panel is to review the data, interpret them, and make recommendations in a manner that is both timely

Table 7.3 Genes in the OncoCarta Panel v1.0.

Gene	Number of mutations	Gene	Number of mutations
<i>ABL1</i>	14	<i>JAK2</i>	1
<i>AKT1</i>	7	<i>KIT</i>	33
<i>AKT2</i>	2	<i>MET</i>	5
<i>BRAF</i>	25	<i>PDGFRA</i>	11
<i>CDK4</i>	2	<i>PIK3CA</i>	14
<i>EGFR</i>	57	<i>HRAS</i>	10
<i>ERBB2</i>	6	<i>KRAS</i>	17
<i>FGFR1</i>	2	<i>NRAS</i>	19
<i>FGFR3</i>	7	<i>RET</i>	6
<i>FLT-3</i>	3		

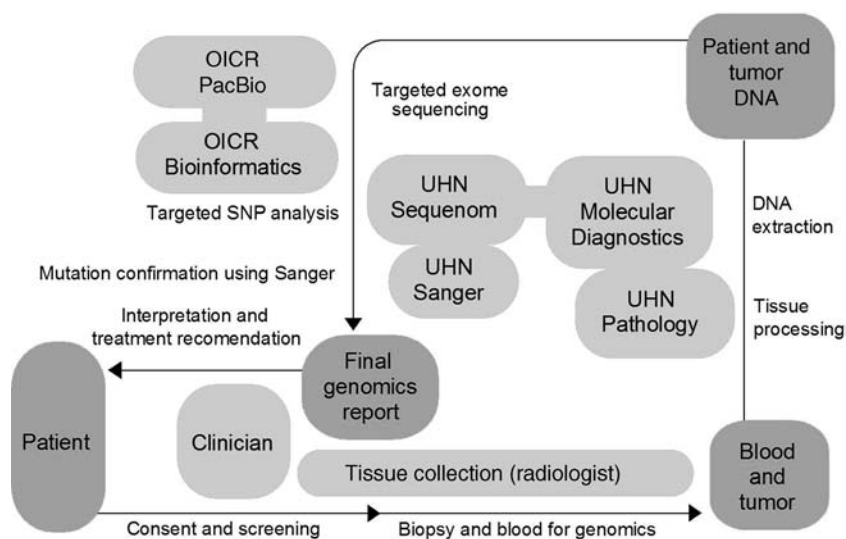


Figure 7.6 Workflow of OICR/PMH MP study.

and adheres to a set of rigorous ethical guidelines that ensure patient privacy, while enabling the rapid dissemination of critical information to relevant parties. The basic workflow for the OICR/PMH study is shown in Figure 7.6. From the initial patient consent until the data is returned to that patient and attending physician, as shown in the workflow cycle, we set an ambitious goal of a 21 day turnaround. The initial report outlined mutations in the fresh tumor biopsy. The clinicians involved in the study reviewed this report, made treatment recommendations for the patient, and were asked to record the impact of the molecular profiling report on their recommended treatments. Follow-up patient observations are to be made every 3 months for 2 years, including efficacy and toxicity related to matched treatments.

The OICR/PMH trial quickly demonstrated the feasibility of real-time molecular profiling in a clinical setting [15]. To meet the target enrollment of 50 patients, a total of 56 patients were approached. All but 5 patients approached gave consent, and a successful biopsy was completed for 49 patients. While median length of time to report findings back to the patient and the practicing clinician from the time of original patient consent was slightly longer than 21 days as targeted, it improved throughout the study.

Out of a total of 49 patients whose biopsies were successfully analyzed by both OncoCarta and PacBio sequencing methods, a total of 19 mutations were identified in 16 patients. The distribution of the tumor types seen in the study and the actionable mutations that were identified is shown in Figure 7.7. Of the 19 mutations identified, 16 of these were deemed actionable by the expert panel. Three previously unknown mutations in three different genes PDGFRA R718 W, AKT1 E341 K, and EGFR Q787 L were identified.

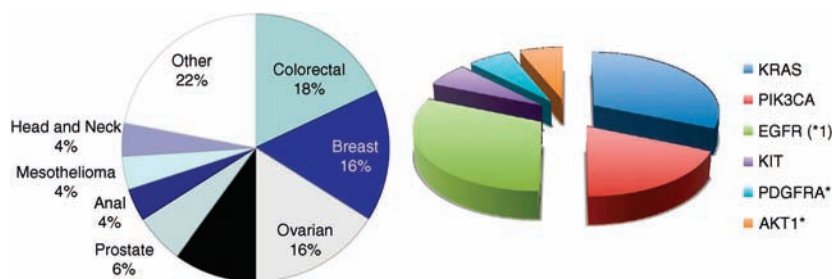


Figure 7.7 Distribution of tumor types and mutations in the OICR/PMH molecular profiling study.

Despite the limited number of patients involved, the study provided the opportunity to draw comparisons between the NGS and the MassARRAY (OncoCarta) platforms. Mutations discovered by the next-generation PacBio approach were validated by OncoCarta in 100% of biopsy and 95% of archival specimens. A slight advantage in the success rate of mutation genotyping in archival DNA was seen within the Sequenom MassARRAY platform in archival specimens, possibly because of the larger amplicons used for the PacBio technology. The primary advantage offered by the NGS platform is, of course, that it affords the ability to identify mutations not found on genotyping panels as, such as the three novel mutations found in the OICR/PMH study. It is important to point out that current clinical laboratory regulations dictate that mutations found by the research laboratory platform should be confirmed in a clinical laboratory before being reported to the patient and attending clinician, if used to guide patient management. In fact, the additional time required for clinical laboratory testing was the most common reason for the delay in reporting beyond the 21 day target.

Some intriguing observations were also made through a comparative analysis between biopsy versus archival specimens. A paired analysis was possible for 34 patients. The degree of genetic concordance between archival and biopsy specimens was 88% (30/34), despite the fact that most biopsied specimens were from metastases and most archived samples were from primary lesions. The results suggest that primary oncogenic driver mutations are generally stable throughout the clonal evolution from the time of collection of archival and biopsied samples. However, the methodology used may not have detected rare clones. Finally, because only 60 exons in 19 genes were assessed focusing on SNVs or short indels, it is likely that there was considerable undetected genetic heterogeneity in these samples.

The most important measure for success of a molecular profiling clinical trial is whether it leads to treatment decisions that positively impact patient outcomes. In the OICR/PMH study, 6 of the 16 patients that were identified with an actionable mutation received treatment based on the identified mutations (Table 7.4). Two of these patients had tumors with PIK3CA alpha mutations, and were enrolled in a clinical trial with a PI3K inhibitor. Of the remaining four patients, two were identified as having a known mutation (KRAS G12D and RET M918T), while the

Table 7.4 Examples where treatment was impacted by OICR/PMH MP trial [17].

Primary tumor of patient	Mutation	Prior clinical experience		Clinical impact of MP trial	
		Number of prior treatment lines	Prior treatments	Matched treatment based on actionable mutation	Response (RECIST)
Colorectal	PIK3CA E542K ^{a)}	4	5-Fluorouracil, leucovorin, oxaliplatin, irinotecan, bevacizumab	Phase I trial: PI3K inhibitor and MEK inhibitor; Cetuximab and Irinotecan	PD; PD
Breast	PIK3CA H1047R ^{a)}	3	Doxorubicin, cyclophosphamide, paclitaxel, trastuzumab, lapatinib, capecitabine, Navelbine	Phase I trial: PI3K inhibitor and MEK inhibitor	PD
Ovary	KRAS G12D ^{a)}	2	Paclitaxel, carboplatin, gamma secretase inhibitor	Phase I trial: PI3K inhibitor and MEK inhibitor	cPR
Breast	AKT1 E341K ^{b)}	4	5-Fluorouracil, epirubicin cyclophosphamide, docetaxel, trastuzumab, tamoxifen, lapatinib, letrozole, capecitabine	Everolimus, Paclitaxel and Trastuzumab	SD
Thyroid (medullary)	RET M918T ^{b)}	2	MET-VEGFR2 inhibitor, Notch inhibitor, cediranib, radiation	Sorafenib	uPR
CUP (squamous cell)	EGFR Q787L ^{c)}	1	Docetaxel, cisplatin, 5-fluorouracil	Erlotinib	uPR

Adapted from Table 4 of Ref. [17].

a) Mutations deemed actionable by expert panel.

b) Patient 3 had KRAS mutant colon cancer based on prior clinical testing. On Study KRAS testing of biopsy sample of liver metastasis and one archived sample identified KRAS wild type; one additional archived sample identified KRAS mutation at 4%.

c) Non-Oncoarta mutation.

RECIST: response evaluation criteria in solid tumors, MET: metastasis, PI3K: phosphoinositide 3-kinase, PD: progressive disease,

NA: not applicable, NSCLC: non-small cell lung cancer, RPLN: retroperitoneal lymph node, Subcut: subcutaneous, cPR: confirmed partial response, SD: stable disease, LN: lymph node, uPR: unconfirmed Partial Response, CUP: carcinoma of unknown primary, GIST: gastrointestinal stromal tumor.

remaining two were shown to have a novel mutation (AKT1 E341K and EGFR Q787L). As an example, a patient with breast cancer, found to have a novel AKT1 mutation received the mTOR inhibitor everolimus in combination with paclitaxel and trastuzumab. Tumor shrinkage was seen after two cycles, before the patient's treatment was discontinued due to complications. Although the responses to the matched treatments seen in this study were temporary, it is important to reemphasize that all of the patients were metastatic, heavily pretreated, and with limited additional treatment options.

Given these considerations, it is apparent that any response seen in a patient group, such as that included in the OICR/PMH study can be viewed with encouragement given the advanced disease status of these patients. These results might, therefore, suggest that a similarly designed molecular profiling study carried out with less heavily pretreated patients with less advanced disease burden might lead to even better response rates when actionable targets are identified. Furthermore, because of various constraints in the OICR/PMH study, not every patient whose mutation corresponded to a known, matched therapy was able to receive the corresponding treatment. For example, two patients were identified with actionable mutations in EGFR (E746-A750 del and L858R) and one with two potentially actionable PI3KA mutations (H1047L and E542K). Matched therapies are now marketed (EGFR inhibitors gefitinib and erlotinib) [18–21] or are in the clinic (in the case of various PI3KA inhibitors) [22,23] that targets these mutations. Importantly, in the future this approach can facilitate the recruitment of patients into clinical trials of emerging and yet-to-be-approved targeted therapies, [15] as, for example, with PI3KA inhibitors.

It is acknowledged that for clinical molecular profiling studies, it is technically more straightforward to focus on actionable mutations that are either point mutations or short insertions and deletions, as was the case in the OICR/PMH study. For future studies, an important desired goal is to be able to expand gene sets to include the other important classes of mutations (e.g., fusion genes and CNVs) that are represented in Figure 7.1. Although several practical hurdles remain in order to be able to evaluate these genes in the time frame that is required in a clinical setting, the speed and low cost of whole genome sequencing has reached the point that evaluating these other classes of mutations along with the types of mutations shown in Table 7.4 may soon be within reach. Furthermore, it is clear that the full potential of clinical molecular profiling will be fully realized when RNA expression and epigenetic aberrations in tumors are evaluated in addition to mutations in DNA. With rapid advances in genomic sequencing platforms with respect to RNA sequencing and being able to evaluate epigenetic signatures (DNA methylation), strategies are now being designed that target these types of tumor signatures as well.

7.4

Some Experiences of Other Clinical Oncology Molecular Profiling Studies

In addition to the OICR/PMH study, various approaches to harness the power of genetic sequencing in clinical molecular profiling studies have also been taken at

other institutions [24–32]. While each of these studies has distinct features due to the tumor type(s) emphasized, genes profiled, and technologies employed, the molecular profiling studies that have been carried out at other institutions share with the OICR/PMH study the goal of genotyping multiple actionable genes in a clinically relevant time frame so as to better impact treatment. One of the earliest clinical trials of this type, a study carried out at Harvard/Massachusetts General Hospital used a highly sensitive “SNaPshot” methodology, with the only major instrumentation being a capillary electrophoresis-automated DNA sequencer. Similar to the OICR/PMH study, an emphasis was placed on evaluating known, actionable mutations, in this case 120 previously characterized mutations in 13 cancer genes, in a “real-time” (2–3 weeks) manner that could also impact treatment decisions [29]. Genetic profiling of 250 primary tumors was consistent with the documented oncogene mutational spectrum and identified rare events in some cancer types. Like the OICR/PMH study, treatment recommendations based on identification of both *EGFR* and *PIK3CA* mutations were made. Also in 2010, the results of a multicenter clinical trial reported by Von Hoff *et al.* [33] provided justification through the clinical impact of molecular profiling. Using a combination of immunohistochemistry and fluorescence *in situ* hybridization on FFPE tissue, and microarray analysis on flash-frozen tissue in 86 patients with refractory metastatic cancer, a molecular genomic target was identified in 98% of 86 patients. Of the 84 patients, 66 were treated according to MP results and in 27% of patients, the MP approach resulted in a longer progression-free survival on a molecular profile-suggested regimen than on the control preadministered regimen. Like the OICR/PMH and MGH/Harvard study, this trial also involved patients with a variety of tumor types (25 different types in total), with breast ($n = 18$), ovarian ($n = 5$), and colorectal ($n = 11$) most heavily represented. It is important to note that many of the targeted therapies available today had not yet been developed at the time when either of these trials had been initiated.

While the above-mentioned clinical studies have evaluated multiple genes in multiple tumor types, other molecular profiling studies have taken the approach of genotyping several genes, but for a single tumor type. In the biomarker-integrated approaches of targeted therapy for lung cancer elimination (BATTLE) trial, 255 chemorefractory non-small cell lung cancer (NSCLC) patients were adaptively randomized to erlotinib, vandetanib (a multitargeted kinase inhibitor), erlotinib plus bexarotene (a retinoic X receptor activator), or sorafenib (a multitargeted and VEGFR inhibitor), based on relevant molecular biomarkers analyzed in fresh core needle biopsy specimens [34]. High-level results from this study showed a 46% 8 week disease control rate and an impressive benefit from sorafenib among mutant-*KRAS* patients, providing evidence for the value of prospective molecular profiling in lung cancer, even for an agent that does not directly target the actionable mutation. In further support of this point, clinical evidence suggests that NSCLC patients that do not express *EGFR* mutations respond better to conventional therapy than targeted tyrosine kinase inhibitor therapy [35], illustrating the value of molecular profiling in avoiding costly and ineffective therapeutic options. In 2012, a systematic genomic testing trial in NSCLC analyzing for

selected mutations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, and *HER2* was successfully carried out on 344 FFPE patient specimens, with a median turnaround time of 31 days [32]. This study also evaluated *ALK* rearrangement using fluorescent *in situ* hybridization or immunohistochemistry. Of the 288 patients diagnosed with stage IV or relapsed metastatic NSCLC, 152 (53%) had at least one identified mutation. Of these, 63% received personal medicine tailored to these mutations, including the *ALK* targeting drug crizotinib that has provided impressive responses in NSCLC patients harboring activating *ALK* rearrangements [36,37]. Therefore, as we have seen earlier, even for relatively rare mutations (e.g., only 2–7% *ALK* rearrangements are present in NSCLC), molecular profiling of multiple genes can rapidly match clinical agents to those patients who are most likely to derive benefit. To underscore the practicality of screening multiple genes at once, a companion diagnostic now commercially available from Quest Diagnostics provides concurrent testing for common activating mutations in *EGFR* (L858R, exon 19 deletions), *KRAS*, and *ALK* (http://www.questdiagnostics.com/testcenter/testguide.action?dc=TS_LungCancerMutation_Panel).

7.5

Identifying Secondary and Novel Mutations through Molecular Profiling

As we have seen, the information gained by genotyping in clinical molecular profiling studies holds great promise for rapidly identifying actionable mutations that can be matched with appropriate targeted agents in an individualized approach to targeted therapy. However, by also harnessing the power of NGS, two additional benefits that clinical molecular profiling studies can provide have been demonstrated. First, NGS can lead to the identification of mutations that are either novel or so rare that no targeted medicine has been evaluated against them. However, because they are present in genes that are already known to develop driver mutations in various cancer types, these novel mutations may play a similar role. The OICR/PMH study revealed novel mutations in genes that have previously established roles in oncology. In the example described earlier, the amino acid mutation (E341K) in *AKT1* that was identified by the PacBio sequencing platform was novel, but its significance could not be inferred from structural biology or by analogy to the functional impact of known, driving mutations that have been validated in other genes. Ultimately, however, knowledge of the existence of this mutation facilitated a rational treatment decision that appeared to have a positive, albeit temporary, clinical impact. Similarly, a novel mutation in the *EGFR* gene (*EGFR* Q787L) was identified by PacBio sequencing during the course of the OICR/PMH study. Its physical proximity to known *EGFR* mutations and similarity to a mutation Q787R predicted to have deleterious consequences [38] suggested it may also play a driver role. As one can imagine, the determination of such novel mutations raises significant challenges for individual or groups of clinician scientists who must render real-time clinical decisions based on this genomic data prior to the generation of laboratory evidence of protein function.

7.6

Understanding and Targeting Resistance Mutations: a Challenge and an Opportunity for NGS

The rapid approval of targeted therapies has followed the achievement of unprecedented responses in both solid and hematologic malignancies that are associated with actionable mutations. However, it has become equally well appreciated that a major challenge arising with the use of targeted therapies is the rapid emergence of mutations that confer drug resistance and, ultimately, treatment failure (Table 7.5) [39]. In the OICR/PMH pilot study, drug-resistant mutations were observed in two individuals treated with targeted agents by comparing the genetic profiles of archival versus biopsied samples. In a patient with colon cancer treated with the EGFR-targeted antibody panitumumab, a KRAS mutation was found in the posttreatment tumor biopsy that was not present in archival samples obtained prior to treatment with the targeted therapy. As preexisting KRAS mutations are known to predict lack of responsiveness to EGFR antibodies, it is not surprising that they also have been shown to emerge during the course of EGFR therapy for colon cancer treatment conferring resistance [11]. In another patient diagnosed with lung cancer and treated with erlotinib, the emergence of the “gatekeeper” EGFR T790M mutation was detected, which is known to be the most common resistance mechanism associated with EGFR-targeting kinase inhibitors [40]. These examples not only show the importance of comparing the genomic profiles of biopsies at different time points, but also suggest that the information may enable clinical decisions to be made based on the types of secondary mutations that emerge.

Undoubtedly, the resistance mutations detected in the OICR/PMH clinical study represent only a small subset of known resistance mutations that have now been identified. In fact, treatment with targeted kinase inhibitors has been shown to lead to the emergence of at least one resistance mutation that can be characterized by genetic sequencing. As more such mutations are identified, the potential benefit of rapid, high-throughput genomic sequencing increases, as such profiling affords the opportunity to address such mechanisms of resistance.

Table 7.6 lists mutations that have been observed in patients treated with targeted therapies, and occur *in the presence of a targeted actionable mutation*. The entries have been classified as being either primary or secondary resistance mutations. For the purposes of our discussion, the distinction between a primary and a secondary resistance mutation is based on its role in the clinical outcome of treatment with a targeted therapy. A primary resistance mechanism can be thought of as one that prevents a response to therapy, even in the presence of an actionable mutation. Conversely, a secondary resistance mutation is one that arises *following* an initially favorable response to targeted therapy and is hence thought to be significantly causative in further disease progression [40]. From both a conceptual and a therapeutic standpoint, particularly when considering the role that genomics can play in developing effective means to have clinical impact, it is fair to say that more headway has been made in the area of secondary mutations than primary.

Table 7.5 Resistance mutations identified in the OICR/PMH study [17].

Patient sample no.	Primary tumor of patient	Identified resistance mutation(s)				Biopsy information	
		Mutation	In biopsy specimen	In archival specimen	Prior targeted treatment	Biopsy specimen site	Archival specimen site
638	Colorectal NSCLC	KRAS G12A	Yes	No	Panitumumab	Liver metastasis	Colorectal primary
		EGFR	Yes	No	Erlotinib	Liver metastasis	lung primary
		L747_E749del	Yes	No			
		EGFR T790M					

Adapted from Table 3 of Ref. [17].

Table 7.6 Examples of resistance mutations to targeted oncology therapies.

Actionable target/ mutation	Tumor type	Resistance mutation	Type of mutation	Primary ver- sus secondary resistance
<i>EGFR</i>	Colorectal	<i>KRAS</i> codon 12, 40	SNP	Both [41]
<i>EGFR</i>	Colorectal	<i>BRAF</i>	SNP	Primary [42]
<i>EGFR</i>	Colorectal	<i>PIK3CA</i> (exon 9 and 20)	SNP	Primary [42]
<i>EGFR</i>	Colorectal	<i>NRAS</i>	SNP	Primary [42]
<i>EGFR</i>	Colorectal	<i>HER-2</i>	Amp	Secondary [43]
<i>EGFR</i>	NSCLC	<i>EGFR</i> T790M	SNP	Secondary [40]
<i>EGFR</i>	NSCLC	<i>MET</i>	Amp	Secondary [40]
<i>EGFR</i>	NSCLC	<i>KRAS</i>		Primary [40]
<i>EGFR</i>	NSCLC	<i>EGFR</i>	Indel	Primary [40]
<i>EGFR</i>	NSCLC	<i>HER2</i>	Amp	Secondary [44]
<i>EGFR</i>	NSCLC	<i>BRAF</i>	SNP	Secondary [45]
<i>EGFR</i>	NSCLC	<i>CRKL</i>	Amp	Secondary [46]
<i>BCR-ABL1</i>	CML	<i>BCR-ABL1</i> T315I, E255K/V, Y253F/H, others	SNP	Secondary [47]
<i>BCR-ABL1</i>	CML	<i>ABCB1</i> (2677 G > T/A)	SNP	Unknown [47]
<i>BCR-ABL1</i>	CML	<i>BCR-ABL1</i>	Amp	Secondary [47]
<i>EM4-ALK</i>	NSCLC	<i>ALK</i> (F1174L, L1196M, G1269A)	SNP	Secondary [48–50]
<i>KIT</i> / <i>PDGFRA</i>	GIST	<i>KRAS</i>		Primary [51]
<i>KIT</i> / <i>PDGFRA</i>	GIST	<i>PDGFRA</i> (D842I, D842V, D842Y, D1842-843IM, DEL I843)	SNP	Secondary [52,53]
<i>KIT</i> / <i>PDGFRA</i>	GIST	<i>KIT</i> (D816H/V, V654A, T670I, D820G, N822K, Y823D, A829P, others)	SNP, indel	Secondary [53–55]
<i>EGFR</i>	Colorectal	<i>EGFR</i> (S492R)		Secondary [56]
<i>HER2</i>	Breast	<i>p95HER2</i>	Deletion	Primary [57]
<i>HER2</i>	Breast	<i>PTEN</i>	Deficiency	Primary [57]
<i>HER2</i>	Breast	<i>PIK3R1/p85</i>	Deletion	Primary
<i>HER2</i>	Breast, gastric	<i>HER2</i> (L755S)	SNP	Secondary [58]
<i>HER2</i>	NSCLC	<i>HER2</i> (L755P)	SNP	Secondary [59]
<i>BRAF</i>	Melanoma	<i>MEK1</i> (C121S, P124S, F129L, others)	SNP	Secondary [60,61]
<i>BRAF</i>	Melanoma	<i>NRAS</i> (Q61K)	SNP	Secondary [62]
<i>BRAF</i>	Melanoma	<i>COT</i>	Amp	Secondary [63]
<i>BRAF</i>	Melanoma	<i>BRAF</i> (p61BRAF)	Deletion	Secondary [59]
<i>BRAF</i>	Melanoma	<i>BRAF</i>	Amp	Secondary [59]
<i>PTCH1</i>	Medulloblastoma	<i>Smo</i> (D473H)	SNP	Secondary [64]

SNP: single-nucleotide polymorphism, Amp: amplification, Indel: short insertion or deletion.

Therefore, we wish to first discuss the topic of secondary mutations before returning to perhaps the even more challenging issue of primary resistance.

7.6.1

Identification and Treatment Strategies for Actionable Secondary Resistance Mutations

Secondary mutations can reside either within the actionable gene itself or in alternative genes. The best-known example of the type of mutation occurring within the same gene as a targeted, actionable mutation is the T315I mutation in the *BCL-ABL* gene in chronic myelogenous leukemia. This “gatekeeper” mutation causes decreased binding of the inhibitor imatinib relative to ATP, enabling tumor cell proliferation in the presence of pharmacological concentrations of the drug. A similar example is the EGFR T790M mutation in lung cancer, which is prevalent among patients who show an initially favorable response to erlotinib, but later develop resistance to treatment.

In contrast to the situation where the secondary resistance develops within the same gene as the actionable mutation, it is clear that many, if not most, genetically detectable mechanisms of secondary resistance to targeted therapies arise through mutations in genes other than the actionable mutation. These secondary mutations can reside either in the same biological pathway or in alternative compensatory pathways to the actionable mutation. For example, as we have found earlier, colorectal patients treated with an anti-EGFR antibody drug develop detectable mutations in *KRAS* [17,65]. Because this mutation arises downstream of the drug target, it produces a compensatory mechanism that negates the growth inhibitory effect of the drug. On the other hand, many aberrant genes arise in separate pathways that confer a redundant role to the drug target. For example, in NSCLC a strong resistance mechanism arises through *MET* amplification, which is seen in about 20% of patients with tumors harboring driving EGFR mutations and who develop secondary resistance to gefitinib or erlotinib [66]. Since coupling of c-Met to ErbB3 leads to aberrant activation of the phosphoinositide 3-kinase (PI3K)/AKT signaling axis, it is not surprising that other means of activation of this pathway have been observed as resistance mechanisms. Similarly, downregulation of *PTEN*, a negative regulator of the PI3K/AKT pathway, has been identified in clinical samples obtained from patients who developed secondary resistance to EGFR inhibitors [46]. Likewise, in colorectal cancer both downregulation of *PTEN* and activating mutations in *PIK3CA* exon 20 mutations have been found to be associated with cetuximab resistance [42].

BRAF-targeted resistance in metastatic melanomas offers an interesting example of secondary resistance mutations. The majority of BRAF-mutant melanoma patients develop resistance to BRAF-targeted inhibitors after a median time of 6–7 months. However, in contrast to the major resistance mechanisms of EGFR-driven lung cancers or *BRC-ABL*-driven CML, no single-nucleotide-activating mutations occur in *BRAF* itself that are *secondary* to the original driving mutations (e.g., V600E, V600K). Instead, a series of mechanisms that melanoma tumors employ

have been discerned to circumvent B-Raf inhibition upstream, downstream, and in different pathways to B-Raf. These mechanisms include receptor tyrosine kinase (RTK) overexpression leading to AKT activation, NRAS mutations, COT overexpression, MEK1-activating mutations, or BRAF alternative splicing or amplifications all of which serve to reactivate the MAPK signaling cascade [59]. Unfortunately, such multifaceted tumor resistance mechanisms make the development of effective targeted therapies that circumvent these resistance mechanisms challenging. However, the complexity of this situation also speaks to the need for rapid genomic characterization of tumors across a wide range of genes in a real-time clinical setting.

Although most genetically characterized targeted therapy resistance mutations currently known are within kinases, an example in a different target class can be found within the 7-transmembrane (7-TM) receptor smoothened (SMO), which is a component of the hedgehog (Hh) pathway and a target for the drug GDC-0449 (vismodegib). Hh signaling pathway is inappropriately activated in certain human cancers, including the aggressive brain tumor medulloblastoma and basal-cell carcinoma associated with Gorlin's syndrome [67–69]. Like other drugs that inhibit Hh signaling by targeting SMO, vismodegib has shown promising antitumor responses in early clinical studies of patients with cancers driven by mutations in this pathway. Unfortunately, in a situation analogous to that of targeted kinase inhibitors, responses to this drug proved to be short-lived due to the emergence of resistance mutations. In 2009, a resistance mutation in the SMO gene corresponding to amino acid SMO-D473H was identified in a biopsy from a patient showing relapse (Table 7.6). The functional consequence of this mutation was demonstrated when cells transfected with the mutation were resistant to growth inhibition by vismodegib. Remarkably, the same mutation was identified in the tumors of mice that had been exposed to vismodegib [64].

The enhanced interplay between preclinical translational and clinical characterization of resistance mutations is fueling an ever more rapid advance in the identification of personalized medicines that target secondary resistance of such mutations. For example, there has been considerable progress in the development of drugs to target secondary mutations identified in patients with CML, lung cancer, and gastrointestinal stromal tumor (GIST). Many of the clinically relevant mutations of *BCR-ABL* imatinib-resistant mutations are inhibited by the FDA-approved ABL family kinase inhibitors dasatinib and nilotinib, with the exception of the T315I gatekeeper amino acid mutations [47]. More recently, the “third-generation” ABL kinase inhibitor ponatinib was approved. Ponatinib is a multi-targeted reversible kinase inhibitor that not only potently inhibits many of the ABL kinase resistance mutations but also effectively targets the T315I resistance mutation [70].

Although few agents have yet approved that effectively inhibit the resistance EGFR mutation T790M, there are a number of irreversible EGFR inhibitors in development. Afatinib (BIBW 2992), which has recently been approved by the FDA, along with neratinib (HKI-272), and pelitinib (EKB-569) contain a Michael acceptor

in their chemical structure that confers the ability to irreversibly alkylate Cys-773 and Cys-805 in the ATP pocket of EGFR and Her-2, respectively. Similarly, canertinib (CI-1033) and dacomitinib (PF00299804) irreversibly inhibit EGFR, HER2, and also covalently bind to Cys-778 of HER4 [66]. In a study involving patients with an activating EGFR mutation, approximately 6% of patients achieved a partial response, with an additional 69% showing disease control rate with median progression-free survival of 4.4 months [66]. Dacomitinib has also shown evidence of activity in a series of clinical trials involving patients with known resistance to both chemotherapy and either erlotinib or gefitinib. In one study involving 50 patients with adenocarcinoma and an additional 16 patients with nonadenocarcinoma NSCLC treated with dacomitinib, 3 patients showed a partial response and 35 had stable disease of 6 weeks or more. Following these encouraging results, both afatinib and dacomitinib have been advanced into phase III development for patients with advanced lung cancer, who have had previous treatment with chemotherapy and reversible targeted EGFR kinase inhibitors. Following impressive results from preclinical studies involving T790M transgene models, combination therapy with cetuximab and afatinib is being explored in patients who have progressed on EGFR inhibitors, with promising early results [66].

There are several additional examples of efforts to address secondary resistance mutations of actionable genes. Mutations in *PDGFRA* that confer resistance to imatinib and sunitinib have been identified in GIST patients. The most common *PDGFRA* mutation in GIST is D842V, which is resistant to both agents. Crenolanib, a selective and potent inhibitor of PDGFRA and PDGFRB was shown to be highly active in biochemical and cell-based studies against PDGFRA D842V and other imatinib-resistant isoforms of this inhibitor. These results suggest that this inhibitor might be clinically efficacious in the context of imatinib-resistant GIST [52]. Ruxolitinib has been approved for myeloproliferative neoplasms driven by the transforming mutated JAK2 V617F kinase. Libraries of mutagenized JAK2 V617F cDNA were used to identify mutations that conferred resistance to ruxolitinib and other JAK2 kinase inhibitors [71]. These results can potentially inform clinical genomics studies as well as the design of new inhibitors of JAK2V617F-driven neoplasms. As a final example of the interplay between clinical and preclinical efforts to address secondary resistance mutations of actionable genes, the *ALK* gene from a biopsy of a patient who developed secondary resistance to the ALK inhibitor crizotinib was sequenced and found to contain a F1174L mutation. This variant of *ALK* was introduced into a RANBP-ALK cell line (RANBP-ALK F1174L) and found to be resistant to crizotinib, but sensitive to another ALK kinase inhibitor TAE-684 [50]. Although TAE-684 itself is a tool rather than clinical compound, this work demonstrates how NGS molecular profiling can enable further drug development as well as patient benefit.

High-throughput genomics is enabling the development of single agents to target not only novel or emerging resistance mechanisms but also combination therapy. For example, since *MET* amplification has been implicated in a significant fraction of NSCLC patients who have developed resistance to EGFR

inhibitors, the concept of combining a MET kinase inhibitor with an EGFR targeting agent has been explored. A clinical study combining the c-MET inhibitor tivantinib (ARQ 197) with erlotinib demonstrated prolonged progression-free survival benefits in patients (16.1 weeks) receiving this combination compared to EGFR monotherapy (9.7 weeks) [66], providing reason to believe that this dual inhibition strategy might provide a strategy to overcome EGFR drug resistance due to MET amplification. Interestingly, in a study by Japanese investigators, a significant (52%) portion of specimens from EGFR-mutant lung cancer patients who had developed acquired resistance to EGFR inhibitors also had the EGFR T790M secondary mutation [66], which raises the possibility that combined treatment with an irreversible EGFR inhibitor and a c-Met inhibitor might be of particular benefit in these cases. A strategy of targeting both EGFR and another signaling pathway has also been used in the design of an ongoing clinical trial, which combines erlotinib with an mTOR inhibitor (everolimus) in chemotherapy-resistant patients. If these results prove promising, given that c-Met signals upstream of mTOR, this combination might also be useful in the case of *MET*-amplified resistance to EGFR therapy. For colon and breast cancer, various combinations involving PI3K, EGFR, and mTOR inhibitors have been proposed in malignancies where resistance mutations are operative in multiple signaling pathways [42]. A considerable amount of preclinical *in vitro* and *in vivo* data has been amassed, suggesting benefits of combining MAPK and PI3K inhibitors in a variety of tumor histology settings [72]. Although these studies are too numerous to detail here adequately, such promising findings have the potential to translate into positive clinical results in the future. In melanoma, a strategy to overcome resistance by combining inhibitors that are at different points in the MAPK signaling pathway has already led to promising results in a phase I/II trial. A group of patients receiving the combination of the B-Raf inhibitor dabrafenib and the MEK inhibitor trametinib was found to show increased progression-free survival of 9.4 months, as compared with 5.8 months in the B-Raf inhibitor monotherapy group [73]. This finding, combined with the fact that the safety profile was favorable for the combination treatment, has spurred advanced clinical trials for this combination.

The encouraging clinical data emerging with second and third-generation kinase inhibitors underscores the potential benefits of NGS methods. As more drugs are discovered that target resistance mutations, patient populations will become increasingly stratified. Systematically profiling tumor samples using highly sensitive methods with low false-positive rates to efficiently identify potentially actionable resistance mutations, and matching patients to the most appropriate agent or combination, has the potential to ensure that these therapies are fully utilized to maximum benefit [69]. In terms of developing the optimal targeted medicine strategies for overcoming secondary resistance, an important question revolves around whether simultaneously targeting targets in targeted therapy naïve patients is optimal, or sequential use of monotherapy-followed addition of agents that target other gene products is

preferred. Through its ability to interrogate many genetic targets simultaneously, it is possible that high-throughput NGS studies that afford comparison of biopsied samples at multiple time points might be able to provide future insights into this question.

7.6.2

Toward the Identification of Actionable Primary Resistance Mutations

Although considerable progress has been made in identifying strategies to overcome secondary resistance, an even more daunting challenge, at least in terms of our progress to date, is that of primary, or “intrinsic” resistance [39]. The problem of primary resistance is best appreciated by considering the suboptimal clinical response rates to targeted agents even when an actionable mutation is detectable in a tumor (Table 7.6). As opposed to the high response rates seen with *BCR-ABL*+ CML and AML to targeted therapy, the response rates in patients with solid tumors that bear an actionable genetic lesion vary from at best 60–80% in the case of erlotinib in lung cancers with actionable EGFR mutations to less than 30% for trastuzumab in HER2 positive breast cancers. Even more discouraging is that, in contrast to the relatively robust response rates observed in mutant *BRAF*-positive melanoma, colorectal patients harboring similar *BRAF* mutations have shown extremely poor response rates (Table 7.7).

In contrast to the situation of secondary (or acquired) resistance, whose causes of emergence can be informed by changes in genetic profiles between pretreatment and posttreatment genetic profiling of tumor biopsies, no such comparisons are available in cases of primary resistance. Therefore, understanding the biology and the relative importance of primary mutations can be far more daunting for developing a clear genomic rationale or an effective therapeutic strategy to address the mechanistic basis for the resistance. Clearly, a key challenge for which high-throughput genomic profiling would appear to be well suited is to be able to evaluate which additional tumor mutations are

Table 7.7 Representative response rates to targeted therapies as single agents.

Actionable mutation	Tumor type	Targeted therapy	Initial response rate (%)
HER2	Breast cancer	Trastuzumab	26 [74]
EGFR	NSCLC	Erlotinib, gefitinib	60–80 [40]
BRAF	Melanoma	Vemurafenib, dabrafenib	~50 [75]
BRAF	Colorectal cancer	Vemurafenib	5 [76]
ALK- <i>EML4</i>	NSCLC	Crizotinib	60 [50]
EGFR wt, KRAS wt	Colorectal	EGFR MAb	35–50

present at the time of initial diagnosis. Armed with this information, clinicians might be able (a) to predict responsiveness to molecular targeted agents so that patients could be stratified to further enhance response rates, and (b) to develop appropriate strategies using combination therapy that might lead to broadly enhanced response rates.

In an encouraging example of how molecular profiling can shed light on mechanisms of primary resistance, the results of a clinical study on 63 patients whose HER2+ tumors had been exposed to trastuzumab was recently published. In this study, patient breast tumor tissue samples were evaluated using a combination of Sequenom MassARRAY genotyping to characterize *PI3KA* mutational status and immunohistochemistry to detect *HER2* and *PTEN* status. These results were then compared to an unselected cohort of archived samples from 73 primary HER2+ tumors from patients not treated with trastuzumab [77]. In the trastuzumab-treated group, it was found that in the large majority of cases (88%), HER2 overexpression persisted in the metastatic tumor following trastuzumab exposure, suggesting that downregulation of HER2 itself was not a mechanism of resistance. However, among cases that were evaluated for *PTEN* or *PI3K* mutation, *PTEN* loss was noted in 59% of patient samples and activating mutations in *PIK3CA* in 29% of such cases, with the combined rate of *PTEN* loss and *PIK3CA* mutation in trastuzumab-refractory tumors at 71%. This number compared with 44% of overall *PTEN* loss and *PI3KCA* mutations in the cohort not exposed to trastuzumab. The study therefore suggests that activation of the Akt/PI3K axis is an important mechanism of secondary resistance, and because mutations in the pathway were prevalent in patients who had never been treated with trastuzumab, it is strongly suggestive that the mechanism likely contributes to primary resistance with trastuzumab. The observation in the OICR/PMH molecular profiling study in which a HER2+ patient whose disease had progressed while on anti-HER2 therapy (lapatinib) was found to have a novel *AKT1* mutation (E341K) is further evidence for the importance of this axis in resistance to trastuzumab. These results imply that for both primary and acquired resistance to trastuzumab, treatment with a therapy targeted against the Akt/PI3K/mTOR pathway might prove beneficial.

It is increasingly apparent that drug activity may be dependent on both mutation and cancer histology. As shown in Figure 7.2 earlier, V600E BRAF mutation occurs in 10–15% of metastatic colorectal cancer. However, as can be seen in Table 7.6, the response rates to BRAF-targeted therapy are dramatically poorer in colorectal cancer compared to melanoma. In colorectal cancer, RTK signaling causes reactivation of the MAPK pathway during BRAF blockade, rendering tumor cells resistant to the drug [76]. These results suggest that the survival advantages of mutant *BRAF* colorectal tumors that are lacking in responsive melanomas during BRAF inhibition occur at the level of protein expression, rather than DNA mutations. Therefore, interrogating DNA mutations alone may be insufficient to be able to predict and address mechanisms of resistance in resistant tumors. Again, high-throughput methods to evaluate resistance mechanisms at the transcriptome and epigenetic level may be required in such instances. A similar situation may exist in many lung cancers, where primary resistance to an

actionable EGFR mutation in many instances has been found to occur not through a genomically characterizable event at the DNA level, but rather through upregulation of extracellular signaling mediator HGF, which signals through c-Met receptors. The elevated c-Met may arise through enhanced production by either tumor cells or fibroblasts that surround the tumor cells [46]. In this and other instances, phosphoproteomics may provide insight into mechanisms of resistance and appropriate targeted therapy by showing the signaling pathways that are activated in resistant tumors. Although an adequate treatment of the methods and applications of epigenomics, proteomics, and phosphoproteomics are beyond the scope of this chapter, it is clear that deep DNA and RNA sequencing afforded by NGS technologies are insufficient to fully understand and address many mechanisms of resistance, particularly primary resistance. Clearly, incorporating such methods alongside NGS DNA sequencing will gain further importance and is likely to become more routine in the future.

In discussing the causes of primary and secondary resistance to targeted therapies, especially in the context of the NGS molecular targeting, the issue of tumor heterogeneity looms both as a challenge and as an opportunity for further progress in identifying, understanding, and treating actionable mutations. Through harnessing the power of whole exome sequencing, it has become increasingly apparent that not only is there heterogeneity between different types of tumors in different patients but also there is heterogeneity within individual tumors within the same patient. A compelling case for this assertion can be found in a recent study involving exome sequencing of 100 breast cancer patients, which found a 73% diversity rate of mutations within their mutational landscape, including 7241 point mutations, 277 indels, 1712 homozygous deletions, and 1751 amplifications. Moreover, in another study, another group found a high rate of somatic mutational differences (63–69%) in separate sections across tumor regions [78]. This degree of heterogeneity also offers possible insights into the emergence of resistance, suggesting that different tumor types harbor resistance clones that may comprise a genetic diversity “fingerprint” of particular tumor histologies. Although the results from the OICR/PMH study revealed high mutational consistency between archival and biopsied metastatic tumors, the limited number of genes included in our study compared to these WES studies should be emphasized. It will be important to see how the comparisons between archival and biopsied genotyping hold as we expand our molecular profiling study to include a larger set of genes, and beyond that as we begin to routinely implement whole genome sequencing into MP clinical practice.

7.7

Concluding Remarks and Future Perspectives

In order to summarize the current and future relationship between genomic sequencing technology and cancer research and treatment, it is helpful to refer to a conceptual framework [8] that organizes cancer genomics into three arms

comprised of technology, translation, and research (Figure 7.8). In this chapter, we have briefly described some of the key genotyping and NGS platforms that have made it possible to rapidly identify actionable mutations, and have gone on to discuss how these technology platforms have been incorporated into clinical strategies to identify these targets in a real-time clinical setting. Indeed, only by incorporating the latest advances in genomic sequencing technology into clinical practice can the full potential of cancer genomic knowledge be harnessed toward the treatment of cancer. Through our discussion of resistance mutations and their increasingly appreciated role in limiting targeted therapy, we hope to have conveyed not only how much progress has been made in understanding the need to fully interrogate the broader cancer genome, but also how much remains to be done to fully harness the potential of personalized medicines that, in the large majority of cases, are limited by preexisting or developing resistance mutations.

To conclude this chapter, we wish to provide a brief perspective on the third and final aspect of cancer genomics shown in Figure 7.6, the role of genome-wide sequencing in the discovery of new genetic mutations, and the identification of interrelationships between mutations that drive the formation, growth, and spread of cancer. Whole genome sequencing studies have now been carried out on several different types of cancers, including glioblastoma [79], prostate [80], melanoma [81], acute myelogenous leukemia [82], and pancreas [83] among others. These efforts have led to the discovery of novel driver mutations and relationships among genes that have provided insights into new treatment strategies.

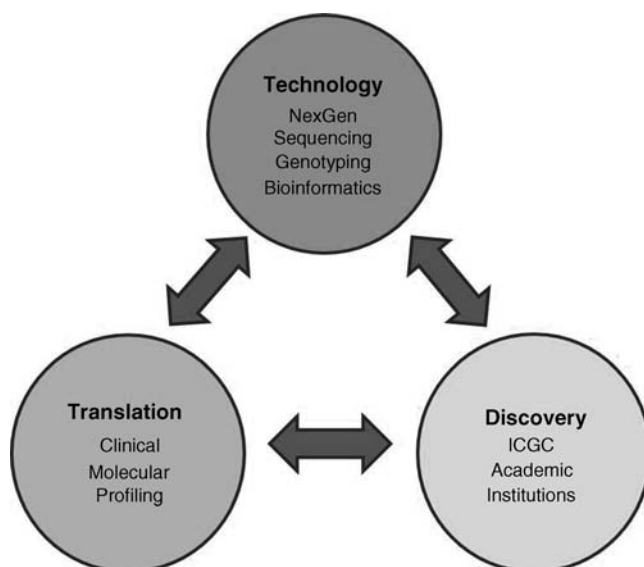


Figure 7.8 The three arms of cancer genomics.

In order to systematically and comprehensively sequence a large number of tumor types in a manner that ensures a standardization of sample treatment and analysis, avoids duplication of efforts, and facilitates the sharing of findings to include a broad spectrum of cancers that incorporate global differences in cancer incidence, the International Cancer Genome Consortium (ICGC) was initiated [12]. The stated goals of the ICGC are as follows: (a) generate comprehensive catalogs of genomic abnormalities (somatic mutations) in 500 tumors from each of 50 different cancer types, (b) ensure high quality and comprehensive data on the full range of somatic mutations, (c) make the data available to the entire research community as rapidly and with as few restrictions as possible, (d) coordinate research efforts so that the interests and priorities of participating nations, organizations, and individuals are addressed, and (e) support the dissemination of new technologies, software, and methods to facilitate data integration and sharing with cancer researchers around the globe. A comprehensive cataloging of cancer genes will include determining somatic abnormalities occurring at a frequency of more than 3% and ideally at a single-nucleotide level. Moreover, using common standards of sample collection, processing, storage, and technology, data from both tumor and matched nontumor tissue will be generated in order to distinguish somatic from inherited sequence variants and aberrations. Finally, extensive transcriptomic and epigenomic data sets from the same tumors from which DNA mutational analyses are conducted will also be generated.

At the time of writing this chapter, the ICGC had received commitments from 16 countries to study over 50 tumor types, and typically 500 tumors and 500 controls per tumor subtype, or the equivalent of over 50,000 human genome projects. A key desired benefit of the ICGC's mission is that by the inclusion of jurisdictions throughout the world, tumor types that are prevalent or are of particular importance in only certain countries or regions are adequately represented for further study. Of special interest to OICR is one of the cancer types for which Canada is responsible, pancreatic cancer-ductal adenocarcinoma (PDAC). Currently, almost 300 matched samples have been obtained, many of which have been successfully implanted into mouse xenografts suitable for further study. By combining the powerful bioinformatics platforms at our disposal with our drug discovery capabilities, we hope to harness the genomic information of these samples to further our efforts to identify and prosecute new targets for PDAC. Of course, these efforts will be carried out in parallel with efforts by various academic, government, and private institutions whose focus will be on this and other tumor types analyzed by the ICGC. It is important to emphasize the long-term nature of this project, and that we expect the successes that are beginning to be felt will continue to be realized for many years to come.

In summary, the progress that continues to be made in the above-mentioned three aspects of clinical genomic research at OICR and other cancer-focused institutions suggests that the era of obtaining comprehensive genomic profiling on every cancer patient, both in a research and in a real-time clinical setting, is moving closer to becoming a reality. Only through utilizing the most advanced genomic sequencing technologies to rapidly identify both known and novel actionable

mutations in cancer patients, both in clinical and in research settings, will we be able to realize the full potential of personalized medicine. Given the progress that has been made in such a short time since the first human genome was sequenced, there is ample reason to feel optimistic about the future of this field.

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8

DNA Damage Repair Pathways and Synthetic Lethality

Simon Ward

8.1

Introduction

With more than 3.2 million new cases and 1.7 million deaths each year, cancer remains an important public health problem in Europe. Given the strong association between cancer risk and age, the global demographic changes due to an aging population will inevitably lead to a major increase in the cancer burden, even if age-specific rates remain constant. However, despite major advances, there remains a substantial unmet need for highly specific drugs that selectively kill cancer cells while sparing other proliferative tissues in the body [1,2].

The development of the hallmark traits of cancer involves a range of mutational events that variously activate oncogenes and disable tumor suppressor pathways that together confer viable dysfunction of the central regulatory networks of the cell [3,4]. The ability of a single cell to acquire the multiple genetic changes that confer these hallmark traits depends on loss of genetic stability early in the tumor cell lineage. This is typically initiated and/or tolerated by defects in the DNA damage response (DDR).

Most, if not all, cancers have defects in one or more DNA damage response and repair pathways [5,6]. These defects underlie the acquisition of the mutations that confer the hallmark traits of cancer, allow the tumor cell to survive oncogene-induced replicative stress without apoptosis, and, following treatment, acquire resistance to cytotoxic therapies. Furthermore, the oncogenesis-associated replication and DNA damage stresses that are common to many (possibly all) cancer cells make it likely that combinations of pathways and subpathways within DDR will be necessary for the robust growth of many, if not all, tumor cells. In turn, this suggests that a key difference between tumor and normal cells will lie in the behavior of the complex networks of DDR pathways [6]. The proposition is that in many cases, cancer cells, and hence patient subgroups, can be specifically targeted by drugs or drug combinations that exploit these intrinsic differences [7–11].

8.2

DNA Damage Response

The ability to effect DNA repair is critical to the viability of all organisms, and complex, interconnected pathways have evolved to ensure genome stability. The integrity of the DNA duplex is continuously threatened by the very process of living, with damage generated through both normal cellular activity (e.g., replication errors) and endogenous (e.g., reactive oxygen species) and exogenous (e.g., environmental pollutants) stresses. These endogenous and exogenous agents can generate large numbers of DNA lesions [12]; for example, sunlight exposure can cause up to 10000 DNA lesions per cell per day [13]. It is of paramount importance that these lesions are repaired to ensure the onward viability of the organism through continued faithful DNA replication. This is achieved by an intricate series of DDR proteins and pathways that are the gateway to the subsequent processes of DNA repair. These DDR proteins control the recruitment and localization, at discrete foci, of various DNA damage sensor, mediator, transducer, and effector proteins, as well as the necessary chromatin remodeling required for DNA repair systems to access the site of damage (Table 8.1 outlines the major classes of DNA damage response proteins) [14,15].

Although there exist many proteins in both the response and repair processes, the fundamental approach remains consistent. Namely, the DNA damage is detected, DDR proteins are recruited to the site of damage, and then the repair is carried out. The DNA damage itself is reversed by a number of major pathways (Table 8.2). For single-strand break repair, these include direct repair (DR) [17], nucleotide excision repair (NER) [18], base excision repair (BER) [19], and mismatch repair (MMR) [20]. Each of these processes requires a number of distinct steps comprising both excision of erroneous DNA bases and incorporation of the desired correct homologous sequence and all use the complementary DNA strand

Table 8.1 Major DNA damage response proteins [16].

Sensor	Transducer	Effector
Detection of and binding to DNA damage	Protein kinase cascades that amplify signal downstream	Execution of DNA repair functions
BRIT1	ATR	LigIV
53BP1	ATM	Artemis
DDB1	PTEN/AKT	Ku70
H2AX	Chk1	Ku80
NBS1	Chk2	Rad51
MDC1	DNA-PK	BRCA2
PARP1	RNF8	Xrcc3
		Rad54
		NER complexes
		Fanconi complexes
		TLS

Table 8.2 Major DNA damage repair proteins and typical lesions repaired for single-strand breaks [11].

Direct repair	Single-strand break repair			Double-strand break repair	
	Nucleotide excision repair	Base excision repair	Mismatch repair	Nonhomologous end joining	Homologous recombination
Nicks caused by DNA ligase	Bulky UV lesions	Oxidative lesions; alkylations	Base mismatches/deletions		
AGT	Helicase	XRCC1	MLH1	Ku70	RPA
	ERCC1	LigIII	ExoI	Ku80	RAD51
	RPA	APEI	MSH2	LigIV	XRCC3
	XP	Pol β		DNA-PK	BRCA2
	ERCC4	PARP		XRCC4	RAD52
					Rad54

as a template. Crucially, unrepaired single-strand breaks can become double-strand breaks at replication forks [8]. Double-strand breaks are more dangerous, with the potential to trigger apoptosis. The entry to both of the main double-strand repair pathways is coordinated by the serine/threonine–protein kinases ATR (ataxia telangiectasia and Rad3 related) [21] and ATM (ataxia telangiectasia mutated) [22] that essentially act to buy time for the DNA repair processes by stalling the cell cycle via two other key serine/threonine–protein kinases Chk1 [23] and Chk2 [24] and activating the process of homologous recombination (HR) [25] and nonhomologous end joining (NHEJ) [26]. Homologous recombination is active mainly during S and G2 cell cycle phases and involves removal of the DNA sequence around the double-strand break and insertion of the original DNA sequence using the sister chromatid as a template. In contrast, nonhomologous end joining can occur throughout the cell cycle, in particular at complementary phases G0 and G1. Furthermore, NHEJ involves the direct ligation of the DNA strands at the break and sometimes at the end trimming to overall create a repair that is less accurate than that achieved by HR.

8.3

Synthetic Lethality

In the quest for safer, personalized cancer drug therapies, the strategy taken has evolved to follow the successful approach of treating many infectious diseases by identifying drug targets that are unique to the target cells – that is, a target-driven therapeutic index [27]. However, as many cancers are driven by loss-of-function mutations in tumor suppressor genes, this clearly does not lend itself to such straightforward strategies. Nevertheless, building on the understanding of DNA

damage and repair, defects in DDR systems create a paradox for cancer cells. On the one hand, defects in DNA damage processes are essential for oncogenesis; on the other hand, the cancer cells require the use of multiple DDR pathways for successful replication and division. Thus, DDR pathway defects not only enable the cancer to evolve but also sensitize cancer cells, making them excellent targets for drug therapy either directed at the aberrant primary pathway or by inhibition of secondary pathways that become critical due to dysfunction of the primary pathway – so-called “synthetic” sensitivity. This can be exploited as a means to improve the therapeutic index of chemotherapeutic approaches.

Synthetic lethality is a frequently discussed approach to achieve this desired ability to enhance the tumor cell specificity and hence patient responder rate [28]. In essence, the concept relies on a combination of mutations in two or more genes giving rise to cell death, when the individual gene mutations do not. Specifically in the case of cancer cells (and for the purposes of this chapter, the scope of synthetic lethality is restricted to this field), this should enable the selective targeting, and hence killing, of tumor cells with specific genetic or epigenetic backgrounds in an environment of untargeted normal cells. The principle for these studies was reported by Bridges in 1922, initially in the fruit fly *Drosophila melanogaster*, in which specific nonallelic genes were found to be lethal when crossed together even though the homozygous parents were unaffected [29]. Subsequent reports from Dobzhansky in 1946 included the first reference to the new term of synthetic lethality [30], and these studies were continued in additional lower organisms including budding yeast using classical genetic studies involving sequential gene mutations [31–33]. Importantly, the two or more genes mutated to drive this synthetic, or conditional, lethality are not necessarily related to each other in an obvious pathway connection, hence requiring both rational and screening approaches to identify such interactions as discussed later.

In its simplest form, as shown in Figure 8.1, a normal cell contains wild type copies of genes A and B. The cell can acquire a mutation to either of these genes,

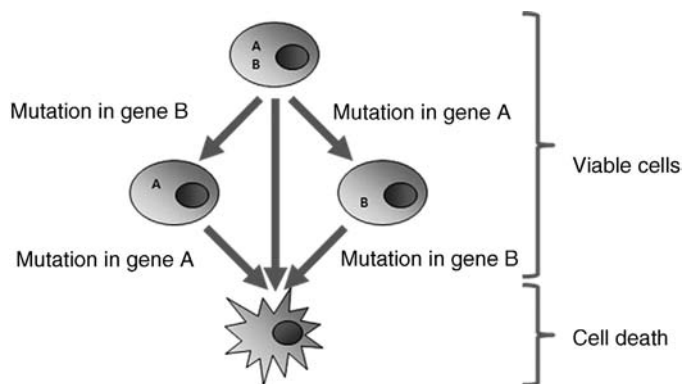


Figure 8.1 Schematic representation of the concept of synthetic lethality.

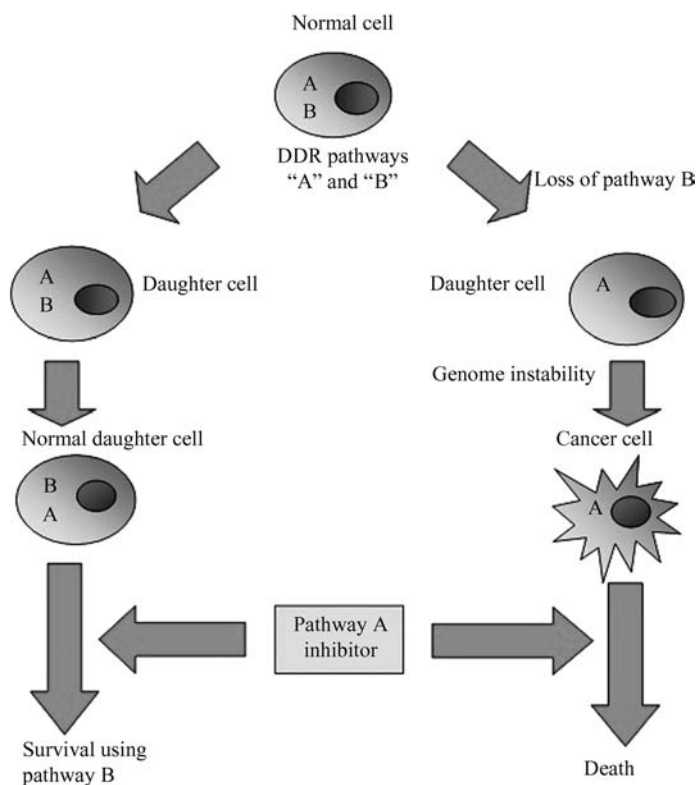


Figure 8.2 Schematic representation of the concept of synthetic lethality with drug molecule.

affording the combination of wild type A with mutant B, or mutant A with wild type B, both of which are viable. However, if the cell acquires mutations in both genes A and B, the cell cannot survive, and hence the combination of the two mutations is deemed synthetically lethal.

With a more specific focus on the potential for drug molecules to exploit this concept of synthetic lethality, Figure 8.2 shows a normal cell with two DDR pathways A and B, for which loss of pathway B by an underlying genetic mutation results in a daughter cell that can subsequently evolve into a cancer cell reliant on pathway A for survival. Treatment of both the normal daughter cells and the cancer cells with an agent that inhibits pathway A will be tolerated in the normal cell (able to use pathway B) but catastrophic for the cancer cell, resulting in the desired selective toxicity.

The principle of synthetic lethal genetic interactions is intriguing as it uncovers the genetic robustness acquired by organisms to defend against a range of intrinsic and extrinsic challenges. This has been demonstrated effectively in *Saccharomyces cerevisiae* in which over 80% of the genes are not required for proliferation in a nutrient-rich medium [34]. This level of genetic or functional buffering is principally afforded by redundancy in various

pathways, in which nonhomologous genes operate nonexclusively [35]. From an evolutionary perspective, this ensures that vital cellular processes are homeostatically maintained by avoiding dependence on single pathway components, and lends itself to the concept of therapeutic targeting within such pathways – in particular to tackle the common loss-of-function tumor suppressor mutations described earlier.

The first intentional exploitation of the principles of synthetic lethality was described by Hartwell and coworkers in 2000 and involved the systematic screening of 76 cancer drugs against 70 mutant isogenic yeast strains [36]. This study aimed to identify synthetic lethal interactions against the yeast mutants that had been chosen to be analogous to gene mutations in human tumor suppressors, and by doing so, uncovered a number of mutations that were more responsive to some of the cancer drugs screened. The underlying rationale for this study had relied on the principle that all tumors result from genome instability, and thus this differential response to the DNA damaging cancer drugs was expected. Specifically, the study uncovered that cisplatin (DNA cross-linking agent) was more toxic in yeast strains with mutations in postreplication repair systems such as *Rad18* and *Rad6* and that mitoxantrone (topoisomerase II poison) was more toxic in yeast strains with defects in double-strand break repair.

A follow-on study published 2 years later screened over 85 000 compounds in yeast strains deficient in double-strand break repair [37]. A total of 126 compounds were identified, which were subsequently identified as topoisomerase type I and type II poisons, in line with the preceding study.

8.4

Lead Case Study: PARP Inhibitors

8.4.1

Introduction

Synthetic lethality presents a particularly attractive approach to tumor targeting, best illustrated by the lethal effect of pharmacological disruption of single-strand break repair by targeting poly(ADP-ribose) polymerase (PARP1) in homologous recombination-defective tumors. PARP1 and PARP2 are members of the PARP superfamily of 17 proteins, first described in 1963 [38], which catalyze the formation of poly(ADP-ribose) from NAD⁺ onto target proteins (and produce nicotinamide as by-product) [39]. This function enables PARP to act as a sensor for single-strand DNA breaks (by DNA binding to a Zn finger in the PARP protein fold) and recruit the requisite DNA repair proteins to the break site. The original working hypothesis is that cells with compromised PARP activity (for example, by inhibition from an exogenous agent) would be deficient in single-strand break repair, which would lead to an accumulation of unrepaired single-strand DNA breaks at stalled replication forks. If unrepaired,

these stalled replication forks can lead to an accumulation of double-strand DNA breaks and subsequently to cell death. One of the main repair functions carried out by homologous recombination proteins is the repair of stalled replication forks, and consequently tumors that carry a mutation – which impairs the cell's ability to conduct homologous recombination, such as those with mutated and impaired BRCA1 or BRCA2 – should be hypersensitive to reduced single-strand break repair capabilities through, for example, PARP inhibition. BRCA1 and BRCA2 proteins are tumor suppressors, are required for homologous recombination, and suppress the genetic instability that can initiate the process of oncogenesis. Furthermore, individuals with inherited mutations in a single copy of either BRCA gene carry a significant lifetime risk of developing breast or ovarian cancer [40–42].

Thus, in normal cells, the impact of PARP inhibition should be buffered by effective homologous recombination, reducing normal cell toxicity, but in homologous recombination of defective BRCA1 and BRCA2 gene mutant cells, PARP inhibition should be profoundly toxic. More recent debate has questioned the mechanistic role of PARP inhibition in either allowing single-strand breaks that form spontaneously to persist or in trapping DNA repair intermediates, particularly during base excision repair – however, both would have the potential to ultimately deliver toxic double-strand breaks [43].

Importantly, PARP inhibition was validated preclinically as an attractive target for synthetic lethality with BRCA1/2 mutants, both in cell lines and xenografts in which cells with BRCA mutations showed considerably greater sensitivity to PARP inhibition [44,45]. Furthermore, PARP1 knockout mice are viable and fertile and do not develop early onset tumors, as might be feared from a deficiency in single-strand break repair, and are more reliant on homologous recombination [46]. However, more recent data questions the true safety of PARP inhibition [47].

8.4.2

Discovery of PARP Inhibitors

The initial preclinical discovery of PARP inhibitors was made following the observations that the by-product of the poly(ADP-ribosylation), nicotinamide, is a weak inhibitor of PARP. Initial templates prepared were derived from these chemical start points and afforded a number of structures (Figure 8.3) that contained the core of the nicotinamide. INO-1001, iniparib, and veliparib have all been progressed into multiple clinical trials as described later. However, despite the understanding described above, PARP inhibitors were originally developed as chemosensitizers and revealed a number of synergies with existing standard cytotoxic drug agents [48].

Subsequent discovery efforts have been divided into two main approaches: either using high-throughput compound library screens with follow-up structure-based drug design or building on the knowledge of the nicotinamide-derived templates to afford a selection of further optimized molecules, which

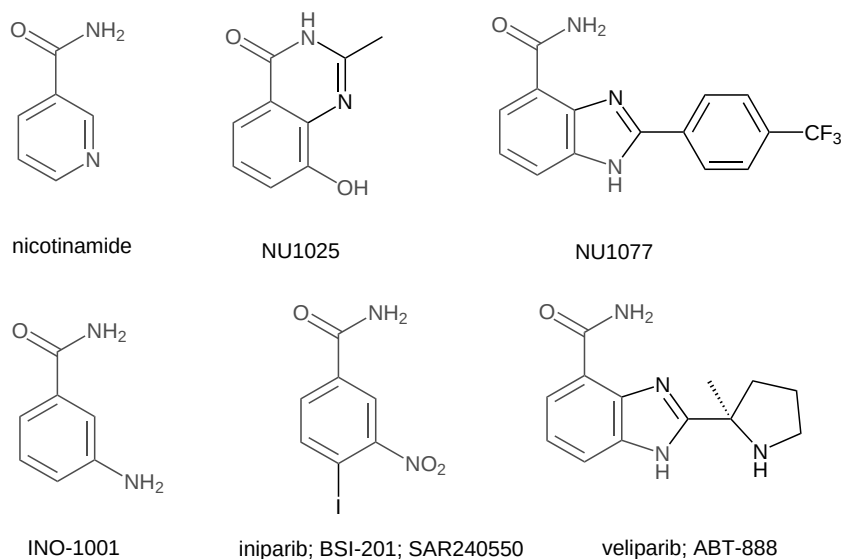


Figure 8.3 Initial nicotinamide-derived PARP inhibitors (with nicotinamide skeleton highlighted through exemplars). INO-1001, iniparib, and veliparib have been studied in clinical trials.

generally possessed greater structural size and complexity as well as improved properties against specific criteria. Figure 8.4 lists all the published structures for later PARP inhibitors that have been progressed into clinical evaluation, and which bear many common features. Many of these molecules, as well as others that have not progressed to clinical evaluation, have been crystallized with PARP revealing detailed information of both the mode of binding and the mechanism of inhibition. Additional mechanistic studies have revealed that the PARP-1 domains Zn1, Zn3, and WGR engage DNA as a monomer forming a network of interdomain contacts that link the DNA damage interface to the catalytic domain [49,50].

8.4.3

Clinical Development of PARP Inhibitors

From an analysis of all trials registered within the website www.clinicaltrials.gov, there are 93 investigations covering the range of PARP inhibitors described above as of 2012. Nearly all of these trials have no reported data, and despite the very large number of registered investigations, none of the molecules has yet reached the stage of being submitted for approval. The first clinical trial took place in 2003, investigating the role of PARP inhibition as a potential chemosensitizer to standard chemotherapy. Some of the subsequent trials utilized the findings published in 2005 of synthetic lethality with BRCA1/2 mutations, whereas others have continued the investigation of combination therapy or monotherapy in the absence of tumor selection (fuller list of trial

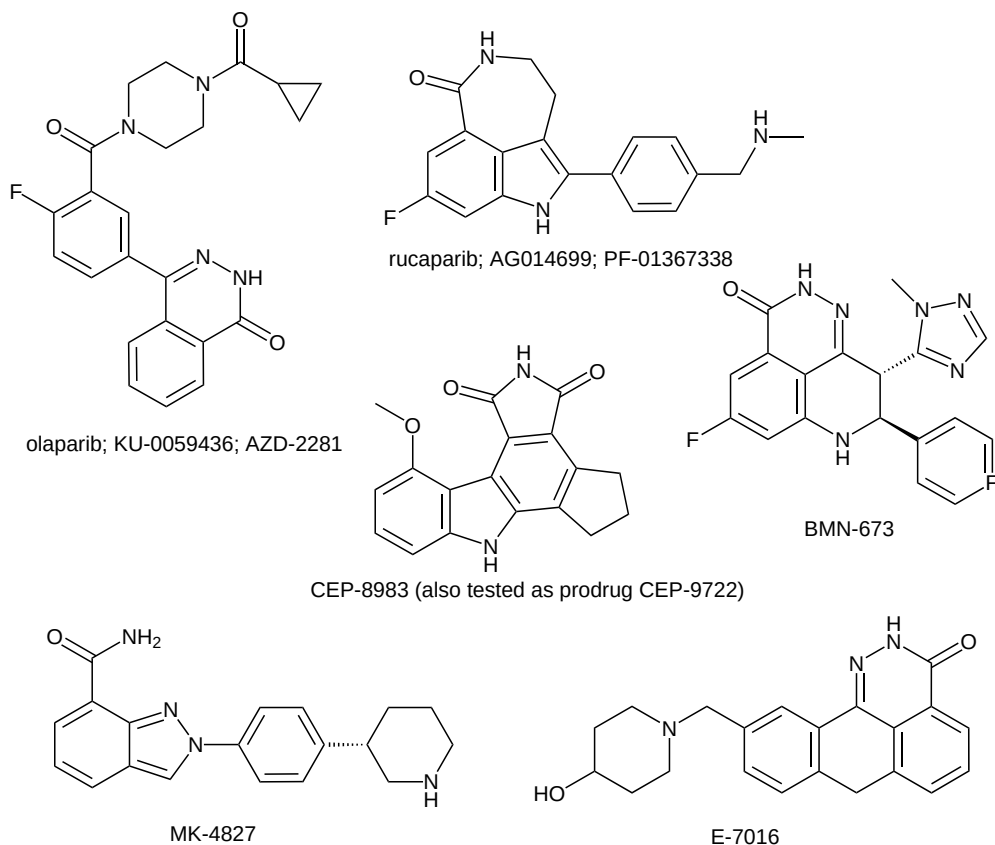


Figure 8.4 Later PARP inhibitors evaluated in clinical trials (structures not released for E7449 and AZD-2461).

details can be found in reviews from Chan *et al.* [9] and Underhill *et al.* [48] that also include references to all published trials for each molecule, which are too extensive to include in this chapter). Initial studies have confirmed that the PARP inhibitors are generally well tolerated and the successful phase II olaparib trial demonstrated that efficacy can be demonstrated clearly and chronically against BRCA-defective breast, ovarian, and prostate cancers, and as such represented the first clinical demonstration of synthetic lethality [51]. However, despite successful phase II efficacy data for iniparib in triple negative breast cancer in combination with carboplatin and gemcitabine, the phase III data did not meet primary endpoints. These data have inevitably led to a questioning of the true therapeutic potential of PARP inhibitors that can be considered unduly pessimistic, particularly given recent data claiming that iniparib is potentially not acting via inhibition of PARP [52]. While this is clearly unexpected given the number of clinical and preclinical studies already carried out using this molecule, it nonetheless serves to make two points. First, that the lack of

effective therapeutic options for several cancers has driven a synthetic lethality “gold rush” where the desire for initial registration as widely as possible seems to have deflected development from the path of obtaining the clearest signal. Second, still, relatively little is known about the desired selectivity profile for PARP inhibitors. Indeed, a recent study reported that many of the PARP inhibitors in ongoing clinical trials, including olaparib, ABT-888, and rucaparib, display equally strong stabilization of PARP1, PARP2, and PARP3 and substantial stabilization of PARP4 (rucaparib also showed substantial stabilization of PARP10, PARP12, PARP15, and PARP16 and olaparib of PARP12, PARP15, and PARP16) [53]. While this broad-spectrum inhibition of PARPs may lead to clinical efficacy, it is clear that future clinical study would be better driven by an informed inhibitory profile across the PARP and tankyrase families, and the current set of molecules in clinical development may be ill equipped to provide this vital information (Table 8.3).

8.4.4

Future for PARP Inhibitors

As a demonstration of the potential importance of understanding the role of PARP selectivity profiles, data published in 2005 demonstrated that PARP1 inhibition should be less toxic to normal cells than PARP2 inhibition (data from clonogenic cell survival using selective PARP silencing) [44]. Recent papers focus considerable attention on the PARP selectivity profiles that are rationalized using extensive protein–ligand crystal structures (as demonstrated by the increasing deposition of PARP ligand structures into the Protein Data Bank) [54,55], and future clinical candidates are to be expected with greater inhibitory selectivity within the PARP family.

However, despite the lack of clear data-supporting selectivity profiles, there has been an explosion of publications surrounding mechanistic interactions of PARP inhibitors with a range of other inhibitors, chemo- and radiotherapeutic approaches. In particular, groups have used siRNA screens (see below) to look for mutations other than BRCA1/2 that would show synthetic lethality with PARP inhibition, a number of which also lie, as would be expected, within the various components of homologous recombination. This search has been focused on identifying other mutations that confer a homologous recombination defective phenotype analogous to that obtained with BRCA1/2 mutations, often described as “BRCAness.” Candidate genes identified to date include PTEN (tumor suppressor gene that negatively regulates the PI3K pathway), DDB1 and XAB2 (genes involved in nucleotide excision repair), CDK1 (cyclin-dependent kinase 1 that plays a key role in cell cycle regulation), and many others within HR and related systems (fuller list in review from 2012) [7].

Important lessons have also been learnt from the current clinical studies that enable informed future study design. In particular, the level of target engagement required to drive clinical benefit has been studied through dose-finding phase II studies combined with attempts to identify markers of PARP inhibition. This

Table 8.3 Clinical trials of PARP inhibitors covering all trials available at www.clinicaltrials.gov as of 2012.

Compound	Number of clinical trials	Highest phase	Approach	Cancer type	Main sponsor(s)
E7016	1	Phase I	Combination with temozolomide	Solid tumors	Eisai
MK-4827	3	Phase II	Single agent	Solid tumors; mantle cell lymphoma	Merck
BMN-673	2	Phase I	Single agent	Solid tumors; hematological malignancy	Biomarin
Rucaparib; CO-338; AG-014699	4	Phase II	Single agent and in combination	Breast, ovarian, other solid tumors	Clovis; CRUK
PF-01367338					
CEP-9722 (CEP-8983 prodrug)	3	Phase II	Single agent and in combination	Solid tumors	Teva/Cephalon
Olaparib; AZD-2281, KU-0059436	24	Phase II	Single agent and in combination	Wide range of solid tumors – breast, ovarian, pancreatic, gastric	AstraZeneca; National Cancer Institute; CRUK; Massachusetts General Hospital; Dana-Farber Cancer Institute
Velaparib, ABT-888	30	Phase II	Single agent and in combination	Wide range of solid tumors – breast, ovarian, pancreatic, gastric	Abbott; National Cancer Institute; University of Michigan Cancer Center
Iniparib, BSI-201	20	Phase III	Single agent and in combination	Wide range of solid tumors – breast, ovarian, glioma, non-small cell lung cancer	Sanofi-Aventis; Centre Val d'Aurelle
INO-1001	4 (+ 2)	Phase II	Single agent and in combination	Breast cancer, melanoma	Inotek; Sanofi-Aventis; Genentech; National Cancer Institute

For INO-1001, two cardiovascular trials are registered in addition to four cancer trials.

correlative biomarker investigation comprised poly(ADP-ribose) polymer and γ H2AX foci formation, the latter originating from rapid phosphorylation of histone H2AX at serine 139 following double-strand DNA breaks. Small γ H2AX foci are associated with cell cycle regulation; medium foci ($0.30\text{--}9.99\mu\text{m}^3$) act as repair platforms and large γ H2AX foci are indicators of DNA double-strand break aggregates and colocalize with DNA double-strand break repair proteins [56]. The formation of both of these surrogate markers can be detected and quantified in tumor tissues as well as peripheral tissues such as plucked eyebrow hair follicles and peripheral blood mononuclear cells [57]. These data will be important for future trial design and dose selection; however, the additional observation of markers of DNA damage in normal cells following PARP inhibitor administration must also clearly be considered in dosing strategies to maximize tolerability.

8.5

Additional Case Studies

Numerous reports of increasing frequency describe findings of synthetic lethality or synthetic sensitivity, mainly in cancer cell lines, between many other pathways, proteins, or therapeutic approaches. Simplistically, these can be divided into those that sensitize cancer cells to a particular chemotherapeutic or radiotherapeutic approach or those that exploit an inherent weakness or dependence in the tumor cell to a specific protein or pathway as outlined above. The following are examples of both the approaches and serve to illustrate both the profound underlying complexity of the systems involved and the evident challenges of progressing any of these to the point of drug registration.

8.5.1

MLH1/MSH2

MLH1 and MSH2 are genes that encode for proteins involved in MMR and other DNA repair pathways. Both are tumor suppressor genes, and germ line mutations result in a predisposition to hereditary nonpolyposis colorectal cancer (comprises 5% of all colorectal cancer cases). Defects in MMR genes are believed to underlie 15% of colorectal cancers [58]. A screen in yeast identified these genes as having potentially synthetic lethal interactions with DNA polymerases, MSH2 with inhibitors of DNA Pol β and MLH1 with inhibitors of DNA Pol γ . Inhibition of both DNA polymerases leads to a rise in 8-oxoguanine oxidative DNA lesions. Furthermore, a rise in the frequency of these lesions was also detected following methotrexate (anti-metabolite and antifolate agent that inhibits DNA and RNA syntheses) treatment in MSH2 deficient cells, thus suggesting a synthetic lethal interaction [59]. More broadly, it has been shown in a variety of MMR-impaired cell lines that inhibition of PINK1 (PTEN-induced putative kinase 1) is synthetically lethal [60]. Together with the MMR-PARP information presented earlier, further approaches to synthetic lethality within the MMR pathway are anticipated (Table 8.4).

Table 8.4 Clinical trials for synthetic lethality agents other than PARP inhibitors (list not exhaustive – lead exemplars only).

Compound	Target	Highest phase	Approach	Cancer type	Main sponsor(s)
CC-115	Dual DNA-PK and TOR kinase inhibitor	Phase I	Single agent	Advanced solid tumors, non-Hodgkin's lymphoma, or multiple myeloma	Celgene
DT01	DNA-PK inhibitor	Phase I	With irradiation	Metastatic melanoma with relapsed cutaneous tumors	DNA therapeutics
LY2606368	Chk 1 inhibitor	Phase I	Single agent	Advanced/metastatic cancer, or squamous cell cancer of the head and neck	Eli Lilly
UCN-01	Nonspecific Chk 1 inhibitor	Phase II (22 trials)	Single or with irinotecan, perifosine, fludarabine, fluorouracil, topotecan, cytarabine, cisplatin, carboplatin, gemcitabine, or prednisone	Wide range of solid and advanced cancers	National Cancer Institute
PF-00477736	Chk1 and Chk2 inhibitor	Phase I	With gemcitabine	Advanced solid tumors	Pfizer
SCH 900776	Chk1 inhibitor	Phase I (three trials)	With gemcitabine, hydroxyurea, or cytarabine	Advanced solid tumours tumors or leukemia	Schering-Plough
X1844	Chk1 inhibitor	Phase I (two trials)	Single agent or with gemcitabine	Advanced solid tumors or leukemia	Exelixis
AZD7762	Chk1 and Chk2 inhibitor	Phase I (three trials)	With gemcitabine or irinotecan	Solid malignancies	AstraZeneca
Rabusertib, LY2603618	Chk 2 inhibitor	Phase II Phase II	With pemetrexed and cisplatin With gemcitabine	Non-small cell lung cancer Pancreatic cancer	Eli Lilly Eli Lilly

(continued)

Table 8.4 (Continued)

Compound	Target	Highest phase	Approach	Cancer type	Main sponsor(s)
Methoxyamine, TRC102	APE1	Phase I	With temozolomide	Advanced solid tumors	Case Comprehensive Cancer Center
		Phase I	With pemetrexed	Advanced or metastatic solid cancer	Tracon Pharmaceuticals
MK-1775	Wee1 inhibitor	Phase II	With carboplatin	Epithelial ovarian cancer	Merck
O ⁶ -benzylguanine	MGMT	Phase II	With carmustine	Glioblastoma	Merck
Lomeguatrib	MGMT	Phase II	With temozolomide	Melanoma	Kudos
Amuvatinib, MP470	Broad-spectrum Tyr kinase + RAD51 inhibitor	Phase II (four trials)	With topotecan, docetaxel, erlotinib, paclitaxel, or carboplatin	Solid malignancies or small-cell lung cancer or lymphoma	Astex

Information obtained from the website www.clinicaltrials.gov as of 2012.

8.5.2

p53-ATM

In wild type p53 cells, inhibition of ATM (Ser/Thr kinase that is recruited and activated by DNA double-strand breaks) results in increased sensitivity to DNA damage and thus in considerably increased toxicity following treatment with cisplatin or topoisomerase II poisons. However, in p53 deficient cells, inhibition of ATM results in a decreased sensitivity to DNA damage and consequently to a reduced sensitivity to DNA-damaging agents. This indicates a clear synthetic lethality between inhibition of ATM and DNA damage, but only if the cells have functional p53 (tumor suppressor protein that regulates the cell cycle) and hence loss of ATM can confer a survival advantage on cancer cells [61]. This example serves to illustrate the complexity of the DDR pathways and the need for both rational and random screening approaches to identify potential synthetic lethal or synthetic sensitive interactions as discussed below.

8.5.3

Chk1-DNA Repair

Checkpoint 1 (Chk1) is a Ser/Thr protein kinase that phosphorylates cdc25, an important phosphatase in cell cycle control and thus regulates entry into mitosis. Chk1 is activated by ATR, and to a lesser extent by ATM, following DNA damage and is responsible for the stability of stalled replication forks. Chk1 inhibitors, such as UNC-01, LY2606368, and AZD7762 (Table 9.4), inhibit Chk1 function, leading to accumulated DNA damage and ultimately to apoptosis and importantly are being evaluated for their potential to potentiate the cytotoxicity of widely used DNA-damaging agents. Early data from the initial molecule, UNC-01, have identified several synergies with DNA-damaging agents, although UNC-01 inhibits many protein kinases other than Chk1, including protein kinase C. Synthetic lethality screening preclinically has also recently uncovered interactions between both Chk2 (using inhibitor CCT241533) and PARP1 [62] and between Chk1 and Erk in leukemia and Src in myeloma [63].

8.5.4

DNA-PK – mTOR

DNA-dependent protein kinase (DNA-PK) is a Ser/Thr kinase (member of the PI3K-related kinase subfamily) that is activated upon DNA damage and plays a key role in repairing double-stranded DNA breaks via the DNA NHEJ pathway. Inhibitors of this kinase have been identified and progressed into clinical development showing potentiation of both radiotherapy and chemotherapy selectively in cancer cells [64]. In particular, CC-115, a dual inhibitor of DNA-PK and mammalian target of rapamycin, mTOR (Ser/Thr kinase widely upregulated in tumor cells and involved in signaling through the PI3K/Akt/mTOR pathway), has been identified with the potential to reduce cellular proliferation of cancer cells

expressing DNA-PK and TOR. In an alternative approach, DT01, currently in phase I, is a preparation of small interfering DNA (siDNA) that mimics double-strand DNA breaks and binds to and activates DNA-PK causing hyperphosphorylation of H2AX, interfering with the cell's ability to repair double strand breaks and hence overcoming resistance to certain mechanisms of radiotherapy and chemotherapy.

8.5.5

DNA Ligases

DNA ligation is an essential part of the replication process and, as such, DNA ligases are involved in nearly all DNA repair pathways. Inhibitors of the various DNA ligases would clearly be expected to potentiate the cytotoxic effects of DNA-damaging agents and to demonstrate potential synthetic sensitivity with DDR defects. Using the published crystal structure of human DNA ligase I complexed with nicked DNA, a virtual screen identified a number of inhibitors of the DNA ligases, which were further characterized and found to potentiate the cytotoxicity of alkylating agents preferentially in cancer cell lines [65].

8.5.6

WEE1

WEE1 is a tyrosine kinase that phosphorylates cyclin-dependent kinase 1 and, by doing so, inactivates the Cdk1/cyclin B complex. Small-molecule inhibitors of WEE1 have been identified that prevent Cdk1 phosphorylation and impair the function of the G2 DNA damage checkpoint, which could potentially be cytotoxic in the context of wider DNA damage (such as caused by chemotherapeutic agents). However, for greater differentiation between cancer cells and normal cells, a potential synthetic lethal interaction exists with p53-deficient tumor cells, as these cells do not have functional G1 checkpoints and so become overreliant on G2 checkpoints for DNA repair cycles. Consequently, WEE1 inhibitors that impair G2 checkpoint function have the potential to render p53-deficient tumors sensitive to DNA-damaging agents [66,67].

8.5.7

APE1

Human AP (apurinic/apyrimidinic) endonuclease, APE1, is the rate-limiting enzyme in the BER pathway for DNA adducts resulting from administration of alkylating agents. As such, inhibition of APE1, and thus of BER, results in an increase in DNA-strand breaks and the potentiation of the cytotoxicity of alkylating agents. Methoxyamine (TRC102) is an indirect inhibitor of APE1 in that it binds to AP sites and prevents Ape1 and Pol β from processing them further and completing repair [68].

8.5.8

MGMT

O⁶-methylguanine-DNA methyltransferase (MGMT) is involved in the direct repair DNA repair pathway, removing O⁶-alkylguanine, the major carcinogenic lesion produced in DNA by alkylating agents. Many inhibitors of MGMT have been produced with the intent to overcome the resistance that develops to DNA-damaging agents, and two of these have entered clinical trials, O⁶-benzylguanine and lomeguatrib. These have progressed to phase II study in combination with carmustine (bischloroethylnitrosourea, a mustard gas used as an alkylating agent) and temozolomide, respectively. However, these agents potentiated the tumor cell cytotoxicity of the DNA-damaging agents, and they also enhanced normal tissue toxicity and induced profound myelosuppression, requiring a reduction of the maximal dose and, as a result, no clear efficacy in the phase II study was obtained [69–71].

8.5.9

RAD51

MP470, amuvatinib, is a nonselective tyrosine kinase inhibitor that inhibits the mutant forms of the stem cell factor receptor c-Kit, inhibiting clinically relevant mutant forms associated with therapy resistance. Amuvatinib also inhibits a broad range of tyrosine kinase receptors including c-Met, Ret oncoprotein, and mutant forms of Flt3 and PDGFR α , which are frequently dysregulated in variety of tumors. It also disrupts DNA repair, potentially through suppression of Rad51, which plays a major role in homologous recombination, and by doing so, further potentiates the action of DNA-damaging agents (Figures 8.5 and 8.6).

8.6

Screening for Synthetic Lethality

From the initial studies reported earlier, a number of synthetic lethal studies have now been carried out in a range of model organisms. In particular, this field advanced enormously with the advent of RNAi screening technologies, allowing studies to move significantly beyond compound library screens. The budding yeast, *Saccharomyces cerevisiae*, remains an attractive model organism, and as such, the *Saccharomyces* Genome Deletion Consortium has created a fully annotated library of all yeast knockout genes [34]. These libraries have been extensively characterized and three main methodologies have emerged – synthetic genetic array (SGA), which was successfully used to screen a single mutant yeast strain against approximately 4700 other mutations by crossbreeding [72], synthetic lethal analysis by microarray (SLAM), which uses bar code sequence tags flanking gene sequence deletion sites to aid data deconvolution [73], and genetic interaction mapping (GIM), which affords increased sensitivity in the identification of synthetic lethal or synthetic sick phenotypes by combining both SGA and SLAM methodologies to identify subtle synthetic and epistatic interactions [74].

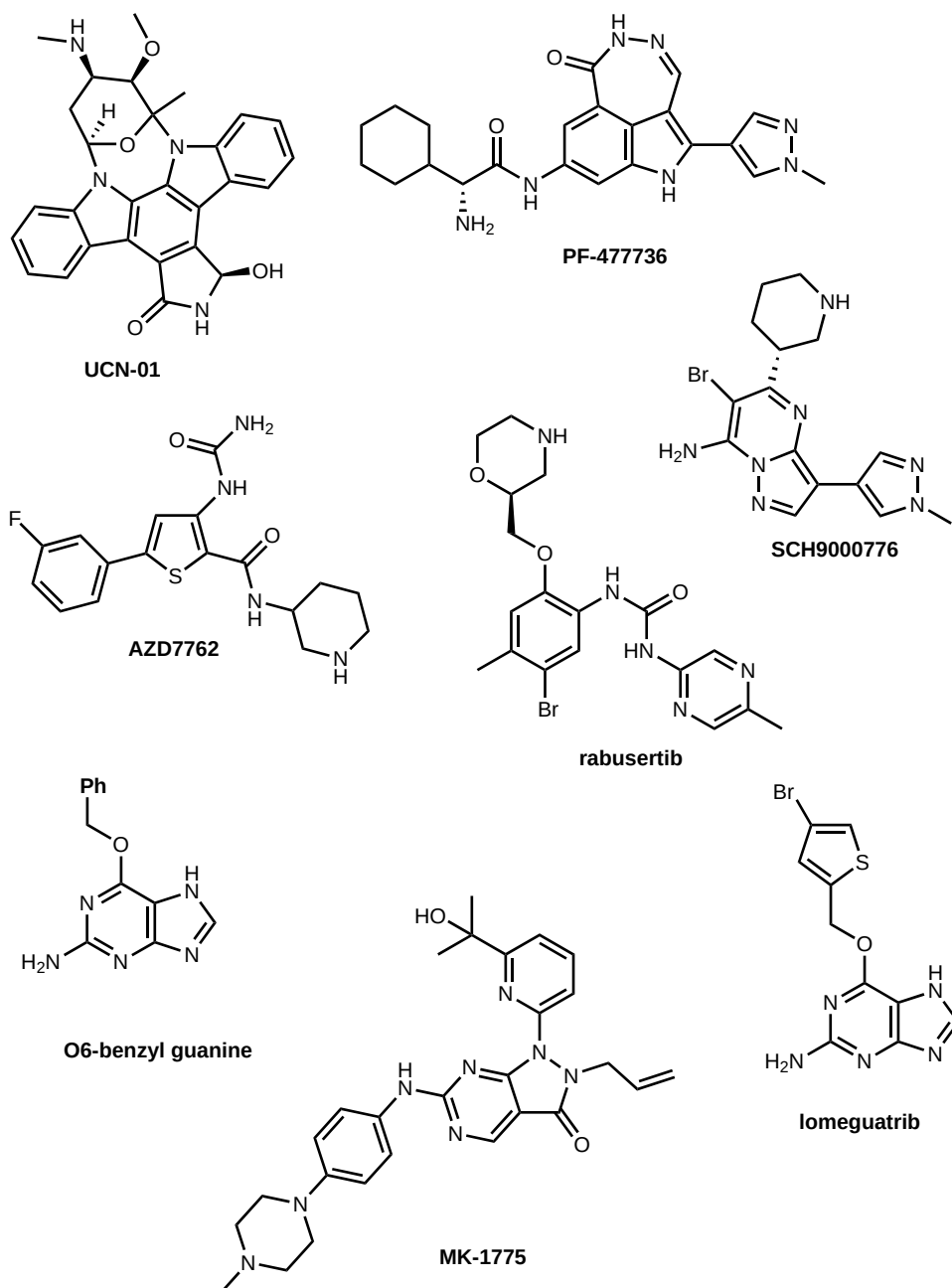


Figure 8.5 Selected structures of agents in clinical trials for synthetic lethality combinations (excluding PARP inhibitors).

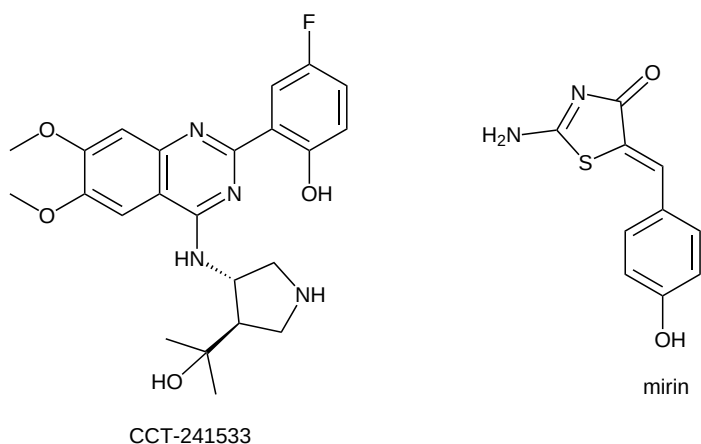


Figure 8.6 Selected structures of preclinical agents for synthetic lethality combinations (excluding PARP inhibitors).

More generally, there are two distinct approaches to link phenotype with genotype that correspond to the classical terms of forward and reverse genetics. For forward genetics, that is, starting with the phenotype and identifying the gene (s) responsible, the RNAi screen is run in genetically variable cancer cell lines looking for genes that when knocked down cause synthetic lethality. This has the advantage of generating data in directly relevant cancer cell lines, but can be difficult to make specific correlations. For reverse genetics, that is, starting with a defined mutation and understanding its effect in terms of phenotype, a specific mutation is engineered and the RNAi screen is run on this isogenic pair, thus simplifying interpretation but lacking the immediate assurance that the mutation will exist in the same state in the cancer cells (Figure 8.7) [75].

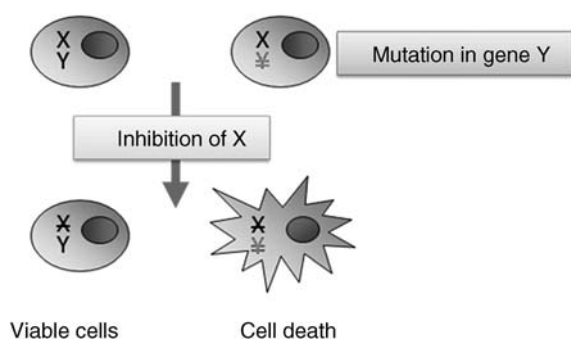


Figure 8.7 Synthetic lethal screen against mutation Y. Cell lines screened for wild type and mutant Y are treated with small-molecule inhibitors or RNAi against protein or gene X.

Single mutations in or inhibition of X and Y are viable, whereas loss of both leads to cell death, allowing identification of selective toxicity of inhibitor or RNAi of X.

Across the various screens used, both siRNA (short duplex RNA) and esiRNA (endoribonuclease prepared-siRNA) have been extensively used for high-throughput assays in individual cells, whereas shRNA (short hairpin vector encoded RNA) methods have been used to screen in pools, using genetically bar coded yeast knockout mutants to allow easy deconvolution of the data obtained [76–79]. Screens have been reported for both isogenic cell line pairs and heterogeneous cell lines against RNAi libraries, compound libraries, or a combination of the two. Regardless of the methodology, all require considerable confirmation through revalidation using follow-up RNAi and compound screens across a range of cell lines and models to ensure validity of the result. The inevitable next step is validation in a mouse xenograft model, which, despite widely acknowledged limitations, still remains widely used and provides required data for progression (or publication) [80].

Such screens have been reported to identify a number of putative synthetic lethal interactions:

8.6.1

RAS

A report in 2009 described an RNAi screen inhibiting approximately 1000 genes of relevance to cancer in 19 RAS mutant cell lines compared with wild type [81,82]. This uncovered a synthetic lethal association of RAS with NF- κ B activator TBK1 that was subsequently used in a mouse lung cancer model to provide support for the development of NF- κ B inhibitory drugs as targeted therapies for the treatment of patients with defined mutations in Kras and p53 [83]. A larger screen reported at the same time, utilizing the high-throughput pooling capacity of shRNA mentioned above, screened approximately 75 000 shRNA vectors against RAS isogenic clone mutants in a DLD1 colorectal cancer cell line [84]. This study uncovered potential synthetic lethal interactions against proteasome function and PLK1, while a later screen in HCT116 cells against 2500 genes uncovered putative synthetic lethality with SNAIL2 [85]. However, the wider observation to draw from the output of these studies is the different answers obtained by related studies. These can be used to highlight the potential shortcomings in RNAi screening – namely incomplete and variable knockdown and nonspecific RNAi toxicity leading to false positives. Furthermore, although isogenic cell line screening has the advantage of simplifying data interpretation, several groups have observed genetic drift [86] between isogenic cell line pairs that is particularly prominent in mutations associated with maintenance of genome stability.

8.6.2

VHL

VHL is a tumor suppressor gene that is associated with a range of rare tumors as well as over 80% of all spontaneous and hereditary kidney tumors. High-throughput shRNA screening in combination with small-molecule screening

was conducted looking for molecules that demonstrated synthetic lethality with VHL. Out of the 100 shRNAs screened against 88 kinases, three were found that preferentially impaired growth of VHL mutants in a dose-dependent manner, namely, CDK6 (catalytic subunit of a protein kinase complex important for cell cycle G1 phase progression and G1/S transition), MET (aberrantly active receptor commonly found in tumors enabling their growth and metastasis), and MAP2K1 (key component of the MAP kinase signal transduction pathway involved in many cellular processes such as proliferation, differentiation, transcription regulation, and development). To validate these findings, available inhibitors of CDK6 were profiled and found to cause preferential toxicity in VHL mutants [87,88].

8.6.3

MRN

The MRN (Mre11-Rad50-Nbs1)-ATM pathway is an essential component of the DDR response. The MRN complex acts as a DNA damage sensor, promotes both HR and NHEJ, and is a key component activator of the ATM kinase. A forward chemical genetic screen identified a number of small-molecule inhibitors of the MRN complex, and for the lead exemplar characterized (mirin), this inhibition of MRN was found to lead selectively to block of ATM kinase action. As expected, treatment with the MRN inhibitor abolished the G2/M checkpoint and homology-dependent repair, and although more potent analogs are required for further development, there is clear therapeutic potential in combination with existing cancer radio- or chemotherapies [89].

8.7

Contextual Synthetic Lethality Screening

In addition to the above screening, which seeks to identify interactions between fixed mutations and protein/gene states, newer approaches have been described that look to exploit the transient features that exist in cancer cells and that still allow for differentiation over normal cells. These transient states can occur in the microenvironment of the cell (changes in pH, oxygen levels, and so on) and/or can be induced by radio- and chemotherapy. Two recent examples highlight developments in this field. Chan *et al.* have used the existing observation that cells exposed to hypoxia have reduced homologous recombination capabilities and demonstrated that PARP inhibitor-treated xenografts displayed increased γ H2AX and cleaved caspase-3 expression in RAD51-deficient hypoxic subregions *in vivo*. This was claimed as a clear example of selective cell killing of HR-defective hypoxic cells *in vivo* and the first example of “contextual” synthetic lethality [90,91]. This idea has been further pursued by seeking to potentiate tumor hypoxia through exogenous agents (such as dichloroacetate) to further promote the selective toxicity of PARP inhibitors [81,91,92].

8.8

Cancer Stem Cells

Currently, much attention is focused on the group of cells within cancers termed cancer stem cells due to their common attributes with stem cells. There are many hypotheses and debates about the role and nature of these cells; however, one of the less contentious assertions is that these cancer stem cells are potentially more resistant to treatment than other cancer cells, and as such, are more likely to survive and be able to regenerate the cancer after radio- or chemotherapy. Empirical evidence for this comes from a number of studies, exemplified by the fact that in breast [93] and colon [94] tumor biopsies taken pre- and postchemotherapy; there are proportionally more cancer stem cells following treatment than before, suggesting that these cells may be more treatment resistant. Understanding the role of DDR in these cancer stem cells may provide a route to tackle one of the root causes of the cancer regrowth and hence disease relapse.

8.9

Conclusions and Future Directions

As for many cancer therapy approaches, the basic hypothesis makes sense. Cancer cells by definition have to distinguish themselves from normal tissue to achieve the various traits that confer oncogenicity, and these distinctive features then offer a means of therapeutic attack. Accepting that essentially all cancer cells have mutations in their DDR pathways means that understanding and harnessing these differences should deliver the long sought after cancer cell selective cytotoxic agent. However, as for all the major strategies that have gone before, the basic biology hypothesis does not translate easily into the clinical setting. This is reinforced in the setting of cancer cells with defective DDR, as the higher mutation frequency of these cells, which opens the therapeutic potential discussed, also leads to a greater propensity to evolve and more quickly develop resistance. The natural conclusion from this propensity is that the likely future therapies, regardless of the initial target precision, will inevitably rely on administration of a cocktail of drugs.

As the current targets under evaluation in the clinic progress, it will be important that lessons are learnt about the most effective way to bring these potentially valuable anticancer medicines to registration and patient use. While the PARP inhibitor story has appropriately excited many scientists and clinicians, it is clear that the initial enthusiasm has been tempered by clinical failures and a growing awareness of the broader complexities of both the potential for efficacy and also for toxicity. Furthermore, it is imperative that the clinical profiling of these synthetic lethality and DDR agents is developed with a modern diagnosis of the disease, rather than the historic anatomical and histological classification systems. Essentially, this requires cancer diagnosis by characterization of the underpinning mutations, allowing the therapeutic

strategy to be focused on tackling the cancer driver mutations. For example, to identify those cancers that should be responsive to PARP1 inhibition, the knowledge that homologous recombination-deficient tumors have a characteristic mutation fingerprint (defined by their reliance on more error prone double-strand break repair mechanisms) should allow clear patient stratification for therapy [95,96].

This approach, which will rely on the development of clinically appropriate biomarkers, should also open up many other possible combinations for targeting synthetically lethal or sensitive combinations than can currently be predicted by an incomplete knowledge of the interacting pathways. Initial signs are encouraging, and include the use of DNA damage-induced nuclear RAD51 foci, which reduce in HR-deficient cells, as a potential biomarker [97]. Notwithstanding the inherent challenges described, the next decade can be predicted to generate ever-increasing attention and effort on both the DDR pathways and the concept of synthetic lethality to bring about much needed and significant improvements in the current standard of care.

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9

Amyloid Chemical Probes and Theranostics: Steps Toward Personalized Medicine in Neurodegenerative Diseases*Maria Laura Bolognesi*

9.1

Introduction

Talking about personalized medicine in neurodegenerative diseases may be a bold step. These diseases are one of the most challenging and frustrating fields of drug discovery. At first glance, this might discount the possibility that personalized medicine will be viable in the field in the near future. But bold steps are called for by both the devastation of these diseases and the continuing failure of drug candidates developed through the current established drug discovery paradigms. Although it puts a heavy burden on our research capacities, we cannot ignore the increasing and substantial rate at which interest in personalized approaches is growing [1].

Neurodegenerative diseases related to dementia place an overwhelming toll on developed societies in terms of human suffering and economic cost. In those countries with longer life expectancies, these diseases are increasing enormously. The global prevalence of dementia is estimated to be as high as 24 million, but it is predicted to double every 20 years through to 2040. Alzheimer's disease (AD) is the leading cause of dementia and is characterized by a progressive cognitive decline, which typically begins with deterioration in memory. Other clinical symptoms include personality changes, disorientation, language impairment, and inability to carry out daily activities. Before death, individuals with this disorder usually become totally dependent upon their caregivers [2].

Drug discovery has not stood helplessly by. In parallel with the rise in cases, drug research has accelerated noticeably in recent decades. However, despite an exponential growth in investment, no effective drug has materialized [3]. The drugs on the market cannot cure AD or even stop it from progressing. The four acetylcholinesterase (AChE) inhibitors currently available achieve only palliative effects during the first stages of disease. They do this by increasing the cholinergic tone. The NMDA inhibitor memantine seems to improve symptoms related to excitotoxicity, but its neuroprotective activity is still debated [4].

Fortunately, the pipeline is not empty. In the last couple of years, patients and scientists have been awaiting the results of several pivotal phase III clinical trials [5–7]. However, the current prospects of success are quite low considering the continuing failure of all the drugs approaching the market. Since the withdrawal of Cognex in 2006, more than 200 AD drug candidates have failed in late-stage clinical trials [8]. In 2012, bapineuzumab and semagacestat joined the list, despite belonging to two different drug classes (a monoclonal antibody and a more conventional small molecule, respectively).

This difficulty in developing effective drugs has been attributed to several factors. These include a still incomplete understanding of AD etiology and the consequent lack of validated targets for therapeutic intervention [9]. Another potential factor is that AD drug candidates have failed because they address a pathology that is already too advanced [10]. This is a direct consequence of the lack of proper biomarkers for earlier diagnosis and follow-up of disease progression [11].

Indeed, the development of biomarkers would be critical for accelerating an AD cure. From a clinical point of view, AD would benefit immensely from a diagnostic biomarker that could define the disease state and predict its course. Biomarkers would also be invaluable for tracking drugs in clinical trials. This, in turn, would lead to more predictable outcomes and enhanced efficacy through the identification and stratification of drug responders. Biomarkers are also critical in the preclinical stages of the drug development process, where they disclose the candidate's pharmacokinetic profile (absorption, distribution, metabolism, excretion, toxicity, and blood–brain barrier (BBB) penetration), proof of principle, dose selection, and efficacy. All these data then guide important decisions in the drug discovery pipeline, such as lead compound selection, optimization, and candidate prioritization [12]. These concepts also apply to the diagnostic development pipeline, whose R&D activities are aligning more and more with those of pharmaceutical industries [13]. All in all, the routine use of biomarkers could be the first step toward an era of personalized medicine to treat AD (Figure 9.1).

9.2

Amyloid Plaques as the Biomarker in AD

In its broadest definition, a biomarker is any measurable biological feature that is useful for diagnosing or predicting a physiological or pathological condition (i.e., a phenotype). For AD, biomarkers are indispensable in clinics for distinguishing individuals with prodromic signs from healthy aging adults. Biomarkers have been described using several modalities, including imaging of cerebral β -amyloid ($A\beta$) deposition, studies of cerebral metabolism with fluoro-deoxy-D-glucose (FDG), magnetic resonance imaging (MRI) scans of brain volume, genotyping of genetic polymorphisms known to be associated with disease risk, and quantification of specific proteins (total tau, phosphorylated tau, and the 42 amino acid form of $A\beta$ ($A\beta_{42}$)) in the cerebrospinal fluid (CSF) or in the plasma [14].

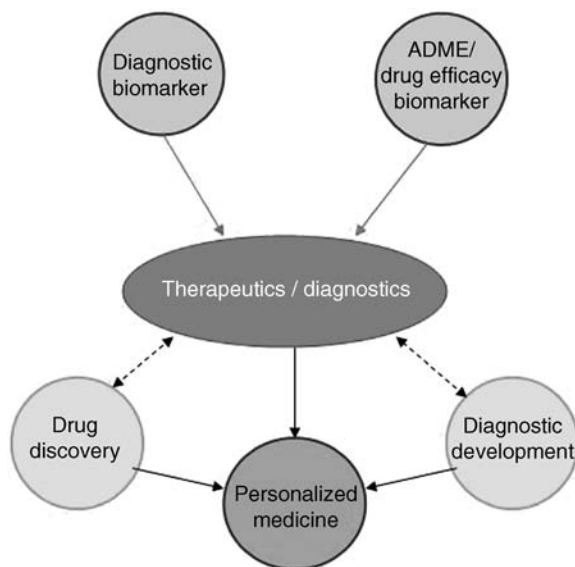


Figure 9.1 The use of biomarkers and targeted therapies inspired by an individual's molecular profile goes in the direction of personalized medicine. Biomarkers will allow researchers to prioritize compounds in the

pipeline and to better select patients during clinical trials. They will also impact the way drugs and diagnostics are developed and medicine is practiced, leading to a more integrated approach.

Of these, the brain deposition of $A\beta$ is a pathological hallmark of AD that is believed to precede clinical symptoms by several years [15]. This peculiar feature makes *in vivo* imaging of $A\beta$ particularly suitable for identifying individuals at risk and in the early stages of AD. Alois Alzheimer was the first to describe what he called “senile plaques” as the signature of his eponymous disease (together with the presence of neurofibrillary tangles and a diffuse neurodegeneration). For more than a century afterward, the disease was diagnosed as autopsy by the histological staining of brain amyloid accumulation. Through the years, the available information has grown enormously. Senile plaques have been characterized at a molecular level as proteinaceous aggregates mainly composed of $A\beta$ peptides. These $A\beta$ peptides are the cleavage product of the amyloid precursor protein (APP). They display an intrinsic tendency to aggregation. It is well established that the transition of $A\beta$ secondary structures from soluble disordered/ α -helix to β -sheet-rich conformers is the trigger of the aggregation process. In fact, β -sheet-rich structures have the tendency to associate with other monomers to form oligomers (dimers, trimers, etc.), protofibrils, and fibrils that then aggregate in plaques. The most widely held view is that aggregation of $A\beta$ peptide in oligomers initiates the vicious cycle that ultimately leads to neuronal death and to the disruption of memory and cognitive functions that characterize the disease [16].

This knowledge has been translated into anti-amyloid therapeutic strategies with putative disease-modifying effects, which are being tested in ongoing clinical trials [17]. Importantly, the tremendous advances in molecular imaging have allowed us to use the biomarker proposed by Alzheimer in living human brain. With an appropriate imaging probe, it is nowadays possible to image the amyloid deposition noninvasively directly in AD patients [18]. The possibility of using amyloid as a biomarker allows, in turn, the identification of preclinical cases as candidates for early intervention. It also allows the effectiveness of anti-amyloid therapy to be followed in individual patients [18].

Although amyloid aggregates have been clearly associated with AD pathology [19], the scientific community has been debating whether or not their presence precisely predicts the course of the disease. If it is not a universally validated biomarker, it has been unequivocally demonstrated that, in familial autosomal dominant AD, the progressive cognitive impairment is associated with brain amyloid deposition, albeit in association with other pathophysiological changes in CSF biochemical markers [20]. Thus, the current diagnostic trend is a combined use of multiple imaging biomarkers, which are not mutually exclusive. Each one has unique strengths and weaknesses [21]. Following the premise that diagnostic imaging of amyloid might advance personalized medicine in AD, some selected amyloid imaging probes will be discussed herein.

9.3

Detecting Amyloid Plaques in Patients: from Alois Alzheimer to Amyvid and Beyond

The use of chemical probes for imaging biomarkers *in vivo* has a long history in drug discovery. PET (positron emission tomography) is the technique used most often to monitor changes in the amyloid deposits in AD patients [22]. Although it may sound pleonastic, it is important to remark that the fundamental prerequisite for any A β imaging agent is that it effectively images A β cerebral deposits. This means that its amount in a given brain area must be quantitatively related to the amount of A β in that area and there must be sufficient signal-to-noise ratio [23]. Another important consideration in amyloid imaging is that the target for all currently available A β imaging agents is fibrillar A β in a β -sheet conformation. As already mentioned, the main culprit of AD pathology has been identified in the A β oligomeric species [16]. Although it is possible that amyloid tracers could bind to oligomers of A β in a β -sheet conformation once they reach a necessary size (probably at least a trimer or tetramer), the *in vivo* signal of amyloid tracers is not directly representative of these species. This is because of their lower concentration with respect to insoluble A β aggregates. However, on the basis of the equilibrium existing between monomers, oligomers, and fibrillar A β , a relationship exists between the amyloid PET signal and oligomer concentration [21].

Until April 2012 (see below), PET imaging of amyloid in AD patients was used only as a research protocol by employing several radiotracers. From a

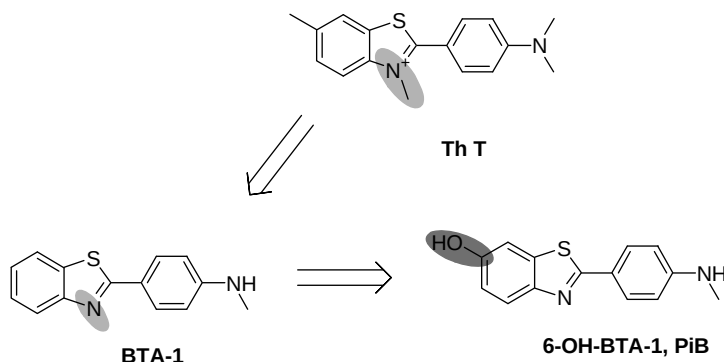


Figure 9.2 Design strategy leading from the *in vitro* dye thioflavin T (ThT) to the radiotracer candidate PiB with optimal properties.

medicinal chemistry perspective, most of them derived from thioflavin-T (ThT) (Figure 9.2), as they share a common benzothiazole pharmacophoric function. ThT is a fluorescent dye used to detect the presence of amyloid fibrils in autaptic tissues and to monitor fibril formation *in vitro* [24]. ThT acts as an amyloid sensor because, upon binding to β -sheet-rich fibrils, it exhibits a red-shift in its fluorescence excitation spectrum with a concomitant emission enhancement. This peculiar behavior stems from two effects that occur upon binding of ThT to the fibrils: (i) steric and electronic stabilization (via charge transfer) of the ground-state charge distribution, and (ii) restriction in the rotational freedom of the carbon-carbon bond between the benzothiazole and aniline rings in its electronically excited state [25]. The ThT behavior was conveniently exploited by LeVine to set up a versatile, inexpensive assay for monitoring fibril formation [26], which is still the most widely used method for monitoring A β aggregation *in vitro*. In addition, ThT revealed a valuable starting point when searching for molecules able to visualize amyloid aggregates *in vivo*. However, it suffers from two major drawbacks that hinder its clinical use in patients. First, ThT has a permanent positive charge and does not easily permeate the BBB. Second, its binding affinity to fibrils is quite low. Several congeners have been synthesized in the search for more lipophilic and potent derivatives. SAR on ThT structure revealed that the benzothiazole could be replaced by a similar aromatic, planar benzofuran, imidazopyridine, benzoxazole, or imidazole scaffold, without dramatically decreasing affinity. In addition, the aniline may be substituted with other flat, rigid moieties (e.g., stilbene) without significantly negative effects. Insertion of a planar styrene group between the benzothiazole and aniline is also well tolerated, while some substitutions, such as bithiophenes, even increase affinity [25]. In 2001, Klunk *et al.* reported that the uncharged ThT analog BTA-1 (Figure 9.2) possessed very good brain entry and a higher affinity for A β deposits in AD brain [27]. A further SAR campaign identified a hydroxylated derivative (*N*-methyl-2-(4'-methylaminophenyl)-6-hydroxybenzothiazole; 6-OH-BTA-1) (Figure 9.2) with clearance

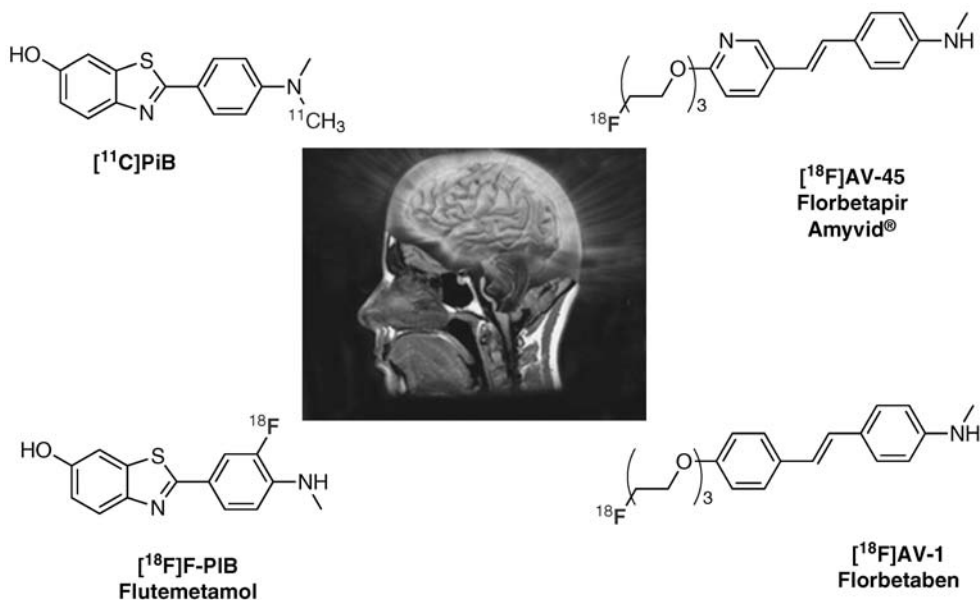


Figure 9.3 Chemical structures of amyloid imaging tracers that have been developed for PET studies in AD patients. The short half-life of the carbon-11 radiolabel has limited the use of PiB to research, whereas a second

generation of tracers labeled with fluorine-18 (flutemetamol, florbetapir, and florbetaben) has made it possible for amyloid PET to enter the clinics.

properties that are optimal for PET radiotracers. Preclinical studies showed that it bound to AD brain with an affinity in the single-digit nanomolar range and, more importantly, entered the brain rapidly and cleared rapidly as well [28]. Therefore, the ¹¹C-labeled *N*-methyl-2-(4'-methylaminophenyl)-6-hydroxybenzothiazole was selected for the first human trial of a benzothiazole amyloid-imaging agent. The compound was given the Uppsala University PET Center code of "Pittsburgh Compound-B" or, simply, PiB, the acronym by which it is universally known. Thanks to its favorable profile, PiB has become the most commonly used PET amyloid agent, adopted in more than 40 research centers worldwide [29] for the direct visualization of brain amyloid plaques in AD-related research studies (Figure 9.3).

Unfortunately, due to its short half-life (20 min), the ¹¹C label on PiB limits its use to major academic PET facilities equipped with cyclotrons and sophisticated radiochemistry infrastructures. To increase the availability of Aβ imaging to all PET facilities worldwide, derivatives of PiB labeled with the longer half-life (110 min) radioisotope ¹⁸F have been developed and evaluated *in vivo*. This shift from a ¹¹C- to ¹⁸F-labeled tracer was expected to significantly enhance the impact of amyloid imaging on research and clinical practice [30]. Flutemetamol ([¹⁸F]F-PiB or GE-067) (Figure 9.3) is a closely related derivative of ¹¹C-PiB,

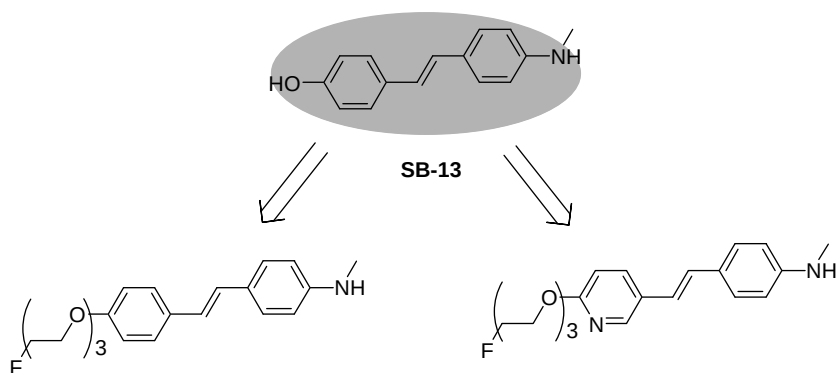


Figure 9.4 Polyethyleneglycol stilbenes and styrylpyridines as cold PET imaging agents. The molecules derive from SB-13 by the insertion of a PEG tether, which allows an easy label with ^{18}F and a suitable lipophilicity.

which differs from the prototype only by the presence of the ^{18}F -fluorine in position 3'. It is currently in a promising phase III clinical trial in Europe, sponsored by GE Healthcare [31].

Similarly, scientists have sought alternative scaffolds to be radiolabeled with ^{18}F , namely, stilbenes and styrylpyridines. These scaffolds also comply with the requirements of planar molecules with extended pi-delocalized systems. They have provided core structures for developing many specific imaging agents for A β plaques [32,33]. Of these, a low-molecular-weight and neutral stilbene derivative, SB-13 (Figure 9.4), demonstrated excellent binding affinity and labeling of A β plaques [34]. A preliminary PET human study using ^{11}C -SB-13 revealed excellent differential increased uptake and retention in the frontal cortex of AD patients (where A β plaques are concentrated), when compared to control subjects [35]. Despite these outstanding preliminary results, no further patient studies using this probe were performed, probably due to the technical limitation associated with ^{11}C . However, these promising data provided incentives to search for SB-13 derivatives more suitably labeled with ^{18}F . Initial attempts were made to develop ^{18}F -labeled probes by adding a fluoroalkyl substituent on either aromatic ring of SB-13. But these provided derivatives that were too lipophilic and showed high nonspecific binding in the brain [30]. To overcome the problem associated with an excessive lipophilicity and to provide a simple ^{18}F labeling procedure, a series of fluoropegylated stilbene derivatives was successfully prepared and tested (Figure 9.4) [36]. The derivative with 3 PEG unit showed high binding to amyloid and an excellent bioavailability, demonstrating that fluorinated PEG was an efficient and effective prosthetic group for ^{18}F labeling. Thus, the same design strategy was applied to the styrylpyridine core structure (Figure 9.4) [37]. As a result of these parallel studies, two structurally similar fluorinated PEG agents are being developed commercially: BAY 94-9172 (florbetaben in clinical trials by Schering/Bayer) [38] and ^{18}F -AV-45 (florbetapir developed by Avid Radiopharmaceuticals as Amyvid) (Figure 9.3) [39]. With the Food and Drug Administration approval of

Amyvid in April 2012, we are now entering the era of clinical amyloid imaging in AD [40]. Hot on the heels of approval for florbetapir, the results of a multicenter phase 3 autopsy trial suggest that florbetaben accurately images plaques and may also prove useful for AD diagnosis.

In addition to the development of amyloid chemical probes, nanotechnology approaches have very recently begun to impact the diagnosis of AD [41]. The majority of efforts are focused on detecting amyloid plaques by MRI using nanoparticles doped with contrast agents, or by nanoparticles tagged with fluorescent probes. Although it is beyond the scope of this chapter, some representative examples will be briefly discussed to highlight the importance of this research to the AD field. Fluorescent–magnetic gamma-Fe₂O₃-rhodamine or gamma-Fe₂O₃-Congo red nanoparticles have been successfully used to label A β fibrils [42]. These fluorescent nanoparticles are promising multimodal imaging agents, which have the great advantage of combining the magnetic and fluorescence imaging into one nanostructured system. This system might enable the early detection of plaques using both MRI and fluorescence microscopy. It therefore holds potential for *in vivo* AD studies.

The use of polymeric nanoparticles is emerging as one of the most promising approaches for brain drug delivery. Nanoparticles composed of a polystyrene core and a degradable PBCA [poly(butyl-2-cyanoacrylate)] shell have been developed as carriers for ThT and thioflavin S. Interestingly, fluorescence spectrophotometric analysis demonstrated that encapsulated dyes exhibited significantly stronger fluorescence than the free forms. The enzymatic degradation of core–shell nanoparticles, as required *in vivo*, was effectively shown after their treatment with esterases *in vitro*. Shells of nanoparticles were dose-dependently degraded, while their polystyrene cores remained intact. In the brain of APP/PS1 mice with age-dependent cortical beta-amyloidosis, both thioflavins selectively targeted fibrillar A β after biodegradation-induced release from their nanoparticle carriers upon intracerebral injection. This study highlights that core–shell nanoparticles with controlled degradation *in vivo* can become versatile tools for tracing A β in the brain [43].

9.4

Same Causes, Same Imaging Agents?

Amyloid aggregates are not a distinctive feature of AD. They are a feature shared by a diverse set of important diseases grouped under the collective name of protein misfolding diseases (PMD).

Striking evidence links amyloid peptides to the pathogenesis of AD, Parkinson's disease, and prion diseases. But it is far from clear how these peptides exert their toxic effects and where in neuronal cells they act. Needless to say, detecting amyloid aggregates and understanding the mechanism of fibrillization in molecular details are very important goals in the diagnostic field. They have therapeutic implications for all PMD. Expanding on the idea that the same pathological mechanism can

be exploited to develop drugs that cure more than one PMD, we might consider that they can inspire the development of similar imaging agents.

Notably, one of the most remarkable features of the PMD group is that many of the aggregated species that are associated with the different pathological conditions have a wide range of characteristics in common. These include various tinctorial properties, such as those associated with ThT and other dyes, as well as structural indicators, such as a characteristic “cross- β ” X-ray fiber diffraction pattern. The aggregates are often fibrillar in appearance and seem to have similar ultrastructures when observed by electron microscopy or atomic force microscopy techniques. This commonality has allowed the recent development of a reagent that can selectively capture diverse misfolded proteins by interacting with a common supramolecular feature of protein aggregates [44]. From a drug discovery perspective, such findings raise fascinating questions about the possibility of common strategies for monitoring the aggregation process and for therapeutic intervention. However, from a diagnostic point of view, specificity is required for a differential diagnosis.

Thus, although this approach seems to be moving away from the idea of personalized medicine (the right drug for the right person administered at the right time), it is an issue that might merit attention.

9.5

Theranostics in AD

One pillar of personalized medicine is the development of companion diagnostics, whereby molecular assays that measure levels of proteins, genes, or specific mutations are used to provide a specific therapy for an individual's condition by stratifying the disease status, selecting the proper medication, and tailoring dosages to that patient's specific needs. The first example of a companion diagnostic was the coapproval of trastuzumab (Herceptin[®]) from Genentech and the HercepTest[®] from Dako in 2000 [45]. Since then, companion diagnostics exist not only in oncology but also across therapeutic areas where they allow doctors to define treatment benefits and identify the “best patients” for a given treatment approach.

In AD, PiB is the only diagnostic that has been used in conjunction with a therapeutic tool. The extent of ¹¹C-PiB PET signal was, in fact, the primary outcome of recent clinical trials of bapineuzumab [46] in the United Kingdom and Finland [47]. In light of the recent failure of bapineuzumab, it is important to highlight that the most likely progress from these efforts will be in stratifying AD subpopulations to set up a more “personalized” clinical study.

Despite the few applications in the neurodegenerative field, it is generally acknowledged that researchers need to develop strategies and tools for combined diagnosis and treatment, in terms of the personalized medicine concept [48]. Theranostics can be such a tool. “Theranostics” was a term coined to epitomize the inseparability of diagnosis and therapy. It refers to the fusion of therapeutic and diagnostic properties in a single agent. The purpose of theranostics is to optimize

the efficacy and safety of therapy, as well as to streamline the entire drug development process [49].

Theranostics are already a reality in cancer treatment [50,51]. Usually they are multifunctional nanomedicines composed of molecular targeting vectors (e.g., peptides) labeled with either diagnostic radionuclides (e.g., positron or gamma emitters) or therapeutic radionuclides for diagnosis and therapy, respectively, of a particular disease, targeted specifically by the vector at its molecular level. Thus, molecular imaging and diagnosis of the disease can be effectively followed by personalized treatment using a single device. In AD, there are only a few such applications, but, in 2008, Gütschow and coworkers reported a pioneer work describing the development of a potential theranostic small molecule for AD in the field of AChE inhibitors [52]. This molecule (PE154) [53] was inspired by considering the so-called “nonclassical” function of enzyme AChE in AD pathogenesis, and in particular in the amyloid pathway.

There is a huge body of evidence to suggest that AChE, similar to other molecular chaperones, accelerates the assembly of A β peptide into amyloid fibrils [54–57]. The peripheral anionic site (PAS) of AChE has been identified as responsible for this proaggregating activity. This has provided the rationale for the development of the so-called dual inhibitors of AChE, that is, molecules targeting both the active site and the PAS of the enzyme. As the relevance of this phenomenon in AD pathogenesis has been demonstrated *in vivo*, its discovery has resulted in a new generation of AChEIs exhibiting two pharmacological properties simultaneously, that is, the enhancement of the cholinergic transmission and the inhibition of A β aggregation [58–60]. On this basis, Gütschow and coworkers envisioned that a fluorescent inhibitor addressing both binding sites of the enzyme could be useful for characterizing AChE inside A β plaques, while the cholinergic activity could be beneficial from a therapeutic point of view [52]. Such a molecule (PE-154) (Figure 9.5), combining both therapeutic and diagnostic properties, emerges as a theranostic for AD. The design of PE-154 was inspired by an established class of heterodimeric cholinesterase inhibitors with a coumarin fluorophore binding the PAS, while a phenyl linker spans the gorge to locate a tacrine-derived moiety at the active site. Using this probe, brain samples from mice and AD patients were successfully stained, although it was found that the compound binds directly to amyloid structures, rather than to cholinesterases inside the plaques. PE-154 showed a concomitant excellent picomolar inhibitory activity against human AChE. In addition, it targeted hippocampal A β deposits in mice after injection of nanoparticles, delivering the fluorescent marker *in vivo* [53].

9.6

Conclusions and Perspectives

As in all therapeutic fields, researchers will have to reconsider the “one-size-fits-all” approach to the development of new drugs against neurodegenerative disease. It is becoming increasingly clear that one therapy will not be optimal for all patients.

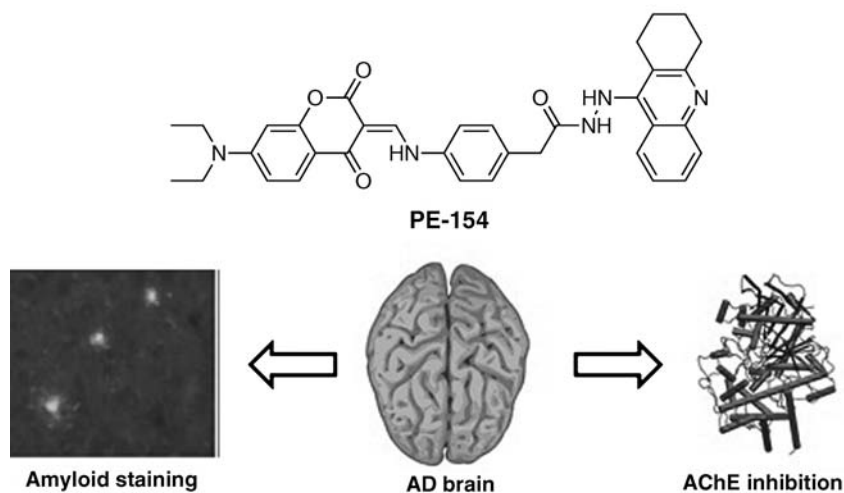


Figure 9.5 The combined diagnostic and therapeutic features of PE154. It detects A β plaques *in vivo* and inhibits tissue-bound AChE with outstanding affinity.

The recent progress in understanding the molecular basis of neurodegenerative mechanisms is opening opportunities for matching therapies to patient populations, thus paving the way for a more personalized medicine.

In this context, biomarkers might be of great help. In addition to their critical role in facilitating drug development, biomarkers can be used for within-patient dose titration during clinical trials, thereby effectively individualizing therapy. For example, a patient without amyloid plaques in the brain is less likely to respond to a drug that targets A β , whereas patients screened into the study with a positive plaque biomarker at baseline may not respond to the low starting dose, therefore warranting escalation to the next dose [61].

At this point, one could argue that amyloid is not the right biomarker in AD. This cannot be excluded. A recent study proposes that the severity of cognitive impairment correlates best with the burden of neocortical neurofibrillary tangles [62]. Thus, the development of tau imaging agents is emerging as an active area of research. Several groups around the world are working to develop imaging agents to assess tau deposition *in vivo*. Although researchers have already disclosed brain-penetrating compounds capable of binding tau aggregates with high affinity, the major barrier to progress remains with the need for tau selectivity relative to A β plaques and other deposits of proteins in cross- β -sheet conformation. In this scenario, we should avoid reproducing the disagreement we have encountered in AD etiology between scientists who believe that A β (baptists) is the major culprit and those who believe that tau is the major culprit (tauists). Rather, we should create an environment where progress in both basic biomedical science and technology can contribute to advancing personalized medicine.

There is an evident need for interactions between basic research and technological research, with clinical science. Such interactions should be encouraged, and fortunately, are already encouraged by all stakeholders involved in drug discovery in Europe and the United States. EMEA and FDA have identified “development of biomarkers” as a high priority in general, and in dementia in particular [63]. At the same time, integrated and interdisciplinary research efforts by academia, the pharmaceutical industry, and the regulatory agencies will be critical for accelerating the discovery and codevelopment of new and more informative AD biomarkers for broad clinical diagnostic use, as well as for the many ongoing clinical trials. In this context, although it is a bold step, the concepts discussed herein suggest that biomarker-based relationships with pathophysiology could increase our overall understanding of this devastating disease and could be important for both optimizing and personalizing AD management.

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10

From Human Genetics to Drug Candidates: An Industrial Perspective on LRRK2 Inhibition as a Treatment for Parkinson's Disease

Haitao Zhu, Hui fen Chen, William Cho, Anthony A. Estrada, and Zachary K. Sweeney

10.1

Introduction

Effective therapies are urgently needed for patients who suffer from neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (PD). Currently available medicines only temporarily address some of the symptoms of these disorders, and they do not impact the underlying rate of neurological decline. In the past decade, genetic studies have been used to understand why certain individuals are more likely to develop a given neurological disorder. By associating a likelihood of developing a disease with the structure or expression of a specific gene, these studies also provide potential protein targets for drug discovery programs.

This chapter focuses on LRRK2, a protein that has been linked to PD in a number of genetic studies. Emphasis has been placed on progress that has been made toward the development of technologies that will enable the discovery and clinical evaluation of LRRK2 kinase inhibitors. As a putatively druggable target for pharmaceutical intervention, LRRK2 is an important test case for the idea that the genetic studies will lead to useful treatments for neurodegenerative diseases. In the last decade, there have been over 500 publications focused on this remarkable protein. However, the myriad tools necessary for the development of LRRK2 inhibitors are still under development, and it is likely that informed clinical trials with these compounds will not begin for several years.

PD is a slowly progressive movement disorder that affects approximately 1–2% of the population over 65 years of age [1]. Patients with advanced disease commonly present with an uncontrolled tremor and have difficulty initiating movement. Depression, insomnia, and gastrointestinal disorders are also frequently associated with PD and substantially impact patient quality of life. The motor deficiencies associated with PD primarily result from the loss of function of neurons in the brain that manufacture the neurotransmitter dopamine. Postmortem examination of the brains of PD patients usually reveals substantial degeneration of dopaminergic neurons. Deposits of insoluble proteins and the loss of neuronal integrity in

regions of the brain not associated with movement control or dopamine signaling are also regularly observed [2].

Dopamine replacement therapy is generally an efficacious approach for relieving the motor symptoms of PD in early stages of the disease. Importantly however, as these drugs do not influence the rate of neuronal degeneration, they generally lose efficacy once a patient's ability to produce and transport dopamine has fallen below a certain threshold. These therapies also do not reduce the nonmotor symptoms of PD. There is therefore a great medical need in disease-modifying therapies [2].

For many years, Parkinson's disease was thought to be primarily environmental in origin. Human genetic studies in the last 15 years have challenged this understanding of the etiology of PD and identified multiple genes to be causal factors for PD [3]. These analyses have also provided alternative targets and approaches for PD drug discovery. It is now accepted that a significant minority of patients that present with PD have known genetic variants that strongly predispose them to the development of this disease. Familial studies have established associations between familial forms of PD with different proteins. However, these familial diseases usually have an unusually early age of onset or are otherwise easily distinguished clinically from sporadic PD.

The most common genetic variants that cause a form of PD closely related to sporadic PD are found in the gene LRRK2 [4]. This association was first discovered in genetic studies of several families with an inherited form of late-onset PD [5,6]. In these families, inheritance of LRRK2-familial PD occurs in an autosomal dominant fashion. The typical age of onset of disease symptoms is about 60, as it is in sporadic PD. The symptoms and course of LRRK2-associated PD are generally very similar or indistinguishable from those of sporadic PD. Postmortem pathology of individuals with LRRK2-familial PD has been variable, but dopaminergic neuron loss and Lewy body formation are usually observed.

Genotyping studies of LRRK2 exonic variants have confirmed that several mutations located in different regions of the protein are causal for the development of PD (Figure 10.1), including N1437H [7], R1441G/C/H [5,6,8], G2019S [9], and I2020T [6]. In addition, a relatively common G2385R mutation has been shown to be a risk factor in the Asian population [10].

Large genome-wide association studies have also identified LRRK2 as a risk factor for the development of PD [11,12]. The association appears to be independent of the presence of the relatively common LRRK2[G2019S] variant. These results further link LRRK2 to the development of nonfamilial, "sporadic"

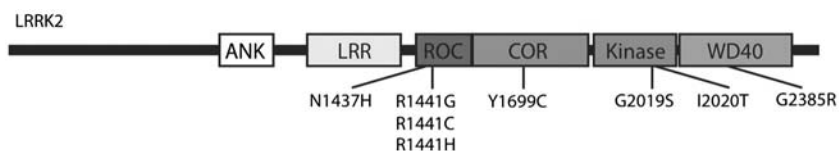


Figure 10.1 Schematic of LRRK2 protein structure with confirmed familial PD mutations and risk factors.

PD, and suggest that modification of the normal function of LRRK2 may be a broadly useful approach to the treatment of this disease.

Supported by the strong genetic evidence linking LRRK2 function to PD disease development, the community of scientists devoted to the development of LRRK2 inhibitors has turned its attention to answering the following questions:

- 1) What does LRRK2 do *in vivo* and what LRRK2-related activities can be monitored and linked to PD disease progression?
- 2) Can inhibitors of this function be identified, and will these inhibitors have pharmacological properties, including *in vivo* safety profiles, that will support their use as PD medicines?
- 3) What cellular and animal models of PD should be used in LRRK2 drug discovery programs? How can studies utilizing these models be expected to further validate LRRK2 as a therapeutic target?
- 4) Will inhibitors of LRRK2 function be useful for slowing the rate of disease progression, and will this intervention have broad utility for PD treatment or will efficacy be limited to individuals with “LRRK2-driven” PD?

10.2

Biochemical Studies of LRRK2 Function

LRRK2 is a serine/threonine kinase and a member of the ROCO family of proteins. This rare class of molecules contains both guanine triphosphatase (GTPase) and kinase domains that are linked by a C-terminal of Roc (COR) domain of unknown function. The N-terminal and C-terminal portions of the protein feature large hydrophobic sequences that are similar to regions in other proteins known to engage in protein–protein interactions. The variations in LRRK2 sequence associated with familial PD are mostly located in the GTPase and kinase domains. The G2019S LRRK2 protein, the most common variant associated with familial PD, contains a hydrophilic serine residue in place of the flexible glycine residue on the kinase activation loop. As analogous modifications in other kinases result in increased kinase activity, it was proposed that this modification could increase the kinase activity of LRRK2.

Immediately following the discovery of the genetic association between LRRK2 and PD, scientists began to investigate the impact that disease-associated variations have on the enzymatic activity of LRRK2. Biochemical evidence that the disease-associated variants were abnormally active (e.g., had increased kinase or GTPase activity) would support genetic evidence that inhibitors of these functions might reduce disease progression.

Various biochemical assays have been developed to measure LRRK2 kinase activity *in vitro*, including autophosphorylation [13], phosphorylation of myelin basic protein [13], and phosphorylation of peptide substrates [14,15]. *In vitro* studies demonstrate that either magnesium or manganese can function as the catalytic

metal ion for phosphate transfer [16,17]. The identity of the metal ion can influence the association of the kinase with ATP and the observed rate of phosphorylation activity. In *in vitro* kinase assays, it is uniformly acknowledged that LRRK2[G2019S] has increased kinase activity compared with wild type. However, there is little consensus with respect to the impact of other LRRK2 PD mutations on kinase activity. This ambiguity likely derives, in part, from the impact of the various substrates and reaction conditions used in the kinase activity assays [18].

The peptide substrate sequence preferences of purified LRRK2 *in vitro* were thoroughly explored by the MRC Dundee group [14,15] and the Pfizer group [19]. Iterative optimization of the peptide sequence led to the conclusion that LRRK2 preferentially phosphorylates threonine-containing peptides over serine containing peptides *in vitro*. The optimized substrate sequence, WWRFYTLRRA (Nictide), was incorporated into the phosphorylated moesin sequence to provide a substrate suitable for use in assays monitoring LRRK2 kinase activity [15].

LRRK2 catalyzes autophosphorylation at a number of different sites *in vitro*, including notable clusters within the GTPase and kinase domains [20–24]. Cookson and coworkers used mass spectrometry to first demonstrate LRRK2 autophosphorylation of T1343 and T1491 within the GTPase domain [20–24]. Ueffing and coworkers identified additional LRRK2 autophosphorylation sites [24]. Phosphospecific antibodies developed toward pThr 1503 [21] and pT1967 [23] recognize specific phosphoepitopes on purified proteins after *in vitro* autophosphorylation reactions. However, the majority of these autophosphorylation sites were not detected *in vivo* [20–24].

The GTPase activity of LRRK2 ROC domain has also been investigated. In general, purified LRRK2 contains measurable GTPase activity [25,26]. As the GTPase domain regulates kinase activity in a number of systems, it was proposed that the ROC domain of LRRK2 might regulate its kinase activity. Indeed, the LRRK2[K1347A] and LRRK2[T1348N] mutations, which are located in the GTPase domain of the protein, have been shown to prevent GTP binding and eliminate kinase activity *in vitro* [27,28]. However, although PD mutations in the GTPase domain (N1437H and R1441G/C/H) have been shown to reduce GTPase activity and/or increase GTP binding [7,29], they have not been consistently shown to affect kinase activity *in vitro* [18]. Furthermore, binding of a nonhydrolyzable GTP analog does not affect kinase activity *in vitro* [30,31]. These data suggest that increased GTP binding may not affect kinase activity, at least *in vitro*. Since GTPase and kinase activities are likely to be affected by protein–protein interactions, whether the LRRK2 ROC domain regulates its kinase activity needs to be investigated *in vivo*.

10.3

Cellular Studies of LRRK2 Function

A more complete understanding of the role of LRRK2 in cellular systems might lead to the discovery of biomarkers and drug targets beyond those identified in

biochemical studies. In addition, the safety and efficacy profile of LRRK2 modulators might be anticipated from studies of LRRK2 function in a cellular environment.

Ectopic expression of mutant LRRK2 in primary cultured neurons or SH-SY5Y cells appears to cause cell death by apoptosis [20,32–36]. A number of laboratories have found that overexpression of LRRK2 reduces neurite length [37–40]. Reduction of the kinase activity of the LRRK2 protein, either by addition of kinase inhibitors or by the introduction of LRRK2 kinase-dead mutations, reduces the effect of LRRK2 overexpression, suggesting that LRRK2 kinase activity is required for cellular toxicity [33,34,37,41–43]. Lee *et al.* found that treatment of cells with the nonselective kinase inhibitors GW5074 and indirubin-3'-monoxime attenuated neurite shortening and cell death induced by overexpression LRRK2[G2019S] [41]. In primary human cortical neurons, the potent and selective LRRK2 kinase inhibitor CZC-25146 inhibited reduction of neurite length caused by transfection with LRRK2[R1441C] [42]. Although neurite shortening in cell cultures is a relatively crude model of PD-related neurodegeneration, these studies do provide some support for the idea that excessive LRRK2 kinase activity may be undesirable.

Cell death and neurite outgrowth cellular assays feature an appealing functional effect associated with LRRK2 kinase activity, but they are not ideal *primary* tools for the discovery and optimization of LRRK2 kinase inhibitors. Cellular pharmacology studies with less potent or selective kinase inhibitors can be misleading, as such compounds often have neurotrophic or neurotoxic effects that are independent of their interactions with LRRK2. Furthermore, in most cases, medicinal chemists must iteratively modify the cellular potency and selectivity of LRRK2 kinase inhibitors starting from moderately selective and potent scaffolds. The individual differences in activity derived from minor structural changes can be small in magnitude, and accurate cellular potency data are indispensable. The most useful cellular kinase assays for drug discovery therefore exploit a direct readout of kinase activity that can be precisely quantified.

It has been recognized for some time that LRRK2 kinase function might be accurately monitored *in vitro* and *in vivo* if a unique and robust phosphorylation marker of kinase activity on LRRK2 or another protein could be identified. As described above, candidate LRRK2 phosphorylation substrates have been identified in a variety of biochemical and cellular systems. Phosphorylation assays are also particularly interesting as they naturally incorporate a biomarker that might be used for *in vivo* studies.

Several groups have described their efforts to assess the potential of 4E-BP, a protein involved in regulation of translation initiation, as a marker of LRRK2 kinase activity in cells. Genetic studies on *Drosophila* by Imai *et al.* ultimately led to the discovery that dLRRK2 can phosphorylate 4E-BP *in vitro* [44]. Overexpression of hLRRK2 was reported to increase the levels of phosphorylated 4E-BP in heterologous cells, while siRNA knockdown of LRRK2 reduced the levels of phosphorylated 4E-BP.

The Merck group also investigated the relationship between LRRK2 kinase activity and 4E-BP phosphorylation [45]. These studies employed selective

inhibitors of LRRK2 kinase activity that were identified from *in vitro* screens. LRRK2 kinase inhibitors unexpectedly increased the phosphorylation of 4E-BP in primary neurons in a dose-dependent manner. Furthermore, time course experiments revealed that up to 24 h is required to observe the full effects of these inhibitors on 4E-BP phosphorylation. These results challenge the link between LRRK2 kinase activity and 4E-BP phosphorylation status in cellular contexts.

A number of academic and industrial laboratories have attempted to determine if LRRK2 phosphorylation can also be used as a biomarker for LRRK2 kinase activity in cells. Alessi and coworkers found that LRRK2 is phosphorylated at Ser910 and Ser935 in cells [46]. Structurally distinct LRRK2 kinase inhibitors induced a dose-dependent dephosphorylation of LRRK2 at these sites [46]. The link between LRRK2 kinase activity and phosphorylation at these sites was further established by experiments in which a designed LRRK2 protein resistant to the kinase inhibitors was not dephosphorylated at Ser910 or Ser935. Interestingly however, dephosphorylated, immunoprecipitated LRRK2 did not autophosphorylate at these residues in *in vitro* experiments. These findings led the authors to propose a model in which LRRK2 kinase activity indirectly regulates phosphorylation of Ser910 and Ser935. Furthermore, association of LRRK2 with 14-3-3 proteins appears to be dependent on the phosphorylation of Ser910 and Ser935. These results have been independently confirmed in BAC transgenic mice overexpressing LRRK2[G2019S] [47].

Autophosphorylation sites on LRRK2 have also been investigated as biomarkers of kinase activity by the Genentech group [48]. Of the many *in vitro* autophosphorylation sites identified, Ser1292 was the only site of that could be confirmed in a cellular context. In contrast to the variable *in vitro* results, in the more relevant cellular context, five LRRK2 variants associated with familial PD (LRRK2[N1437H], LRRK2[R1441G], LRRK2[R1441C], LRRK2[G2019S], and LRRK2[I2020T]) displayed elevated autophosphorylation activity. Studies using transgenic mice confirmed that levels of phosphorylated protein were higher in animals expressing LRRK2[G2019S] protein than in transgenic animals expressing equivalent amounts of wild type LRRK2. Interestingly, mutation of Ser1292 to alanine ameliorates the neurite outgrowth defects induced by LRRK2 PD mutations in cultured primary neurons. These results suggest that increased autophosphorylation of Ser1292 is a common feature for LRRK2-familial PD mutations and that phosphorylation at this residue may contribute to the toxicity mechanisms underlying LRRK2 PD pathogenesis.

LRRK2[pSer1292] was used to develop a cell-based assay to measure LRRK2 kinase activity. A large number of structurally diverse LRRK2 kinase inhibitors were interrogated using this system. Cellular IC₅₀ values obtained in these studies were highly correlated with LRRK2 inhibition constants obtained in biochemical screens. Brain-penetrable LRRK2 inhibitors could also be used to reduce levels of LRRK2[pSer1292] following oral administration in BAC transgenic mice expressing LRRK2[G2019S] protein. A clear relationship was observed between levels of LRRK2[pSer1292] and unbound brain drug concentrations. Extension of these findings to human tissue samples could enable clinical pharmacodynamic studies

of LRRK2 kinase inhibitors, and would make antibodies against LRRK2[pS1292] useful tools for conducting clinical trials with LRRK2 kinase inhibitors.

To summarize, cellular studies of LRRK2 function have now provided important technologies and insights into LRRK2 function. These systems have confirmed that many of the PD-associated LRRK2 variants have higher kinase activity (as judged by pSer1292 phosphorylation) than wild type LRRK2. From a broad functional perspective, the kinase activity of LRRK2 is reproducibly associated with reduced cellular viability and growth and LRRK2 kinase inhibition can reduce these deficits. Finally, and perhaps more importantly, a biomarker of LRRK2 function useful for measuring inhibition of LRRK2 enzymatic activity in animals has been discovered.

10.4

Animal Models of LRRK2 Function

Many animal models have been developed to recapitulate the pathophysiological features of Parkinson's disease. One class of animal models is based on damaging dopaminergic neurons in rodents or nonhuman primates by administering neurotoxins such as MPTP, 6-hydroxy-dopamine, and rotenone [49]. In general, these models present significant loss of dopaminergic neurons in the substantia nigra and show deficits in locomotive behavior that resemble movement disabilities found in PD patients. These models have been useful for preclinical testing of dopamine replacement drugs designed to relieve the motor symptoms of Parkinson's disease. However, they are not good models for testing disease-modifying drugs because the rapid course of neurodegeneration in these models does not resemble the gradual, age-dependent neurodegeneration of PD patients.

Another major class of PD animal models relies on the genetic manipulation of PD gene expression in mice using transgenic or viral vector expression systems. Several transgenic mouse models have been created to express LRRK2[G2019S] or LRRK2[R1441G] proteins [50–54]. These animal models display little or no loss of dopaminergic neurons in the substantia nigra even after extensive aging. Alterations of dopamine physiology in the brain have been observed in many of these animals. Locomotive behavioral defects were identified in some of the animal models. As the phenotypes observed in these models are subtle, it will be challenging to use these models for preclinical validation of drugs that modulate LRRK2 function. Viral vector-mediated expression of LRRK2[G2019S] has been reported to cause dopaminergic neuron death in both mice and rats [41,55]. Both kinase-dead mutation and a nonselective LRRK2 kinase inhibitor have been shown to ameliorate the phenotypes, suggesting that LRRK2 kinase activity is involved. However, more selective LRRK2 kinase inhibitors need to be tested in these systems in order to confirm the initial findings.

LRRK2 knockout mouse models have been generated by multiple groups [52,56–58]. The central nervous system of the LRRK2 knockout mice appears to be

normal. No functional impairment of dopaminergic system has been identified, although subtle locomotive behavioral abnormalities were observed in one model [58]. In the periphery, pathological phenotypes were found in the lung and kidney [52,57]. Specifically, a marked increase in the number and size of secondary lysosomes in kidney proximal tubule cells and lamellar bodies in lung type II cells were observed. Importantly, kinase-dead knock-in mouse showed a similar pathology in the kidney but not in the lung. Furthermore, both kinase-dead knock-in mice and wild type mice treated with an LRRK2 inhibitor showed a marked reduction of LRRK2 protein levels [52]. These data suggest that potential adverse consequences of LRRK2 inhibition in the periphery should be taken into consideration when developing LRRK2 kinase inhibitors for clinical studies.

10.5

Clinical Studies of LRRK2-Associated PD and Future Prospects

The clinical characteristics of LRRK2-associated PD are largely identical to sporadic PD cases [59]. From a treatment perspective, several options exist for the symptomatic treatment of familial or sporadic Parkinson's disease, including L-DOPA formulations, dopamine agonists, COMT inhibitors, MAO-B inhibitors, anticholinergic agents, and amantadine (reviewed in Ref. [60]). These symptomatic treatments are inadequate for the long-term treatment of PD because of drug side effects, limited efficacy for the reduction of nonmotor symptoms, and gradual loss of efficacy.

Disease-modifying treatments would not only offer symptomatic relief for motor symptoms but may also treat nonmotor deficits and exhibit long-lasting, clinically meaningful impacts on patient function. LRRK2 inhibition may represent a novel mechanism for disease-modifying treatment of PD, not only in LRRK2 carriers but also in sporadic PD, depending on the nature and degree to which LRRK2 may be involved in the pathophysiology of sporadic PD.

Demonstration of disease modification offers many challenges in clinical trial design and execution. A key challenge is that the evidence required to demonstrate disease modification is not clearly defined. The FDA has not provided explicit guidance; however, the EMEA has provided some perspective on this issue. According to the Committee for Medicinal Products for Human Use (CHMP), in order to claim that a given treatment is "disease modifying," a two-step procedure is foreseen: (1) a delay in clinical measures of disease progression should be shown and (2) an effect on the underlying pathophysiology process that correlates to meaningful and persistent changes in clinical function should be demonstrated (EMA Guideline on Clinical Investigation of Medicinal Products in the Treatment of Parkinson's Disease, Doc. Ref. CPMP/EWP/563/95 Rev.1, 2008). As the mechanism by which normal or mutant LRRK2 function interacts with PD-related pathophysiology remains unclear, the identification of biomarkers that demonstrate an effect on LRRK2-specific pathophysiology process will be difficult.

In the absence of a clear LRRK2-associated biomarker, a more general biomarker of PD progression could be incorporated into a trial that aims to demonstrate

disease-modifying efficacy. One of the most widely studied biomarker strategies for measuring PD disease progression is dopaminergic system neuroimaging. Various surrogates for visualizing dopaminergic neuronal integrity have been evaluated, including ^{18}F -DOPA uptake for assessing striatal DAT function by PET, PET and SPECT ligands for direct visualization of the DAT transporter, and PET studies using VMAT ligands (reviewed in Ref. [61,62]). Some of these techniques have already been used to study LRRK2 mutation carriers [63–66], and preliminary results suggest that the imaging characteristics of symptomatic LRRK2 mutation carriers are not dissimilar from those of sporadic PD. An important caveat is that imaging endpoints as surrogates for DA neuron integrity or function may be confounded by direct pharmacological effects or regulatory effects on the molecular targets of the imaging ligands [67–69]. It is unknown how an inhibitor of LRRK2 activity may interact with the molecular targets of these imaging probes. Therefore, an imaging study to demonstrate that an LRRK2-targeted treatment reduces DA neuron degeneration may need to be preceded by a careful molecular study to rule out the effects of LRRK2 inhibition on regulation or expression of DAT or VMAT in DA neurons.

An additional challenge to using imaging biomarkers to measure drug-related modification of disease progression is the slow rate of disease progression relative to imaging endpoint variability. For example, the rate of disease progression using β -CIT SPECT is approximately 5–10% per year [62,70], while the test–retest variance for these techniques may be in a similar range [71,72]. Therefore, measurable change in disease progression that exceeds the variability of the imaging endpoint would likely occur only after 2 years. Furthermore, to measure an intervention-related *reduction* in disease progression would require an even longer trial, or a trial that is much larger, to detect a modest effect in this rate of decline.

Lengthy clinical trials required to demonstrate disease modification will likely lead to participant dropouts. This will be especially problematic if a novel LRRK2 inhibitor is explored as a single-agent therapy. A placebo-controlled LRRK2 inhibitor monotherapy trial is probably not ethically feasible in people with advanced disease since the course of disease progression is well known, and treatments (at least symptomatic treatments) are currently available. Therefore, a monotherapy treatment trial would need to enroll newly diagnosed or recently symptomatic PD patients. The DATATOP study in such early PD patients encountered dropout rates (that is, the point at which clinical assessment determined that trial participants required levodopa treatment) of approximately 20–40% per year, depending on the treatment to which the participant was assigned [73]. For a multiyear study, this dropout rate will be problematic for a monotherapy trial. Mitigation strategies may include testing a novel LRRK2 inhibitor as an adjunct to standard of care therapy. However, as previously mentioned, the potential effects of concomitant medications on imaging endpoints may be of concern. Alternatively, a clinical trial may enroll participants with known LRRK2 mutations prior to the onset of symptoms. However, there may be ethical challenges to introducing “treatments,” with their associated treatment risks, to

those who do not yet manifest any signs or symptoms of disease. Even if ethically feasible, demonstration of efficacy would likely either require lengthy trials to demonstrate prevention or delay of clinical onset, or would be based solely on a biomarker change. A trial based solely on a biomarker change may be insufficient to demonstrate efficacy in the eyes of regulators.

Finally, a clinical trial with a novel LRRK2 inhibitor may prove to be operationally difficult. Demonstration of efficacy with a novel LRRK2 inhibitor would be most likely to succeed in a population of pathogenic LRRK2 mutation carriers. LRRK2 mutations, although the most common genetic mutations associated with PD, are rare. The most common LRRK2 mutation (G2019S) is estimated to represent only 1% of all PD cases [74]. This prevalence rate is too small to make genetic screening practical. Therefore, a clinical trial will require access to known populations of people carrying LRRK2 mutations. The Michael J. Fox Foundation is making efforts to form a consortium of clinical investigators to coalesce LRRK2 patients and data worldwide for potential study (<https://www.michaeljfox.org/foundation/publication-detail.html?id=275>). Several important questions exist regarding how this valuable resource will be utilized. Will this population of patients be distributed across the multiple research groups who are actively pursuing research programs in LRRK2? Will access to these patients be managed such that there is a competitive process to select the “best” drug candidate to be tested in this population? Several significant LRRK2 populations have been identified outside of the US, particularly the Arab Berber population in North Africa [75]. It remains to be seen if a clinical study, especially one incorporating technologically demanding biomarker measure, is possible in North African countries.

In summary, LRRK2 inhibition offers hope for a disease-modifying therapy for PD. However, there are multiple operational and scientific hurdles to conducting meaningful disease-modifying clinical trials. Success will depend on identification of a robust biomarker of PD progression or, even better, a biomarker that directly measures LRRK2-relevant biology. In addition, evidence that LRRK2 inhibition will benefit sporadic cases of PD will greatly increase the feasibility of well-powered clinical trials.

10.6

Small-Molecule Inhibitors of LRRK2

The substantial efforts to study the activity of LRRK2 *in vitro* and *in vivo* have provided scientists interested in discovering new medicines with technologies for the expression and isolation of various forms of the protein. It is clear from these studies that the LRRK2[G2019S] protein possesses increased kinase activity. This supports a therapeutic strategy for the treatment of LRRK2-associated PD designed to reduce the kinase activity of LRRK2. A number of clinically efficacious inhibitors of kinase function have been developed in the last 20 years. In the process of discovering these molecules, the pharmaceutical industry has acquired a variety of

methods for rapidly identifying potent and selective ATP-competitive kinase inhibitors. Simultaneous to these developments, efficient strategies for discovering molecules that are capable of crossing the blood–brain barrier and accessing targets in the central nervous system have been introduced. Inhibition of the kinase activity of LRRK2 in the brain of humans therefore seems to be an eminently feasible proposition. The combination of target “druggability” and genetic support for this therapeutic strategy has made LRRK2 a unique target for the treatment of PD and stimulated drug discovery efforts in nearly every major pharmaceutical company.

Effective small-molecule LRRK2 inhibitors are necessary for a variety of fundamental *in vitro* and *in vivo* studies interrogating the consequences of inhibiting LRRK2 function. In order to be useful in cellular studies of LRRK2 function, an inhibitor should have excellent biochemical and cellular potency and kinase specificity. An LRRK2 inhibitor useful for *in vivo* experiments as well as cellular experiments must also have pharmacokinetic properties that enable LRRK2 function to be inhibited in the brain and other tissues for a substantial portion of a dosing interval. Finally, an LRRK2 inhibitor suitable for clinical development needs to display a low risk of inducing drug–drug interactions and a minimal risk of inducing genetic abnormalities.

10.7

Structural Models of the LRRK2 Kinase Domain

LRRK2 kinase domain crystal structures are not currently publicly available. In order to gain structural understanding of the functional effects of LRRK2 mutations and potential binding modes of small-molecule inhibitors, researchers have developed three-dimensional models of the LRRK2 kinase domain. The most crucial elements for building these models are the choice of structural templates and sequence alignment. Because of the low sequence similarity between LRRK2 and other protein kinases, it is not obvious which structures are the most appropriate templates for homology modeling. Based on the kinome phylogenetic tree analysis by Manning *et al.*, the LRRK2 kinase domain belongs to the tyrosine kinase-like (TKL) subfamily of serine/threonine protein kinases [76]. Several models have been constructed using TKL kinase structures such as B-Raf [77,78], TGF-beta activated kinase 1 (TAK1) [79], and TGF-beta receptor kinase 1 as templates [80].

LRRK2 homology models have also been constructed using structural templates outside of the TKL subfamily. Mata *et al.* developed a model using the tyrosine kinase LCK as a template based on sequence homology and availability of structures with bound substrates [81]. The Gray group reported an LRRK2 model built using an anaplastic lymphoma kinase (ALK, tyrosine kinase subfamily) crystal structure [82].

Nichols *et al.* [15] and Chen *et al.* [83] employed an inhibition profile-based approach in template selection for their homology models. Nichols *et al.* used

ROCK1 from the AGC subfamily as a template based on the observation that some selective ROCK1 kinase inhibitors displayed similar activity for LRRK2. This ROCK1-derived model was used to predict the binding mode of the kinase inhibitor H-1152 by superimposing the protein backbone C α atoms of the LRRK2 model with the ROCK1/H-1152 crystal structure (PDB code: 3d9v). Chen *et al.* utilized a JAK2 crystal structure as a template based on the analysis of in-house kinase profiling data of LRRK2 inhibitors identified in a high-throughput screening campaign. The JAK2-based LRRK2 homology model proved to be very robust in predicting binding modes and guiding the optimization of structurally diverse LRRK2 inhibitors.

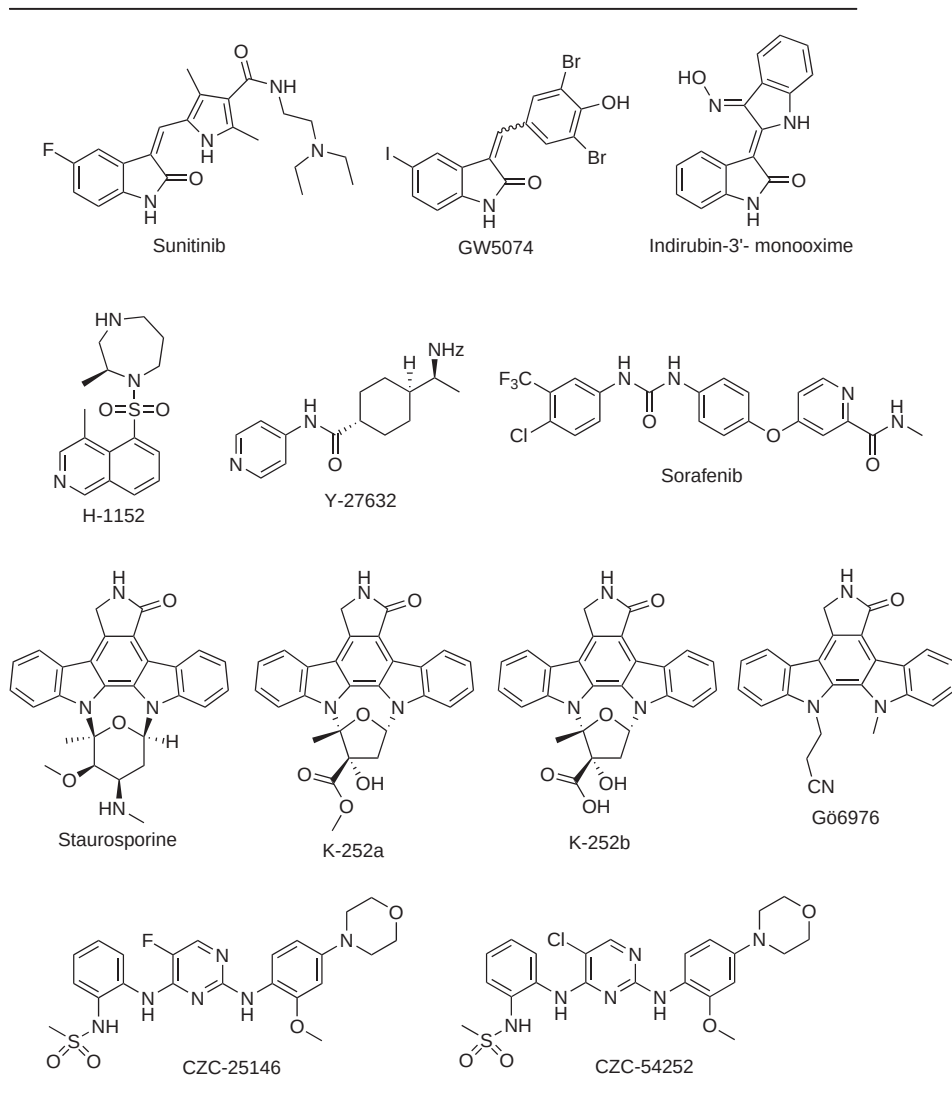
Recently, Gilsbach *et al.* reported several *Dictyostelium discoideum* Roco4 kinase domain crystal structures with both wild type and PD-related mutant proteins [84]. Roco4 is a member of the Roco family and has a similar domain topology to LRRK2. The Roco4 kinase domain likely shares both sequence and structural similarity to LRRK2 [78], suggesting that Roco4 structures may be used as structural templates for building LRRK2 homology models. The utility of homology models built using the Roco4 structures remains to be demonstrated.

10.8

Strategies Used to Identify LRRK2 Kinase Inhibitors (Overview)

As highlighted in a recent review, several groups have identified LRRK2 inhibitors from high-throughput or targeted biochemical screening [85]. The earliest approaches to identifying small-molecule inhibitors of the LRRK2 kinase involved screening nonselective kinase inhibitors. Nichols *et al.* were the first group to demonstrate the inhibition of LRRK2[G2019S] using the known promiscuous kinase inhibitor sunitinib and selected ROCK inhibitors such as H-1152 and Y-27632 (Table 10.1) [15,85]. Broad-spectrum kinase inhibitors, such as sorafenib, staurosporine, and structurally related derivatives K-252 a/b and Gö6976, were also reported as low micromolar to low nanomolar inhibitors of LRRK2[G2019S] (Table 10.1) [17,41,86]. Using a targeted screening approach, Lee *et al.* tested a set of 84 kinase and phosphatase inhibitors, which resulted in the identification of indirubin-3'-monoxime and Raf inhibitor GW5074 as moderately potent LRRK2 inhibitors (Table 10.1) [41]. LRRK2 small-molecule inhibitors that were discovered using the above-mentioned approaches served critical roles in the exploration of the relationship between wild type LRRK2 and various LRRK2 mutations, full-length versus truncated LRRK2 constructs, and validation of *in vitro* high-throughput screening (HTS) assays.

In vivo protection against LRRK2 toxicity was also demonstrated using GW5074 and indirubin-3'-monoxime using an HSV amplicon-based mouse model of LRRK2 [41]. More recently, GW5074 and sorafenib were shown to protect against G2019S LRRK2-induced neurodegeneration *in vivo* in *Caenorhabditis elegans* and *Drosophila* models [87]. Taken together, these results suggested that LRRK2 small-molecule inhibitors could potentially have a disease-modifying effect. However, as these

Table 10.1 Broad-spectrum LRRK2 inhibitors and LRRK2 inhibitors discovered through targeted screening.

Name	Biochemical IC ₅₀ (nM)		Substrate	<i>In vivo</i> model	References
	Wild type LRRK2	LRRK2 [G2019S]			
Sunitinib	79; 15	19; 26	Nictide; LRRKtide		[15,46,85]
H-1152	244	600; 150	GST-Moesin; Nictide		[15,46]

(continued)

Table 10.1 (Continued)

Name	Biochemical IC ₅₀ (nM)		Substrate	<i>In vivo</i> model	References
	Wild type LRRK2	LRRK2 [G2019S]			
Y-27632	2300	1800; 1000	GST-Moesin; Nictide		[15]
Staurosporine	~1; 2; 8.2; 40	0.2; 1.8; 40	GST-Moesin; LRRKtide; MBP		[17,41,85]
K-252a	~25; 3.6	2.8	LRRKtide		[17,85]
K-252b	~50		LRRKtide		[17]
Gö6976	~250		LRRKtide		[17]
Indirubin-3'-monoxime	4830	1310	MBP	HSV	[41]
GW5074	~500; 3150	880	LRRKtide; MBP	HSV; <i>C. elegans</i> ; <i>Drosophila</i>	[17,41]
Sorafenib	5580	1230	MBP	<i>C. elegans</i> ; <i>Drosophila</i>	[41]
CZC-25146	4.8	6.9	LRRKtide	CD-1 mice	[88]
CZC-54252	1.3	1.9	LRRKtide	CD-1 mice	[88]

compounds are known to inhibit a number of different kinases, it is difficult to attribute their neuroprotective effects entirely to LRRK2 inhibition.

Ramsden *et al.* and Hopf *et al.* employed a quantitative chemoproteomics method to screen for inhibitors that would interfere with the association between an immobilized sunitinib derivative and LRRK2 [42,88]. This effort resulted in the discovery of CZC-25146 and CZC-54252 (Table 10.1) as single-digit nanomolar inhibitors of LRRK2[G2019S] with moderate selectivity (potent inhibition of 5/184 and 10/184 kinases at 2 μ M, respectively) [89]. CZC-25146 demonstrated a good mouse pharmacokinetic profile, but did not significantly penetrate the blood–brain barrier (4%) (Table 10.2). CZC-25146 attenuated LRRK2[G2019S]-induced neuronal injury in both primary rodent and primary human neurons *in vitro*. These results further support the potential utility of selective LRRK2 inhibitors for PD therapies.

ATP-competitive kinase inhibitors are often assayed for activity against large panels of kinase inhibitors in biochemical assays. This methodology produces large amounts of data that can be used to quickly identify selective, potent inhibitors of proteins of interest [90,91]. Using this method, Alessi and coworkers have identified the potent and selective inhibitor LRRK2-IN-1 (Table 10.3), [92] which is structurally similar to a previously reported BMK1/ERK5 inhibitor, [93] and ALK inhibitor TAE684 (Table 10.3) [82]. LRRK2-IN-1 is highly potent against LRRK2 [G2019S] (6 nM) and selectively binds to LRRK2 at 10 μ M when tested against >470 distinct kinases. LRRK2-IN-1 inhibits LRRK2 kinase activity *in vivo*, and the administration of LRRK2-IN-1 to mice leads to the dephosphorylation of Ser910 and Ser935 in the kidney. This compound also reduces 14-3-3 binding to

Table 10.2 *In vivo* pharmacokinetic profiles of reported LRRK2 inhibitors.

Name	MW	TPSA	cLogP	Species	IV Cl (mL/ min/kg)	IV $T_{1/2}$ (h)	F (%)	Total B/P	B_u/P_u ^{a)}	References
CZC-25146	489	117	3.5	Mouse	2.3	1.6	24	Neg ^{b)}		[88]
LRRK2-IN-1	571	97	2.9	Mouse	5.6	1.7	49	Neg ^{b)}		[92]
TAE684	614	102	5.8	Mouse	17	11.3	83	2.3		[81]
G7080	378	88	1.9	Mouse		0.23, 0.13	67	1.4	0.6	[82,106]
				Rat		0.5	64	0.23	0.2	[105]
GSK2578215A	399	64	4.3	Mouse	30	1.1	12	2.4		[95]
G1023	411	88	2.1	Rat	24	1.2	80	0.9	0.5	[105]
G7915	443	88	2.8	Rat	8.3	3.1	40	1.0	0.5	[105]
				Cyno	11	7.7	24		0.6	[105]

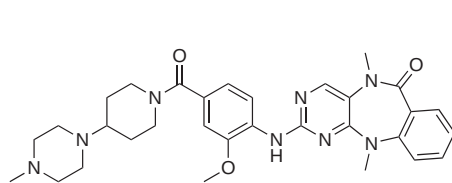
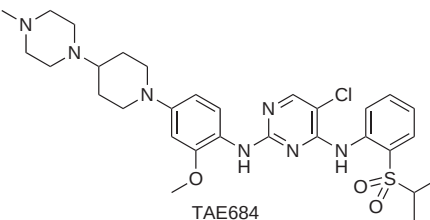
a) Unbound brain/unbound plasma AUC ratio.

b) Negligible brain penetration reported.

endogenous LRRK2 in neuroblastoma SHSY5Y cells derived from humans and mouse Swiss3T2 cells [92]. LRRK2-IN-1 appears to be a useful tool compound for *in vivo* studies. The compound has low plasma clearance in mice (5 mL/min/kg), a half-life of 4.5 h, and good bioavailability (%F = 49) (Table 10.2). Unfortunately, the physicochemical properties of LRRK2-IN-1 (MW 571) and TPSA (97 Å²) are generally inconsistent with CNS penetration [94]. Indeed, following intraperitoneal (IP) injection of LRRK2-IN-1 in mice, no dephosphorylation of Ser910 and Ser935 was observed in the brain [92].

TAE684 inhibits LRRK2[G2019S] with a biochemical IC₅₀ of 6 nM and inhibits *in vivo* phosphorylation of WT and G2019S-mutated LRRK2 at Ser910 and Ser935 in the periphery of mice at 100–300 nM following oral dosing [82]. Unlike LRRK2-IN-1

Table 10.3 LRRK2 inhibitors discovered through high-throughput kinase profiling.

 LRRK2-IN-1  TAE684					
Biochemical IC ₅₀ (nM)					
Name	Wild type LRRK2	LRRK2 [G2019S]	Substrate	<i>In vivo</i> model	Reference
LRRK2-IN-1	13	6	Nictide	C57BL/6 mice	[92]
TAE684	7.8	6.1	Nictide	C57BL/6 mice	[81]

however, TAE684 inhibits multiple kinases (reported K_d values less than 100 nM for six kinases). TAE684 also demonstrated a favorable mouse PK profile with a half-life of 11 h and excellent oral bioavailability (%F = 83) (Table 10.2). More importantly, TAE684 can penetrate the blood–brain barrier (brain to plasma (B/P) ratio of 2). While the MW, TPSA, and presence of basic nitrogen atoms is similar between LRRK2-IN-1 and TAE684, the increased total brain penetration for TAE684 could be attributed to (a) increase in lipophilicity and inherent permeability (cLogP for LRRK2-IN-1 = 2.9 versus 5.8 for TAE684) and/or (b) replacement of two amide functionalities in LRRK2-IN-1. Additionally, the sulfone moiety introduced in TAE684 should be able to form an intramolecular hydrogen bond with the 4-anilino N—H, thereby potentially improving passive permeability. Unfortunately, despite the improved total brain penetration of TAE684, this inhibitor did not demonstrate *in vivo* inhibition of LRRK2 kinase activity in the brain. It is possible that the highly lipophilic compound has very low unbound concentrations in the brain due to high protein binding in this compartment [82].

The final approach that has been used for the discovery of LRRK2 kinase inhibitors involves medium- to high-throughput screening of commercial and proprietary libraries. To date, this method has provided the most promising chemical matter. GSK scientists have recently reported a benzyloxy benzamide series exemplified by GSK2578215A (Table 10.4) [95–97]. GSK2578215A

Table 10.4 LRRK2 inhibitors discovered through medium- to high-throughput screening.

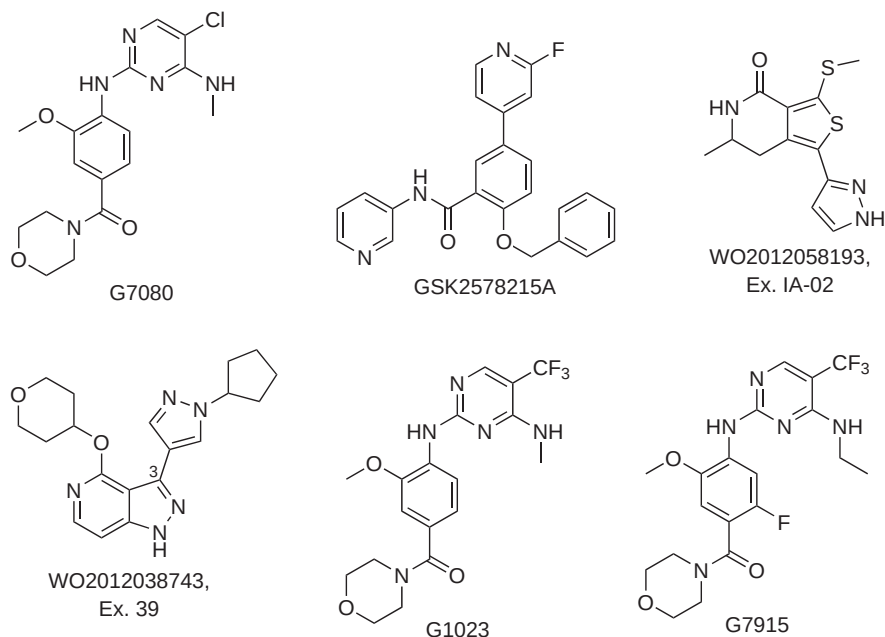


Table 10.4 (Continued)

Name	Biochemical IC ₅₀ (nM)		Cellular IC ₅₀ (nM) S1292 autophos ^[48]	Substrate	In vivo model	References
	Wild type LRRK2	LRRK2 [G2019S]				
G7080		3	29	LRRKtide		[82,104–106]
GSK2578215A	10.9	8.9		Nictide	Male C57 BL/6 mice	[95,96]
WO2012058193, Ex.IA'-02		<5000		LRRKtide		[99]
WO2012038743, Ex.39		6		LRRKtide		[101]
G1023		2	9	LRRKtide	LRRK2 Tg mice	[82,105]
G7915		1	9	LRRKtide	LRRK2 Tg mice	[105]

exhibited an IC₅₀ of 8.9 nM for inhibition of LRRK2[G2019S] with good levels of overall kinase selectivity (3/460 kinases showed moderate inhibition at 10 μ M). Mouse PK experiments revealed that GSK2578215A has a half-life of 1.1 h, low bioavailability (%F = 12), and a total B/P ratio of 2.4 (Table 10.2). *In vitro* cellular studies with GSK2578215A demonstrated dose-dependent Ser910 and Ser935 dephosphorylation in both WT and LRRK2[G2019S] stably transfected into HEK293 cells, dephosphorylation of endogenous LRRK2 in human lymphoblastoid cells derived from a PD patient homozygous for the G2019S mutation, and dephosphorylation of endogenous LRRK2 in mouse Swiss 3T3 cells. However, similar to TAE684, upon IP injection of GSK2578215A at 100 mg/kg in normal mice, dephosphorylation of Ser910 and Ser935 was only observed in the periphery (kidney and spleen) with no brain inhibition [95]. Upon examination of the physicochemical properties of GSK2578215A and other inhibitors in the published patent reports from GSK, several compounds including GSK2578215A display favorable MW (<400) and TPSA (<80) ranges for CNS-penetrant small molecules and are void of basic nitrogens. However, as was the case with TAE684, GSK2578215A is fairly lipophilic (cLogP = 4.3), which could potentially result in a significantly lower unbound brain to unbound plasma (B_u/P_u) ratio due to high protein binding and a small amount of free drug that is able to interact with LRRK2 in the brain.

Published patent reports have also surfaced from Merck [98,99], MRCT [100], MRCT-Genentech [101,102], and Genentech [103,104] reporting structurally diverse LRRK2 kinase inhibitors. The Merck patent applications disclose a series of pyrazole thieno dihydropyridones (Table 10.4) and pyrazole thienocyclohexanones with LRRK2 IC₅₀ values less than 5 μ M when tested in an LRRK2[G2019S] Lanthascreen assay. The MRCT- and MRCT-Genentech-published patent applications reveal a series of potent pyrazolopyridines with consistent single-digit

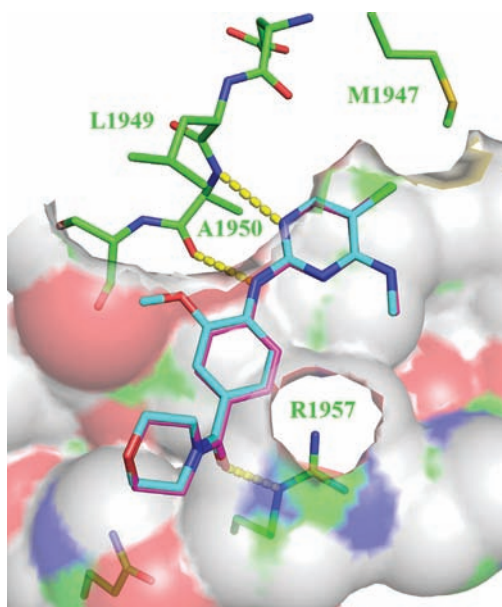


Figure 10.2 Overlay of docking models of GNE diaminopyrimidine HTS hit (magenta) and selective lead G7080 (cyan) in the ATP binding site of LRRK2 homology model (green). Intermolecular hydrogen bond

interactions between ligand and protein are shown as yellow dashed lines. Part of the protein interaction surface is shown and colored by atom properties with carbon atoms in white.

nanomolar inhibition of LRRK2[G2019S] (Table 10.4). Docking studies suggest that these inhibitors bind to the hinge through the pyrazole moiety of the pyrazolopyridine and access a selectivity pocket through substitution at the 3-position of the pyrazole ring. The dissemination of compound profiling and characterization beyond structure and biochemical activity from these applications, however, is still in its infancy.

Two literature reports have now been published that describe a series of highly potent, selective, metabolically stable, and brain-penetrable aminopyrimidine inhibitors that were initially disclosed in Genentech's 2011 patent application [83,104,105]. In lieu of LRRK2 crystal structures, Chen *et al.* [83] disclosed a JAK2-based LRRK2 homology model that was used to assess the binding modes of the diaminopyrimidine inhibitors. The predicted binding mode of a diaminopyrimidine HTS hit in LRRK2 is shown in Figure 10.2. The 2,4-diaminopyrimidine core binds to the adenine site and interacts with the kinase hinge through a pair of hydrogen bonds to the backbone amide —NH and carbonyl oxygen of Ala1950. The C-5 chlorine atom of the core forms favorable van der Waals interactions with the Met1947 gatekeeper side chain, and the C-4 *N*-methyl fills a hydrophobic cavity. The aniline ring binds in a flat hydrophobic cleft along the hinge with the 4-morpholine amide group pointing toward solvent. Based on the docking model, a

small cavity was identified near residue Leu1949 where small substituents could be tolerated to improve overall selectivity of the series. For example, addition of a methoxy group on the 2-position of the aniline ring led to the discovery of the highly selective compound G7080 (Figure 10.2 and Table 10.4), which inhibits only wild type and LRRK2[G2019S] at greater than 50% in a 63 member Invitrogen selectivity panel at 1 μ M concentration. G7080 was found to cross the blood–brain barrier with total B/P AUC ratios and B_u/P_u AUC ratios of 1.4 and 0.61, respectively, in wild type, and 2.9 and 1.3, respectively, in P-gp/BCRP knockout mice (Table 10.2).

G7080 was shown by Gray and coworkers to potently inhibit phosphorylation of wild type LRRK2 and LRRK2[G2019S] at 0.3–1.0 μ M in cells and *in vivo* phosphorylation of Ser910 and Ser935 in mouse spleen, kidney, and brain after IP dosing at 50 and 100 mg/kg [106]. Following detailed *in vivo* selectivity studies monitoring 150 kinases using mouse brain and spleen from inhibitor-treated animals at increasing dose levels using a chemical proteomics approach, KiNativ further confirmed that G7080 is a highly selective LRRK2 inhibitor.

Optimization of G7080 by Estrada *et al.* resulted in the discovery of a series of highly optimized aminopyrimidines [105]. High levels of *in vitro* and *in vivo* activities were achieved through iterative measurement of inhibition of LRRK2 autophosphorylation at Ser1292. Representative of this series of inhibitors, G1023 and G7915 (Table 10.4), display single-digit nanomolar biochemical and cellular activity. Invitrogen kinase selectivity profiling of G1023 at 0.1 μ M (0/178 kinases with >50% inhibition) and 1 μ M (0/63 kinases with >80% inhibition) and KinomeScan selectivity profiling of G7915 (3/451 kinases with >65% probe displacement) suggest that these compounds are also highly selective and specific inhibitors of LRRK2. Extensive PK profiling of several diaminopyrimidines is also reported along with *in vitro*–*in vivo* stability and permeability correlations. G1023 and G7915 demonstrate good rat PK profiles (Table 10.2) with a desirable balance between *in vivo* stability, oral exposure, and brain penetration (B_u/P_u = 0.5 for both). Furthermore, G7915 possesses a comparable cynomolgus monkey PK profile (Table 10.2) with a B_u/P_u of 0.6. This is the first report of higher species PK for an LRRK2 small-molecule inhibitor. *In vivo* inhibition of LRRK2 autophosphorylation in both the periphery (spleen) and brain (hippocampus) of LRRK2[G2019S] transgenic mice with multiple compounds was also demonstrated for the first time. G7915 showed robust concentration-dependent knockdown of pLRRK2 with oral dosing at both 15 and 50 mg/kg. Using a pharmacodynamic inhibition model, a calculated *in vivo* unbound brain IC_{50} of 7 nM was reported for G7915, which is consistent with the cellular IC_{50} (9 nM). G7915 also showed clean responses in *in vitro* and *in vivo* genotoxicity assays, reversible and time-dependent inhibition assays, good exposure and tolerability in rodent safety studies, and good peripheral and brain exposure in 7 day cynomolgus monkey studies. As a result, G7915 and other compounds from this reported series of diaminopyrimidine inhibitors should be capable of answering key preclinical

questions surrounding the consequences of inhibiting LRRK2 kinase activity in the periphery and brain.

The remarkable selectivity and structural diversity of the reported LRRK2 kinase inhibitors suggest that it should be possible to develop compounds useful for determining the impact of LRRK2 kinase inhibition on animal models of PD. Many of the early LRRK2 small-molecule inhibitors that appeared in the literature were either nonselective and/or did not sufficiently cross the blood–brain barrier. This underlies the challenge that CNS-targeted kinase programs must tackle in synthesizing inhibitors that possess the appropriate balance of size, lipophilicity, and polarity. Potent and selective inhibitors that only lack brain penetration however, could still prove to be valuable as LRRK2 is expressed in the lung, spleen, kidney, monocytes, and other tissues, and an association between LRRK2 and Crohn’s disease, cancer, immunology, and leprosy has been suggested [45,107]. More recently, a series of brain-penetrable and highly selective aminopyrimidine inhibitors has been reported with good B_u/P_u ratios. These compounds might serve as useful tools for understanding the consequences of inhibiting LRRK2 function in the brain and other tissues [105].

The advancement of multiple, structurally diverse chemical series into efficacy and toxicology studies in both rodents and higher species will be important milestones toward the discovery of LRRK2-targeted medicines. As the prospective patient population will most likely be administered drugs for several years, chronic dosing studies including detailed histopathology analyses must be conducted to gauge the toxicological implications of inhibiting LRRK2 for extended periods.

10.9

Conclusions

A number of additional studies have confirmed the genetic association between LRRK2 and PD in the relatively short time since the LRRK2 protein was discovered. For example, scientists have discovered activities of LRRK2 that can be monitored *in vivo*, and a variety of inhibitors of LRRK2 kinase function have been identified. Several of these compounds have good selectivity and are able to inhibit LRRK2 kinase activity in the brain.

Further validation of LRRK2 as a therapeutic target has been frustratingly slow however. Most LRRK2-based animal models of PD have weak and variable phenotypes, and no selective LRRK2 kinase inhibitor has been demonstrated to reduce these deficits. Consensus regarding the true cellular function(s) of LRRK2 has not yet emerged. Given the complications inherent to the development of medicines that slow the progression of neurodegenerative diseases, further understanding in this area will be critical for the progression of LRRK2 inhibitors into informed clinical studies. Precompetitive information sharing and collaboration mediated by nonprofit interest groups such as the Michael J. Fox Foundation represent one promising method by which the pharmaceutical industry might work efficiently to meet the unmet medical needs of Parkinson’s disease patients.

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11

Therapeutic Potential of Kinases in Asthma

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11.1

Introduction

Asthma is one the most prevalent chronic diseases, affecting roughly 300 million people worldwide [1]. The disorder is characterized by airway hyperresponsiveness (AHR), inflammation, airway remodeling, and reversible airway obstruction. Symptomatically, asthma manifests itself by episodes of wheezing, breathlessness, chest tightness, and coughing. The current standard of care for asthma is pharmacological treatment with inhaled corticosteroids (ICSs), inhaled long-acting beta-2 agonists (LABAs), or leukotriene antagonists. Although these therapies are quite effective for mild to moderate asthma sufferers, a significant proportion of patients with severe asthma do not adequately respond to corticosteroids [2]. This may be partly due to the etiology of asthma being multifactorial and associated with multiple hereditary components (i.e., genetic variations) as well as environmental factors (i.e., allergens and viral infections). Several methods for classifying asthma have been proposed over the years [3–7]. USA national guidelines have used the severity of the symptoms to divide asthma into four categories: intermittent, mild persistent, moderate persistent, or severe persistent [3]. The method is based on the amount of treatment required to control the clinical characteristics of the disease (e.g., the frequency of symptoms and forced expiratory volume in 1 s (FEV1)). More recently, numerous attempts have emerged that aim to classify asthma according to the pattern of airway inflammation. For example, Gibson and coworkers have proposed a classification into four categories based on inflammatory subtypes in induced sputum: neutrophilic asthma, eosinophilic asthma, mixed asthma, and paucigranulocytic asthma [4]. Others have divided asthma patients into Th2-high and Th2-low subjects, depending on the level of Th2 inflammation in the lung [7]. Although the heterogeneity of the disease makes the identification of a “one-size fits all treatment” difficult, it also provides a framework to develop personalized treatments for asthmatic patients [8]. To date, only a small number of genes have been identified that significantly predict response to the current asthma

medications and examples of genetic testing for asthma are lacking [9]. This may change in the light of recently published studies. In a notable genome-wide association investigation by Tantisira *et al.*, the authors identified *GLCCI1* as a gene influencing the pharmacological response to inhaled glucocorticoid in asthma [10]. Another recent clinical study showed that patients with a high level of the matrix protein periostin demonstrated greater improvements in lung function with the anti-IL-13 monoclonal antibody, lebrikizumab, than patients with low periostin levels [11]. These findings have the potential to form the basis for the development of personalized therapy in asthma in the future.

The pathophysiology of asthma involves complex interplays between a variety of immune cells (e.g., Th2 cells, B cells, mast cells, eosinophils, and neutrophils) and structural cells (e.g., epithelial and smooth muscle cells) [12]. These interactions are orchestrated by a range of cytokines and other mediators that regulate the action, proliferation, and migration of the effector cells. Protein kinases are key regulators of a wide array of cytokine-induced signals and have long been recognized as potentially important targets for asthma [13–15]. In the field of medical oncology, selective kinase inhibitors have emerged as an important class of anticancer agents, especially useful for the treatment of patients stratified based on specific genomic biomarkers [16]. Notably, the recently (2011) launched vemurafenib (Zelboraf) received Food and Drug Administration (FDA/FDA) approval for the treatment of late-stage melanoma. Zelboraf is specifically indicated for the treatment of patients with melanoma whose tumors express a gene mutation called BRAF V600E, and it is marketed with a companion BRAF V600E diagnostic test to identify the subset of patients most likely to benefit from the drug. It has not been established as yet whether or not such a strategy could be transposed to the treatment of phenotypically well-defined asthmatic populations. However, it has been postulated that kinase activity increases were responsible for the resistance to glucocorticoid treatment [17,18]. Another study also reported abnormal phosphorylation of protein tyrosine kinases in lung biopsies from severe asthmatics [19]. These observations support the concept that kinases could be appropriate targets for personalized health care treatment in asthma. The overall suitability of kinase inhibitors as asthma drugs, however, will depend on a fine balance of expression, function, disease relevance, and toxicity potential. We discuss herein the role of the most promising protein kinases in asthma and review the current progress of the major kinase inhibitors currently in clinical development.

11.2

Mitogen-Activated Protein Kinases

Many external triggers of the inflammatory response seen in asthma (such as viral and bacterial infections, allergens, cytokines, and growth factors) can activate intracellular kinases following binding to transmembrane receptors on responsive

cells. This leads to a rapid amplification of the initiating signal due to the number of enzymes involved in each kinase cascade. One kinase cascade responsible for the inflammatory response is the mitogen-activated protein kinase (MAPK) pathway [20]. The MAPK family includes three distinct stress-activated protein kinase pathways: p38MAP kinase (p38), c-Jun N-terminal kinase (JNK), and extracellular regulating kinase (ERK). The ERK pathway is predominantly activated by mitogenic and proliferative stimuli, whereas the JNK and p38 pathways respond to environmental stress [21]. MAPKs, which are activated by dual phosphorylation on threonine and tyrosine by upstream kinases, promote inflammation through activation of proinflammatory transcription factors such as activating protein-1 (AP-1) and nuclear factor κ B (NF- κ B). Enhanced activation of p38 MAPKs, JNK, and ERK has been proposed to play a role in steroid-insensitive asthma [22]. Therefore, MAPK inhibition appears to be a good strategy for the treatment of asthma.

However, despite intensive research efforts, no MAPK inhibitor has moved beyond phase III trials for the treatment of inflammatory indications [23]. This is, for the most part, a result of unacceptable safety profiles. The toxicities have been varied and are believed to arise from different off-target effects [23,24]. Nonetheless, the potential to deliver an inhibitor by inhalation to reduce systemic exposure gives some reason to be optimistic that this family of kinase inhibitors could address the unmet medical need in asthma [25]. In this section, each of the three kinases will be considered in more detail.

11.2.1

p38

p38 exists as four isoforms in mammals, α , β , γ , and δ . p38 α and p38 β are ubiquitously expressed, p38 γ is predominantly expressed in cardiac and smooth muscle, and p38 δ is found mainly in the testes, pancreas, and small intestine. Of the four isoforms, p38 α has been best characterized. This isoform is involved in regulating the synthesis of proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α), and has therefore been targeted by pharmaceutical companies for the development of drugs to treat inflammation indications. P38 α inhibitors currently in late-phase development include talmapi-mod (SCIO-469 (1)), developed by Scios for multiple myeloma and rheumatoid arthritis, and VX-702 (2), developed by Vertex for rheumatoid arthritis. Both compounds are in phase II clinical trials. See Figure 11.1 for the structures of the p38 inhibitors.

Among the reports describing asthma as an indication, the p38 inhibitor ML3403 (3), which is a structural analog of a well-documented and prototypical p38 inhibitor SB203580 (4), shows promise. SB203580 and ML3403 are both selective inhibitors of p38 α and β , which inhibit the catalytic activity of p38 by competitive binding in the ATP pocket. SB203580 inhibits many inflammatory cytokines, chemokines, and inflammatory enzymes, and was shown to attenuate BAL TNF α production in an ovalbumin challenged rat model of asthma [26]. While SB203580

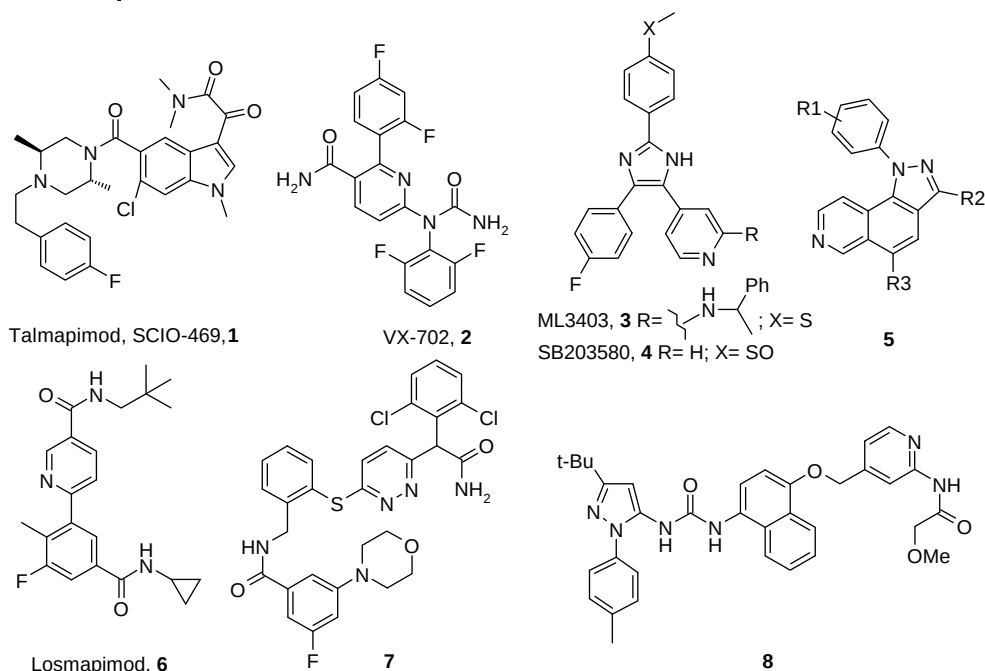


Figure 11.1 p38 inhibitors.

cannot be used clinically due to liver toxicity, ML3403 binds with reduced activity to liver cytochrome P450 enzymes and as such might be more appropriate in a clinical setting. ML3403 binds to both active and inactive p38 with high affinity (p38 active form IC_{50} 14.7 nM, inactive form IC_{50} 20.8 nM), and inhibits p38-mediated airway smooth muscle (ASM) synthetic function to an equivalent degree as SB203580 [27]. Therefore, ML3403 appears to be a promising lead candidate for treating airway inflammation.

A much more advanced p38 inhibitor, UR-13870 from Palau Pharma, is an orally active, low molecular weight compound that has shown efficacy in experimental models of rheumatoid arthritis, psoriasis, and neuropathic pain. This compound has potential therapeutic value in other inflammatory diseases such as asthma and COPD. While the structure and isoform selectivity of UR-13870 are unknown, a generic representation of the series to which it belongs has been disclosed (5). The compound has successfully completed two phase I clinical trials in healthy volunteers and according to a Palau Pharma press release, it showed a good safety profile, excellent oral bioavailability, and potent and long-lasting inhibition of p38 kinase activity in humans. They postulate that the distinctive PK/PD profile with a sustained inhibition of $\text{TNF}\alpha$ over 24 h at all doses makes UR-13870 a promising therapeutic option for clinical use [28].

Losmapimod (6), a p38 inhibitor from GSK originally designed for rheumatoid arthritis but with potential for other inflammatory indications as well, was

efficacious in the rat PG-PS model for arthritis with an ED_{50} of 0.03 mg/kg, as well as in the murine collagen-induced arthritis (CIA) model with complete suppression of arthritis at 20 mg/kg p.o. BID [29]. Recently, two publications have been disclosed for newer p38 MAPK inhibitors. One from Pulmagen Therapeutics shows pyridazine (**7**) to have p38 $IC_{50} < 10$ nM [30]. The second, from Respivot, claims that urea (**8**) is a potent inhibitor of p38 subtypes α and γ (IC_{50} 5 and 402 nM, respectively) with good efficacy in an *in vitro* model of antiinflammatory activity (LPS-induced TNF α release from differentiated U937 cells and THP-1 cells) [31]. In addition, **7** does not exhibit overt cellular toxicity at 10 μ g/ml.

These recent reports of different subtype selective p38 inhibitors are encouraging with respect to future prospects for a personalized medicine strategy. It has been shown that p38 MAPK- γ protein expression is higher in patients with severe asthma than in healthy subjects [24]. Further analysis with p38 γ overexpression in U937 cells supported the finding that p38 γ in severe asthma is likely to be one of the molecular mechanisms of steroid insensitivity. In fact, inhibition of p38 MAPK- γ phosphorylation by LABAs (e.g., formoterol) restored corticosteroid sensitivity in IL-2/IL-4-treated PBMCs [24].

11.2.2

JNK

c-Jun N-terminal kinase consists of three isoforms, encoded by three different genes, of which the JNK1 and JNK2 isoforms are widely distributed, while JNK3 is mainly located in neuronal tissue [32]. Prototypical SP-600125 (**9**) (Figure 11.2) is a competitive and selective inhibitor of JNK types 1 and 2. It is between 300- and 500-fold more selective for JNK compared with other kinases, such as p38. Furthermore, it has been shown that in acute and chronic animal models of asthma, SP-600125 (30 mg/kg) reduces bronchoalveolar lavage accumulation of eosinophils and lymphocytes, cytokine release, serum IgE production, and smooth muscle proliferation after repeated allergen exposure [33]. Celgene was

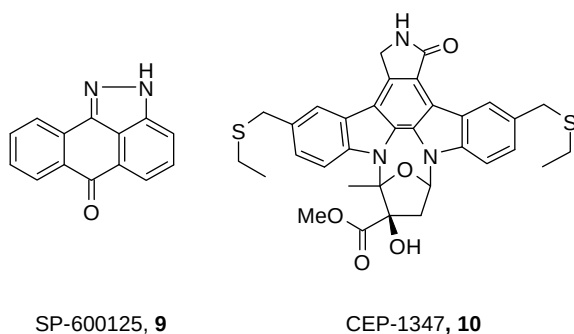


Figure 11.2 JNK inhibitors.

developing it for the treatment of rheumatoid arthritis, allergic asthma, cerebral ischemia, allotransplant rejection, and cancer. However, development appears to have been discontinued as no recent progress for this compound has been reported, and now it is used primarily as a tool compound.

CEP-1347 (KT-7515 (**10**)), which acts via inhibition of mixed lineage kinases (MLKs) to inhibit the JNK pathway, is an orally active, semisynthetic inhibitor of key kinases within the stress-activated protein kinase pathway, which was under development by Cephalon (now Teva) for the treatment of asthma. However, no development has been reported since 2009.

Clearly, JNK inhibitors have the potential to treat asthma. Importantly for the potential personalization of healthcare, significantly greater expression of phosphorylated c-JUN and phosphorylated JNK has been identified in corticosteroid-resistant subjects compared with corticosteroid-sensitive subjects. Corticosteroids suppressed the phosphorylation of c-JUN and JNK in the corticosteroid-sensitive group, but enhanced the phosphorylation of c-JUN and JNK in the corticosteroid-resistant group. Therefore, resistance to corticosteroids in asthmatic subjects may be caused, at least in part, by failure to suppress JNK phosphorylation, leading to ineffective suppression of c-JUN N-phosphorylation [34].

11.2.3

ERK

There are two isoforms of extracellular regulated kinase (ERK) in vertebrates, ERK1 and ERK2, which are 84% identical at the amino acid level. Observations are that ERK1 and ERK2 are very similar in function. Indeed, ERK1 and ERK2 are expressed in most tissues, including hematopoietic cells such as T, B, and dendritic cells, and airway smooth muscle cells [35].

While there are no ERK1/2 kinase inhibitors, two recent patents from Takeda have been disclosed that describe MEK1 inhibitors in which asthma is cited as a possible indication. MEK1 is a dual specificity protein kinase that phosphorylates and activates ERK1 and ERK2. Therefore, one can infer that an ERK1/2 inhibitor would have a similar effect as an MEK1 inhibitor. The first patent refers to indolizines (and quinolizines) such as **11** (Figure 11.3) with an IC_{50} of ≤ 100 nM

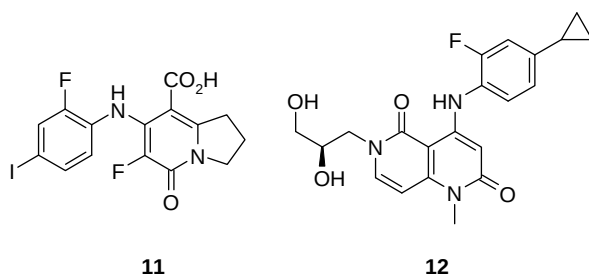


Figure 11.3 JNK and ERK1/2 pathway inhibitors.

against MEK1 [36]. The second discloses naphthyridine-dione (**12**) with an IC_{50} of ≤ 7 nM against MEK1 [37]. ERK activation is enhanced in the airway tissues of patients with severe asthma and is particularly apparent in airway smooth muscle even after treatment with oral and inhaled glucocorticoids [38].

11.3

Nonreceptor Protein Tyrosine Kinases

11.3.1

Syk

Spleen tyrosine kinase (Syk) has ample evidence supporting its potential utility in asthma. Syk is a key mediator of immunoreceptor signaling, and it has long been postulated that interfering with its function will impact a variety of disease states, including allergy and asthma [39]. Syk is expected to play a key role in IgE-mediated responses in allergic asthma and rhinitis. The activation of immune pathways in response to environmental allergens leads to increased levels of IgE and subsequent binding to receptors on mast cells and basophils, which can lead to either the exacerbation or the initial trigger of an asthma attack. There is ample evidence to support the potential use of Syk inhibitors to treat diseases. While the Syk knockout mice are not viable, a recent study was reported in which an induced Syk deletion in mice enabled the deletion of Syk in all organs of the adults [40]. This represented an important study, as the perinatal lethality of Syk knockout mice has previously demonstrated a critical need for Syk in embryogenesis, and also prompted speculation that prohibitive on-target toxicity could emerge during chronic, selective Syk inhibition [41]. Fortunately, the deletion in adult mice demonstrated that there was no impairment of general health, at least in the short term. There is still some risk that other toxicity might arise during chronic dosing, but it is not overt.

Syk has been pharmacologically inhibited by both small- and large-molecule therapeutics. An inhaled, large-molecule antisense oligonucleotide (ExcellairTM) has reached phase II clinical trials for the treatment of asthma. The 21 day phase I trial was designed primarily to determine the tolerance to the treatment and showed no serious adverse events. However, it was also noted that 75% of patients with asthma either reported an improved ability to breathe freely, or a reduced need to use their rescue inhaler [42]. Several small-molecule inhibitors of spleen tyrosine kinase have been reported to be useful for treating asthma. The most advanced of these was Rigel Pharmaceutical's R343 inhaled syk inhibitor (**13**) (Figure 11.4) for the treatment of allergic asthma [43]. R343 was reported to be highly selective for Syk and entered a series of phase I clinical trials, first in normal healthy adults, then later in mildly asthmatic adults. The initial results from these clinical trials showed that R343 is well tolerated and offers improvement in both the early- and late-phase asthmatic responses following an allergen challenge. Although these data are promising, high doses

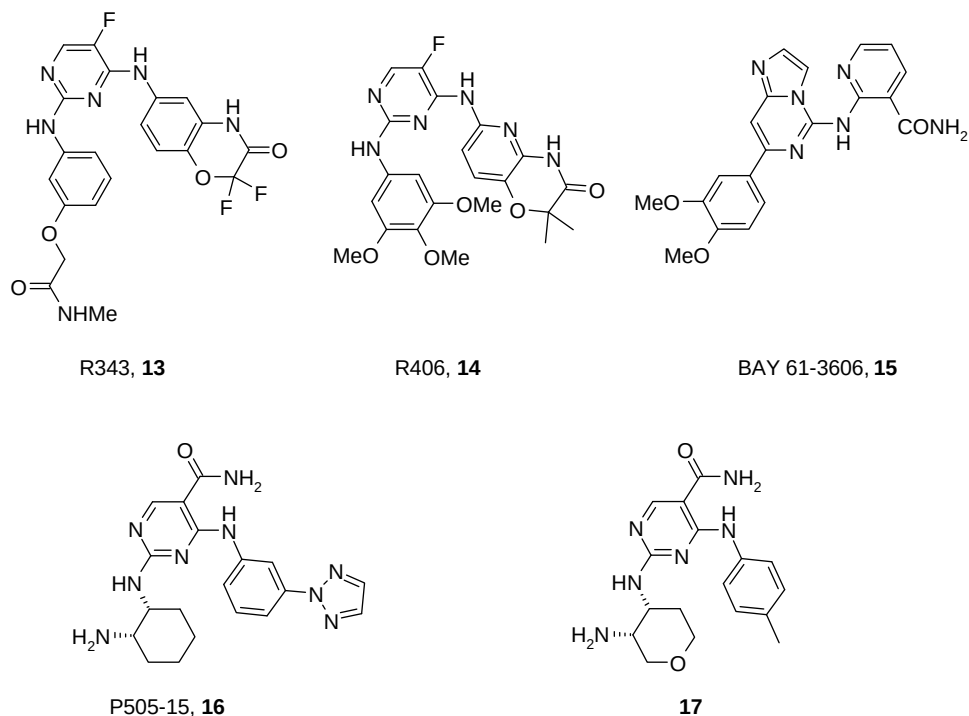


Figure 11.4 Syk inhibitors.

of R343 are required to achieve efficacy, which may preclude combination and codosing with other therapies, and could ultimately limit the utility of this molecule. However, if a subset of asthma patients were identified that would uniquely respond to Syk inhibitor therapy, a personalized approach might offer stronger prospects for this molecule. Rigel advanced into phase II trials with R343. However, in August 2013 they announced that it had failed this mid-stage study. Another Rigel compound from the same chemical scaffold, R406 (**14**), was also used to demonstrate a functional role for Syk in the development of mast cell- and IgE-mediated airway hyperresponsiveness and airway inflammation in mice, and these results indicated that inhibition of Syk could be an excellent option for the treatment of allergic asthma. While this molecule is in clinical trials for several diseases, it has not yet been advanced for the treatment of asthma [44].

Another noteworthy small-molecule inhibitor of Syk that has demonstrated promising efficacy in animal models is BAY 61-3606 (**15**), which was one of the first Syk inhibitors reported to block antigen-induced airway inflammation in rodents [45].

A caveat to the apparently encouraging animal and clinical data for the small molecules is that despite initial assertions that these were selective inhibitors of Syk, they have in fact been found quite promiscuous kinase inhibitors. It is possible that some efficacy is augmented by off-target kinase activity, and that

the efficacy observed in the clinic and in animal models might only be partly due to Syk inhibition. However, similar responses observed during pharmacological inhibition and genetic deletion studies are encouraging. The search for Syk inhibitors has been progressing for some years, and a large number of diverse chemical scaffolds can be found in the patent and biomedical literature. It is difficult to judge how specific for Syk many of these scaffolds are, but Portola pharmaceuticals claim to have the most advanced, selective inhibitor (P505-15 (**16**)), although so far, it has not been examined in any asthma models [46,47]. GSK recently described a potent and selective tool molecule **17** from the same scaffold, although this molecule was not advanced into the clinic due to a scaffold-specific mutagenicity risk [48]. It is likely that more selective Syk inhibitors will emerge as drug companies seek to harness the power of Syk inhibition while avoiding some of the toxicities that result from the current, limited field of advanced candidates.

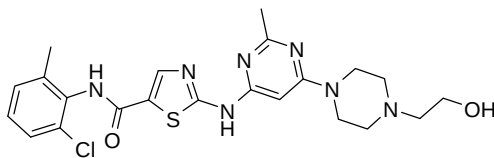
11.3.2

Lck

Lymphocyte-specific protein tyrosine kinase (Lck) is a member of the Src family of tyrosine kinases, and is critical in the activation of T cells. T-cell activation is necessary for cell-mediated immunity. On antigen binding to the T-cell receptor, a signaling cascade is initiated by Lck that ultimately leads to the release of cytokines, proliferation of cells, and cell survival. Inhibition of Lck has the potential to have a significant impact on this process, as it is the first kinase in the T-cell signaling cascade [49]. The clinical value of inhibition of T-cell activation would likely address a wide variety of diseases of the immune system, including asthma. However, despite tremendous effort, success in finding a compound to enter the clinic has been elusive [50].

The challenge of generating selective Lck inhibitors is momentous. In contrast to the Syk family of kinases that includes just two members (Zap70 being the second), the Src kinases comprise a family of 11. All these siblings have very similar ATP-binding pockets (the active site of a kinase), and several of them (Src, Fyn, and Yes) are ubiquitously expressed in the human body. Thus, any Lck inhibitor will need very high specificity or else show prohibitive toxicity profiles. Lck is expressed primarily in lymphoid cells so a selective Lck inhibitor might show a reasonable safety profile, provided there are no on-target toxicities [51]. This is not assured though, and it has been suggested that eye, heart, kidney, and other toxicities might arise as a result of on-target systemic Lck inhibition [51]. The consequences of this: most Pharma companies have moved away from this target. One can speculate that perhaps asthma, with a local, inhaled delivery approach, might still offer some opportunities, particularly if future studies were to identify a cohort of asthma patients that appeared suited to T-cell pathway inhibition.

While specific inhibitors are lacking, there are a handful of nonselective Lck inhibitors either on the market, or that have advanced through clinical trials.

Dasatinib, **18****Figure 11.5** Lck inhibitor.

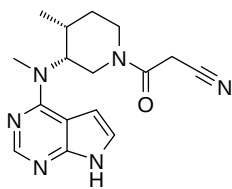
Dasatinib (Sprycel (**18**)) (Figure 11.5) is a protein tyrosine kinase inhibitor that targets Src family kinases and Abl family kinases and is approved for use in the treatment of chronic myeloid leukemia. It is a potent inhibitor of Lck [52]. Similarly, a Bruton's tyrosine kinase inhibitor being developed by Pharmacycyclics (PCI-32765 (**21**)) (Figure 11.8), primarily for the treatment of some lymphomas and leukemias, is also active against Lck with an IC_{50} of 33 nM [53]. These starting scaffolds might yet provide starting points for designing more specific inhibitors of Lck, and give reason to be hopeful that powerful drugs that specifically target Lck might yet emerge.

11.3.3

JAK

The Janus kinase (JAK) family of nonreceptor protein tyrosine kinases (JAK1, JAK2, JAK3, TYK2) plays a critical role in intracellular signal transduction by mediating phosphorylation of the transcription factor STAT. Although most members of the JAK family are ubiquitously expressed, JAK3 expression is primarily limited to hematopoietic cells such as activated T and B lymphocytes and natural killer (NK) cells [54,55]. JAK3, in combination with JAK1, regulates cytokine signaling through association with the common γ -chain cytokine receptors for interleukins IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. It has been shown that JAK3 is abundantly expressed in mast cells and plays an important role in IgE receptor-mediated mast cell activation and degranulation [56]. JAK3 also regulates biological responses of dendritic cells, T cells, macrophages, and B cells, which, along with mast cells, are implicated in the pathogenesis of allergic asthma. A recent study has provided evidence of a role for IL-9 in controlling lung mast cell numbers and regulating airway remodeling in response to chronic allergen challenge [57]. This establishes a link among IL-9, mast cells, and fibrosis of the airways, and suggests that a modulator of the IL-9 pathway, such as a JAK3 antagonist, could lead to a reduction in chronic inflammation and improved lung function in patients with asthma.

Several small-molecule JAK inhibitors are in late-stage clinical trials for various therapeutic indications. The most advanced compound targeted for immunological disorders such as rheumatoid arthritis, psoriasis, and transplant rejection is Pfizer's tofacitinib (also known as CP-690550 (**19**)) (Figure 11.6), a pan JAK

Tofacitinib, **19****Figure 11.6** JAK inhibitor.

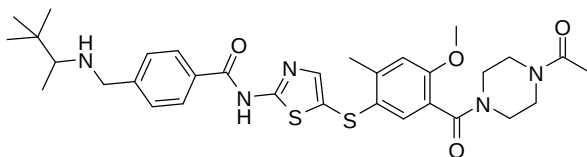
inhibitor. This was recommended for use in the treatment of rheumatoid arthritis by the US FDA in 2012. While Pfizer has not announced any intention to initiate clinical trials in asthma, this molecule has shown efficacy in a murine model of pulmonary eosinophilia thereby representing a potential novel therapy for diseases associated with this condition such as allergic (eosinophilic) asthma [58].

11.3.4

ITK

IL-2-inducible T cell kinase (ITK) belongs to the TEC family of nonreceptor tyrosine kinases that also includes Btk, Tec, and Txk. It acts downstream of signaling pathways in multiple types of immune cells including mast cells, T cells, and NK cells. ITK has been shown to play an important role in T-cell differentiation to Th2 cells, IL-2 production, and T-cell proliferation [59,60]. Mice lacking ITK display an impaired or altered immune response, largely attributed to abnormal development of Th2 response [61]. Further knockout experiments suggested that ITK is involved in both the acute-phase response and late-phase response of diseases such as allergic asthma where aberrant Th2 cell response has been implicated [62].

In 2004, Bristol-Myers Squibb reported several series of selective small-molecule ITK inhibitors, illustrated by BMS-509744 (**20**) (ITK IC_{50} = 19 nM) (Figure 11.7) based on aminothiazole scaffolds. Since then, multiple other companies have investigated inhibitors of this kinase including Boehringer Ingelheim, Pfizer, Vertex, and GlaxoSmithKline. Despite the vast improvements in potency, selectivity, and pharmacokinetic properties resulting from these efforts, there are few

BMS-509744, **20****Figure 11.7** ITK inhibitor.

reports of ITK inhibitors entering the clinic in general and none targeting asthma as a primary indication [63].

In a recent investigation on the association between genetic variants of the ITK gene and asthma, 31 single-nucleotide polymorphisms (SNPs) were genotyped using asthmatic and healthy control groups [64]. Comparison of the data suggested a relationship between ITK polymorphisms of $-196C > T$ and the susceptibility of subjects to development of asthma. This information suggests that ITK might be a relevant target for a personalized approach to the treatment of asthma.

11.3.5

Btk

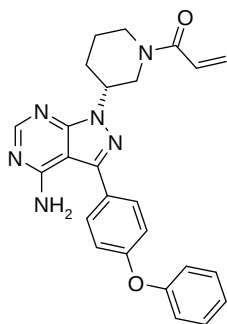
Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase, which is preferentially expressed in a variety of hematopoietic cells, including B cells, mast cells, macrophages, and monocytes, but not in T cells [65]. In B cells, Btk plays a key role in the BCR-activated signaling pathway and its absence has been shown to significantly impair cellular development and proliferation processes [66–68]. In humans, mutations in the BTK gene cause X-linked agammaglobulinemia, a genetic disease characterized by a lack of peripheral B cells and low levels of serum Ig [69,70]. Based on these immunosuppressant effects, Btk has received a lot of attention for the treatment of conditions such as rheumatoid arthritis with several compounds reported in the clinical development of these diseases [71,72]. In parallel, studies have highlighted the pivotal role of Btk in the regulation of other cell types critically involved in the development of allergic asthma. In particular, the absence of Btk severely impairs FcεRI- dependent mast cell responses with regard to the production of allergic cytokines and degranulation [73,74], whereas it has also been shown that Btk is required for IgE-mediated activation of human basophils [75]. Taken together, the involvement of Btk in B cell and mast cell signaling pathways suggests that the kinase is a potential candidate for interventions to regulate mast cell-mediated allergic disorders such as asthma [68].

Although no Btk inhibitor has been reported in clinical development specifically for asthma, several have been reported in clinical evaluation for inflammatory diseases and B-cell malignancies. The most advanced candidate is Ibrutinib (PCI-32765 (**21**)) (Figure 11.8), an irreversible inhibitor developed by Pharmacyclics and currently in phase III trials for the treatment of chronic lymphocytic leukemia (CLL) and other diseases [76]. Two other clinical molecules of unknown structures, ONO-4059 from Ono and AVL-292 from Avila/Celgene, are listed in phase I trials for the treatment of CLL or rheumatoid arthritis.

11.4

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are cell surface enzymes that function as switches for many inflammatory signal transduction pathways. Upon engagement by an

Ibrutinib, **21****Figure 11.8** Btk inhibitor.

external stimulus (e.g., a cytokine or a growth factor), receptor tyrosine kinases activate an intracellular signal, which ultimately leads to the activation of regulatory genes controlling the growth and activation of a variety of immune cells. Several of these kinases have been recognized as playing relevant roles in the pathophysiology of asthma and will be discussed in this section [77].

11.4.1

EGFR

Epidermal growth factor receptor (EGFR) is a member of the ErbB (erythroblastic leukemia viral oncogene homolog) family of kinases, a superfamily that also includes the closely related tyrosine kinases: ErbB-2, ErbB-3, and ErbB-4. The ErbB receptors are expressed in various tissues of epithelial, mesenchymal, and neuronal origin and have received a lot of attention due to their important role in human cancer [78]. Several small-molecule ErbB inhibitors have been approved for cancer treatment, either by selectively targeting EGFR (gefitinib (**22**) and erlotinib (**23**)) (Figure 11.9) or as dual EGFR/ErbB2 inhibitors (lapatinib (**24**)) [79]. For breast and lung cancers, the tumor expression levels of EGFR and ErbB2 have been used to stratify patients who might benefit from inhibitors of these kinases. A data analysis of five clinical trials showed that EGFR-positive patients had much improved response rates to gefitinib and erlotinib than EGFR-negative patients [80]. Similarly, lapatinib has been specifically approved for selected patient populations with HER2 overexpressing breast cancer [81]. These two examples showed that personalized healthcare approaches based on genotyping combined with the use of targeted EGFR inhibitors could be developed.

This is encouraging in the context of personalized medicines for asthma, since EGFR has also been examined in this disease. The expression of EGF and the immunoreactivity of EGFR have been shown to be more elevated in the airway epithelium of chronic asthmatic patients and to correlate with disease severity

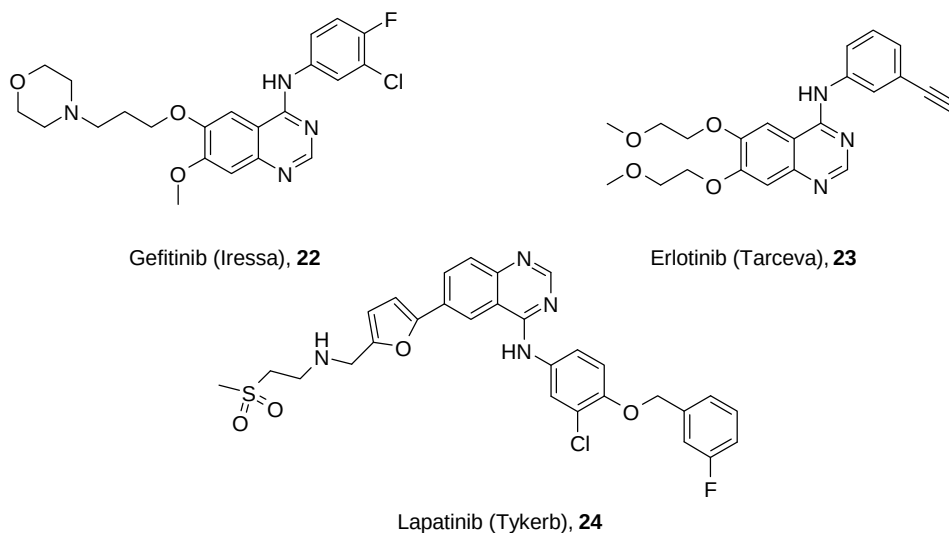


Figure 11.9 EGFR inhibitors.

[82–84]. Activation of EGFR *in vitro* has been linked to pharmacological effects relevant to severe asthma such as proliferation of human airway smooth muscle [85] and human airway epithelial cells [86], mucin production [87], or airway remodeling [83]. In particular, studies on normal human airway epithelial (NHBE) cells with the selective tool molecules, AG1478 and BIBX1522, showed that inhibition of EGFR prevents the synthesis of mucin and attenuates IL-13-induced NHBE cell proliferation [86,87]. A recent investigation demonstrated that EGFR receptor signaling mediates airway hyperreactivity and remodeling in a murine house dust mite model of chronic asthma [88]. In these studies, the investigators demonstrated that airway hyperresponsiveness, airway smooth muscle thickening, and goblet cell metaplasia were all reduced in sensitized mice following erlotinib treatment. However, an *in vitro* study on asthmatic bronchial mucosa showed that blockade of EGFR may actually retard tissue repair and augment airway remodeling [83]. This conflicting result suggests that while blocking EGFR may be beneficial to certain features of asthma, it maybe to the detriment of important repair mechanisms. To date, no EGFR inhibitor has progressed to clinical development to shed more light on the usefulness of EGFR inhibitors for the treatment of asthma.

11.4.2

c-Kit

c-Kit is a member of the tyrosine kinase family of growth factor receptors, which is activated by stem cell factor (SCF) [89]. Although no selective inhibitor has yet been developed, c-Kit has been targeted by several agents with multikinase activity, such as imatinib (25), sunitinib (26), and masitinib (27) (Figure 11.10) [90].

dimer – may contribute to the susceptibility for more severe asthma [107]. These results suggest that determination of PDGFR- α promoter polymorphism may be a useful approach in determining the severity and allergic status of childhood asthma.

Although no selective inhibitor has been successfully developed, this tyrosine kinase is also targeted by imatinib, masitinib, and sunitinib, previously discussed as c-Kit inhibitors. It is not clear how much the inhibition of PDGFR contributes to the effect on asthma of these molecules.

11.4.4

VEGFR

Vascular endothelial growth factor (VEGF) is a critical angiogenic factor implicated in blood vessel formation during growth and development as well as in response to tissue injury and repair. VEGF is the ligand for three receptor tyrosine kinases known as VEGF receptor-1, VEGF receptor-2, and VEGF receptor-3 (VEGFR1–3). Activation of VEGFRs induces multiple cellular processes, including cell migration, survival, and proliferation, which are common to other growth factor receptors, such as the PDGFRs and the EGFRs and, as such, have been studied primarily for cancer [109]. In asthma, several studies point to a contribution of VEGF to airway responsiveness and airway remodeling. Elevated levels of VEGF have been detected in several asthmatic populations [110,111]. A clinical study showed that a 6-month treatment with budesonide/formoterol can decrease the expression of VEGF and VEGFR1 as well as airway remodeling in moderate asthmatic patients [112]. There is also some evidence that genetic polymorphisms in VEGF are associated with decline in lung function [113], suggesting that genotyping of these variations could be used as a biomarker approach to identify individuals who could benefit from VEGF-targeting therapies. Preclinical studies in a murine model examined the distinct roles of VEGFR-1 and VEGFR-2 in noneosinophilic asthma. The results showed that T-cell priming to LPS-containing allergens depends on VEGFR-1 signaling and that the subsequent Th17 polarization was VEGFR-2 dependent, suggesting that pharmacological blockage of either of these receptors could be of interest for the treatment of noneosinophilic asthma [114]. Taken together, these studies provide evidence that targeting VEGFR could be a possible strategy to develop personalized medications for asthma. A more thorough understanding of the true potential of VEGFR in asthma, however, would benefit from clinical studies conducted with selective VEGFR-1 or VEGFR-2 kinase inhibitors, neither of which is currently in clinical development for asthma.

11.5

Phosphatidylinositol-3 Kinases

Phosphatidylinositol-3 kinases (PI3Ks) are a family of intracellular signal transducer enzymes that phosphorylate the 3-hydroxyl group of the inositol ring of

phosphatidylinositol (PtdIns). Based on primary structure, regulation, and *in vitro* lipid substrate specificity, PI3Ks are divided into three different classes termed class I, class II, and class III. Most pharmaceutical research has focused on class I PI3Ks, which can further subdivided into class IA and IB according to their signaling pathways and regulatory proteins. Class IA comprises PI3K α , PI3K β , and PI3K δ , all of which respond to signaling through receptor tyrosine kinases. PI3K γ on the other hand is activated though GPCRs and belongs to class IB. The expression pattern of these four isoforms is also different: PI3K α and PI3K β are ubiquitously expressed whereas PI3K γ and PI3K δ appear to be mainly restricted to leukocytes and as such have been investigated as potential targets for inflammatory diseases, including asthma [115,116].

Studies either by pharmacological inhibition or genetic deletions showed that PI3K δ plays a key role in the activation of T cells, B cells, and mast cells, all of which are immune cells known to play a role in allergic asthma [117]. Thus, selective inhibition of PI3K δ signal transduction pathway by IC87114 (**28**) (Figure 11.11) effectively reduced OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgE levels, goblet cell hyperplasia, and AHR in a mouse asthma model [118]. PI3K δ has also been studied in the context of severe asthma, which is characterized by glucocorticoid unresponsiveness and probably involves a strong component of oxidative stress. Marwick *et al.* showed that blockade of PI3K with a broad-spectrum inhibitor restores glucocorticoid function in an oxidative stress *in vitro* assay [119]. In a mouse

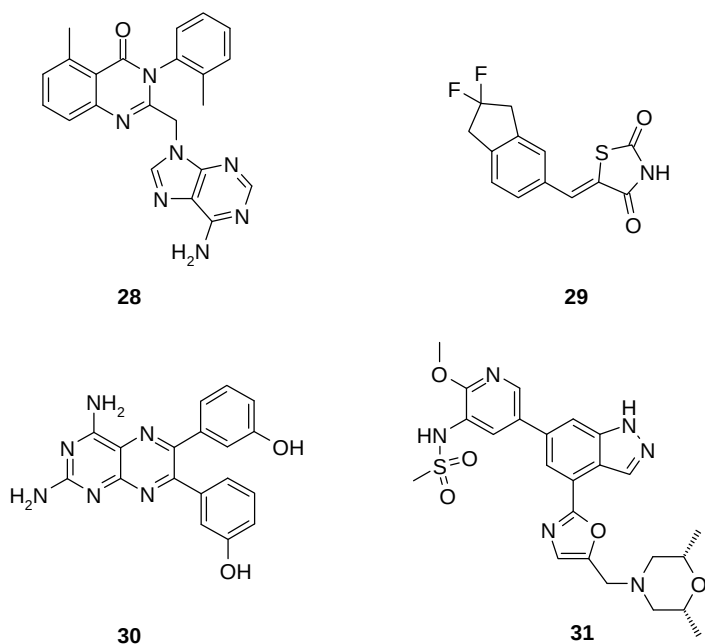


Figure 11.11 PI3K γ and δ inhibitors.

cigarette smoke model, the same authors showed that the reduced glucocorticoid function observed in wild type animals was reestablished in PI3K δ kinase dead knock-in mice but not in PI3K γ knockout mice. Collectively, these studies support PI3K δ as a target for both the treatment of allergic and severe asthma.

PI3K γ has also been examined specifically for asthma. In a study using mouse lung slices, Jiang *et al.* showed that the selective PI3K γ inhibitor AS-604850 (**29**) inhibited acetylcholine (ACh)-induced airway contraction [120]. Subsequently, the same authors reported a similar finding *in vivo* when treating IL-13 challenged mice with **29** [121]. Taken together, these studies suggest that inhibition of PI3K γ may counteract the airway contraction that contributes to the AHR in asthma. Doukas *et al.* investigated the possibility of inhibiting both isoforms at once [122]. In a murine asthma model, the authors showed that an aerosolized formulation of the dual PI3K γ/δ inhibitor, TG100-115 (**30**), markedly reduced asthmatic symptoms, including both pulmonary eosinophilia and AHR.

Although a dozen companies have reported some preclinical activity related to PI3K γ or PI3K δ , only a handful of compounds have progressed in clinical development for treating inflammatory diseases. CAL-263 in phase I trials by Gilead Sciences (originating from Calistoga Pharmaceuticals) is a selective PI3K δ inhibitor that is being examined for allergic rhinitis.¹⁾ IPI-145 (formerly INK-1197, unknown structure), which has also entered phase I clinical trials, is a compound from a series of orally active, small-molecule dual PI3K δ/γ inhibitors under development by Intellikine for the treatment of asthma and other immune-mediated inflammatory diseases, as well as hematological cancers [123]. GlaxoSmithKline has listed in its pipeline the selective PI3K δ inhibitor GSK2269557 (possible structure **31**) in phase I for the treatment of asthma. This compound has a pIC₅₀ value over 7 in the PI3K δ , and has at least 10-fold selectivity over PI3K α , PI3K β , and/or PI3K γ [124].

11.6

AGC Kinases

11.6.1

PKC

Protein kinase C (PKC), rather than being a single kinase as its name might imply, is actually a family of 15 isozymes in humans. This family of serine/threonine kinases, which belongs to the AGC superfamily of kinases, has been well explored since its characterization over 30 years ago. The PKC isoforms can be grouped into three distinct subclasses mainly based on their mode of activation. The classical isoforms (PKC α , PKC β 1, PKC β 2, PKC γ) are activated by binding with diacylglycerol (DAG) and calcium (Ca²⁺) at the C1 and C2 domains, respectively. On the

1) Study to investigate effects of CAL-263 in subjects with allergic rhinitis exposed to allergen in an environmental chamber, <http://clinicaltrials.gov/ct2/show/NCT01066611>.

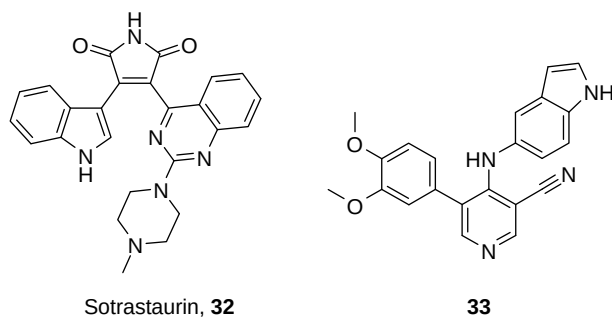


Figure 11.12 PKC inhibitors.

other hand, the novel isoforms (PKC δ , PKC ϵ , PKC η , PKC θ) are activated by DAG alone while the atypical isoforms (PKC ζ , PKC ι/λ) require neither of these activating agents [125,126].

Although the classical PKC isoforms were early targets of kinase inhibitor research, the novel isoforms are currently in the forefront. Due to its expression in mast cells and its critical role in regulation of T-cell receptor signaling to IL-2 production and expression, PKC θ has become an attractive, novel therapeutic target for T cell-mediated diseases such as asthma [127,128]. In addition, recent *in vitro* studies have implicated PKC δ and PKC ζ in eosinophil recruitment and migration, thus leading to the speculation that they might have therapeutic potential for treating allergic airway inflammation [129].

The most advanced PKC inhibitors are all staurosporine analogs with midostaurin, enzastaurin, and ruboxistaurin, all in late-stage clinical trials. These nonselective inhibitors are being targeted toward oncology indications. The related analog sotrastaurin (**32**) (Figure 11.12), also a pan PKC inhibitor, is being developed by Novartis for transplant rejection. Wyeth has reported several series of PKC inhibitors including the 4-amino-3-cyanopyridine **33** that was reported to be potent and extremely selective for the PKC family of kinases, but only moderately selective within the family [130]. Notably, this compound showed potency in an assay measuring decreased IL-2 production from activated murine T cells. Thus, the lack of selectivity within the PKC family is probably the highest barrier to the development of a viable inhibitor for asthma from this kinase class.

11.6.2

ROCK

Rho-associated coiled coil containing protein kinase (ROCK) also belongs to the AGC superfamily of serine–threonine protein kinases. Two ROCK isoforms, ROCK1 and ROCK2, have been identified to date. These isoforms bear >90% homology in the kinase domain and both are ubiquitously expressed in various human tissues. ROCK is a major downstream effector of the monomeric GTP-binding protein RhoA that is

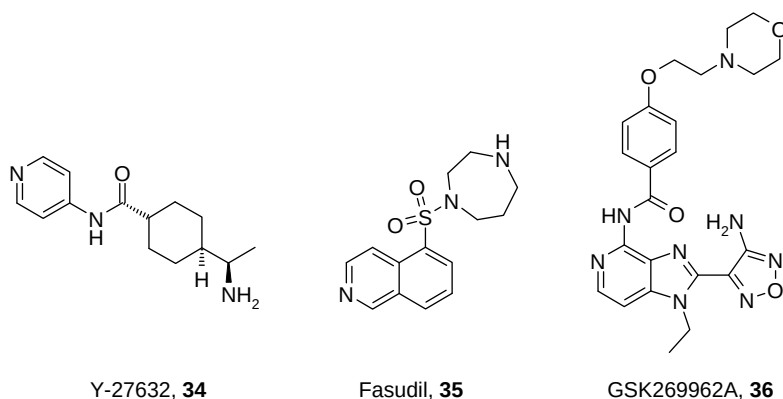


Figure 11.13 ROCK inhibitors.

highly expressed in airway smooth muscle cells. ROCK is activated by direct interaction of the C-terminal Rho-binding domain with GTP-bound RhoA [131].

Activated ROCK phosphorylates myosin phosphatase targeting subunit 1 (MYPT1) that subsequently attenuates myosin light chain (MLC) phosphatase activity. The result of this inactivation is Ca^{2+} sensitization and ultimately enhanced contraction and cytoskeletal reorganization in airway smooth muscle cells [132]. Since reduced responsiveness to β -adrenergic receptor agonists is thought to occur in part by sensitization to intracellular Ca^{2+} , ROCK has been associated with this phenomenon [132–134]. The ROCK inhibitors Y-27632 (**34**) and fasudil (**35**) (Figure 11.13) have been shown to attenuate the effects of agonist-induced airway hyperresponsiveness including smooth muscle contraction and β -adrenergic desensitization in a variety of animal models [135].

ROCK has also been implicated in the control of airway smooth muscle cell proliferation and migration, which are the main pathological features of airway remodeling brought about by chronic inflammation in the lung. Eosinophil infiltration around the airway may also be affected by this kinase. Treatment with **34** or **35** suppressed eosinophil recruitment in OVA-sensitized mice and **34** suppressed the proliferation of human bronchial smooth muscle cells that have been stimulated with serum [135].

While **34** and **35** have been used historically for validation of ROCK as a therapeutic target, these inhibitors are lacking both the potency and kinase selectivity to be advanced as drug candidates. Several inhibitors with improved potency and selectivity have emerged from research efforts within industry and academia and have been studied for their pharmacological effects in numerous disease areas [136,137]. One such example is the novel aminofurazan-based inhibitor GSK269962A (**36**). This demonstrated excellent potency and ROCK selectivity and was found to block the generation of inflammatory cytokines in lipopolysaccharide-stimulated monocytes and induce the dose-dependent relaxation of smooth muscle tissue in models of vasodilation [138]. The wealth of *in vivo* biology findings indicate that RhoA/ROCK plays a central role in the

pathophysiology of asthma and suggests that ROCK inhibitors may offer potential as a therapeutic target for this disease.

11.7

I κ B Kinase

NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcription factor that regulates cytokine and chemokine production in various inflammatory diseases. It is also often active in cancer, and its suppression can limit the proliferation of cancer cells. While it is highly expressed in inflammatory bowel disease, arthritis, atherosclerosis, and many other diseases, it is included in this chapter due to its chronic expression in asthma sufferers. While there are several potential therapeutic strategies that may be useful in downregulating NF- κ B to treat asthma, one of the critical kinases of relevance is I κ B kinase (IKK). IKK phosphorylates the inhibitory I κ B α protein (inhibitor of kappa B). I κ B α is normally bound to NF- κ B as part of a protein complex. In the complex, the nuclear localization signals are masked, and so the NF- κ B remains in an inactive state in the cytoplasm. Once IKK phosphorylates I κ B α , the inhibitory protein dissociates, allowing the NF- κ B to move to the nucleus and activate transcription. This, among other things, leads to a powerful inflammatory response. Treatment of mice with an IKK inhibitor (BAY 11-7085) dose dependently inhibited inflammation in an ovalbumin-induced model of asthma [139].

The IKK complex is composed of three subunits: α , β , and γ . The IKK- γ subunit plays a regulatory role, and the other two subunits are kinases. While the IKK- α and IKK- β units are structurally similar, their functions are different. IKK- β is essential for rapid activation of the pathway initiated by proinflammatory signals (TNF α or lipopolysaccharide (LPS)) [140]. Thus, inhibitors of IKK- β have the potential to treat asthma by suppressing NF- κ B activation and therefore limiting the production of inflammatory cytokines.

There have been extensive efforts over the years to generate selective IKK- β inhibitors for the treatment of inflammatory disease [141], and some have met with moderate success, reaching clinical trials. It has been suggested that IKK inhibition may suppress corticosteroid resistant asthma, so it would fill an important niche in the market [142]. IMD-0354 (37) (Figure 11.14), an IKK- β inhibitor developed by the Institute of Medicinal Molecular Design of Tokyo, ameliorated airway hyperresponsiveness and reduced mucus and eosinophilia in ovalbumin-sensitized mice, a model of allergic asthma [143].

However, IKK- β knockout mice are embryonic lethal, suffering from liver degeneration [144], so the safety of IKK- β inhibitors could limit their usefulness in chronic diseases such as asthma. If treatment of the general asthma population did limit or prevent the uptake of an IKK- β inhibitor, personalized healthcare might help identify those patients where the risk to reward ratio might be acceptable. If a sufficient therapeutic window cannot be achieved through an orally administered, systemic inhibitor, it might also be necessary to seek topical, inhaled delivery.

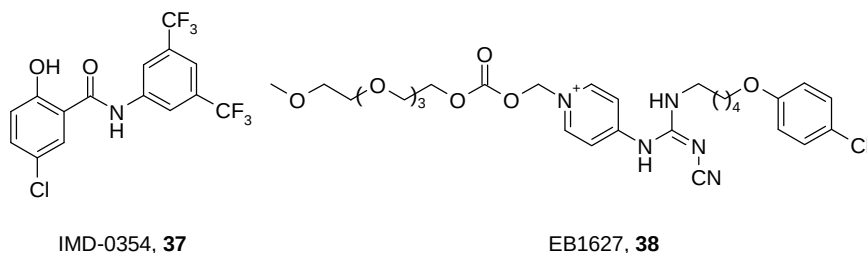


Figure 11.14 IKK- β inhibitors.

Indeed, tissue specific deletion of IKK in bronchial tissue generated viable mice that showed reduced inflammation and levels of airway mucus, airway eosinophils, and peribronchial CD4+ cells after ovalbumin challenge [145].

Several companies including Pfizer, GSK, and AstraZeneca have investigated IKK- β for asthma but, to the best of our knowledge, no IKK- β inhibitors have ever reached clinical development for this indication. The importance of the NF- κ B pathway in multiple diseases prohibits a discussion of all the inhibitors that have been reported to be in preclinical testing. A handful of companies have reached the clinic with IKK- β inhibitors for other indications, including Leo Pharma (EB1627 (**38**)) [146].

11.8

Other Kinases

11.8.1

SphK

The sphingolipid metabolic pathway regulates the levels of the potent bioactive messengers ceramide and sphingosine-1 phosphate (S1P) in all eukaryotic cell membranes [147]. These messengers have been shown to be key players in a variety of physiological processes, such as cell differentiation, proliferation, apoptosis, migration, and angiogenesis. Sphingosine kinase (SphK) is an intracellular kinase, which converts sphingosine into sphingosine-1 phosphate and as such is a key regulator of the levels of sphingosine, S1P, and ceramide, an *N*-acetyl derivative of sphingosine. As these three metabolites are interconvertible, it has been proposed that it is not their absolute amounts but rather their relative levels that determine their physiological effects [147].

In the context of asthma, the sphingolipid pathway has attracted interest due to its potential role in lung inflammation [148]. In human subjects, it was reported that the levels of S1P were dramatically increased in the airways of allergen challenged asthmatic patients [149]. *In vitro*, it has been shown that stimulation of the IgE-receptor Fc ϵ RI on mast cells activates the SphK signaling pathway, leading to increase in S1P mast cells levels [150]. In turn, S1P has been shown to activate the MAPK pathway resulting in mast cell degranulation and release of leukotrienes

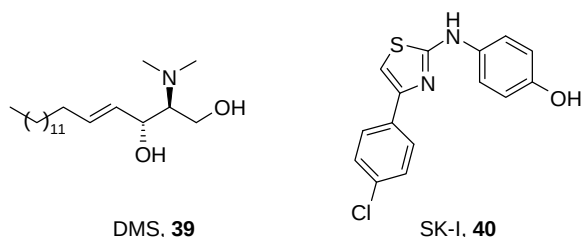


Figure 11.15 Tool SphK inhibitors.

[151]. Furthermore, it has been shown that exposure of airway smooth muscles to S1P results in airway hyperreactivity mediated by RhoA [152]. In preclinical models of allergic asthma, tool SphK inhibitors such as *N,N*-dimethylsphingosine (DMS) (**39**) or SK-I (**40**) (Figure 11.15), have been shown to significantly reduce airway inflammation, lung mucus production, and airway hyperresponsiveness, all key processes underlying the pathophysiology of asthma [153,154]. It is interesting to note that during these *in vivo* studies, the delivery of the inhibitors was carried out via the inhaled route, thereby providing a topical delivery to the lungs and probably minimizing systemic exposure.

Although to date, no SpHK inhibitor has been reported in development, these studies suggest that blockade of SpHK activity may prove useful for the treatment of allergic asthma.

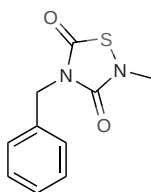
11.8.2

GSK-3 β

Glycogen synthase kinase-3 (GSK-3) is a serine–threonine protein kinase, originally discovered due to its ability to phosphorylate and inactivate glycogen synthase to modulate blood glucose levels. GSK-3 is active in cells under resting conditions and is one of the few kinases that are actually inhibited by phosphorylation, rather than activated [155]. In mammalian cells, there are two isoforms, alpha and beta.

GSK-3 α is involved in the control of several regulatory proteins including glycogen synthase, Myb, from the myeloblastosis family of transcription factors, and c-Jun, and is of particular interest in central nervous system diseases, perhaps most notably for its ability to phosphorylate tau, the principal component of neurofibrillary tangles in Alzheimer's disease.

GSK-3 β is able to regulate nuclear transcription factor- κ B (see above), so it is no surprise that inhibitors of GSK-3 β have been investigated and linked to asthma. While the exact molecular mechanism by which GSK-3 β activates NF- κ B remains nebulous, the downstream effects have been demonstrated in animal models of asthma. Chemical inhibition of GSK-3 β with a selective small-molecule inhibitor reduced the severity of ovalbumin-induced allergic asthma [156]. In these studies, the effects of intravenous injection of a selective GSK-3 β inhibitor, (TDZD-8 (**41**)) (Figure 11.16) on ovalbumin-sensitized mice were reported. These included inhibition of eosinophilia and airway mucus production, and reduced hyperresponsiveness to inhaled



TDZD-8, 41

Figure 11.16 GSK-3 β inhibitor.

methacholine. The authors concluded that inhibition of GSK-3 β may provide a novel means for the treatment of allergic airway inflammation.

In contrast, the airway remodeling that occurs in severe asthma is reported to partly result from upregulation of TGF β 1-driven extracellular matrix production. Studies have shown that stimulation of airway smooth muscle cells with TGF β 1 leads to inhibition of GSK-3, implying that inhibition would promote smooth muscle hypertrophy and exacerbate asthma [157]. Other authors studying potential causes of severe asthma found that GSK-3 β is a regulator of airway smooth muscle cell size. Increased airway smooth muscle mass is a characteristic finding in severe asthma, suggesting that GSK-3 β may play a role in asthmatic airway remodeling [158]. The authors suggest that this is the likely role of GSK-3 β in asthma. In addition, airway myocytes from ovalbumin-treated mice showed increased cell size and the inactive, phosphorylated form of GSK-3 β , suggesting that inhibition of GSK-3 β might contribute to the airway smooth muscle hypertrophy [159].

Ultimately, further studies are likely needed to reach a clearer understanding of the role of GSK-3 β in different subsets of asthmatic patients. The evidence for a different role of GSK-3 β in different types of asthma implies a strong need for a personalized approach. Understanding the subsets of asthma sufferers that would respond, and those that would not or actually see their disease symptoms worsen, might enable us to identify another weapon to attack asthma and enable personalized therapy. While there are GSK-3 β inhibitors in the clinic for other indications, none are currently being studied for the treatment of asthma, so the ultimate impact on personalized treatments can only be anticipated. A cautious approach is warranted, given the wide role of GSK-3 in biological function: GSK-3 β knockout mice die late in development, while GSK-3 α knockout mice are viable. GSK-3 is highly expressed throughout the body, and particularly in the brain, and studies have highlighted a critical role of both isoforms in brain development and neurological disease [155].

11.9

Conclusions: Future Directions

Asthma is a disease with an etiology that is poorly understood, but clearly influenced by both genetic and environmental factors. Predictive models of asthma treatment response are not yet a reality, but are viable goals for the near future.

Since the first genome-wide association study (GWAS) that identified a gene associated with childhood asthma in 2007 [160], multiple other genes have been identified that are related to lung function and asthma. As research and technology inevitably progress, we are likely on the cusp of a major breakthrough in our ability to predict who will suffer from asthma, how serious it will be, and, ultimately, what ideal therapeutic strategy should be followed. The current treatment paradigm, in which most patients are treated first with inhaled corticosteroids, and then further drug therapy is gradually added until the disease can be adequately controlled, may be reaching an end. This “one drug fits all” approach will be gradually replaced as specific therapeutics become available to treat small, clearly stratified populations. Instead of using multiple drugs concurrently to treat or control severe asthma, we will be able to predict in advance which drugs will work, significantly reducing the drug burden.

The future of kinase inhibitors for asthma appears challenging, but promising. Some are progressing well in the clinic, notably Palau Pharma’s p38 inhibitor, and several PI3K δ inhibitors. It is possible that these could be coupled with diagnostic tests that would help to identify patients most likely to benefit or have improved therapeutic indexes. The impact of the personalized medicine approach is sure to come, as more is learned about the associations of some of these kinases (e.g., ITK, PDGFR, and VEGFR) with the severity of asthma and particular patient subpopulations. Based on the current literature, isoform selective p38 inhibitors appear to have great potential in steroid insensitive asthma patients. Likewise, JNK, ERK, and IKK inhibitors might also soon find specific use in steroid insensitive patients. Genetic associations between asthma and several of the kinases discussed in this chapter (e.g., ITK, PDGFR, and VEGFR) are encouraging and suggest that there is strong potential to find a personalized strategy. For other kinases, there remain unanswered questions or ambiguity, and more research will be needed for them to emerge as potential partners for personalized approaches to treat this pervasive disease.

Given the importance of all the kinases described in this chapter in asthma, it seems only a matter of time before drugs targeting these kinases are identified. As our understanding of the disease expands, we will likely be able to select the best drug treatment from our toolbox based on diagnostic tests. As we continue to identify critical pathways in specific populations of asthma sufferers, we will be able to select better targets, including kinases, and relevant patient populations in which to run new clinical trials. Ultimately, it is hoped that these advances will result in improved and more efficient ways to treat this heterogeneous disease.

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12

Developing Targeted PET Tracers in the Era of Personalized Medicine

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12.1

Imaging and Pharmacodynamics Biomarkers in Drug Development

The development of effective therapeutics, from target identification and clinical evaluation and ultimately to approval, is a slow and costly endeavor. The time and expenditure to bring a new drug to the market depends on the nature of the drug candidate (small-molecule or biologic) as well as the clinical indication and targeted patient population. These trials are often more than 5–10 years duration and can cost millions of dollars [1]. Furthermore, the true cost the industry needs to bear must account for the cost incurred from failed trials. Estimates of drug development costs for a new molecular entity (NME) to reach the market, incorporating the high failure rate, can be higher than \$10 billion. In the period from 2005 to 2009, the development of a new CNS drug was, on average, 10 years, the clinical development of an antineoplastic drug was approximately 7–8 years, and the time for clinical development of an anti-HIV antiviral was about 5 years [1a]. The bulk of the development costs are incurred in the later phases of clinical trials. Registration trials (pivotal trials) are required to establish that the therapeutic is both effective, bringing patient benefit by extending life or improving the quality of life, and safe. These trials often require many thousands or even tens of thousands of patients and are performed at multiple centers and often across multiple countries. It seems intuitive, therefore, that any technique that has the ability to increase our confidence in early clinical development that the experimental agent is hitting its target and producing the biological response consistent with its known mode of action will improve our decision making by selecting the best agents to advance into pivotal trials. In this way the pharmaceutical industry can manage opportunity cost by selecting drug candidates with the highest chance of clinical success. In this respect, radiological imaging techniques hold the promise to influence the drug development process [2].

It has been also recognized by the regulatory authorities that novel technologies such as imaging will help in the clinical evaluation of the effectiveness and safety of putative treatments and could thereby reduce the development costs and expedite the availability of new therapies [3]. Nevertheless, these imaging

techniques remain pharmacodynamics (PD) readouts of biological activity and cannot be substitutes for conventional clinical outcome measures. With rare exception (e.g., the use of X-ray to determine the degree of bone erosion), validation as surrogate measures of outcome and therefore acceptance by the regulatory authorities as alternatives to conventional outcome measures has not happened. Clinical validation is a big hurdle, and, until this is met, imaging will only be used by the pharmaceutical companies to support internal decision making during the early stages of clinical drug development.

Clinical imaging biomarkers require the use of a noninvasive robust technique to measure biological phenomena deep inside the body; hence techniques based on the use of radiolabeled tracers are a prime choice for physiological and pathophysiological measurements complemented with computed tomography (CT) and magnetic resonance imaging (MRI) techniques for measuring anatomical changes. The idea to use a radioactive isotope to track an exogenous substance in a living organism was conceived by George de Hevesy at the beginning of twentieth century. A few decades later, in 1943, de Hevesy was awarded with the Nobel Prize for development of radioactive tracers to study chemical and biological processes. The distribution of radioactivity in living systems could be recorded using various techniques, detecting ionizing radiation emitted by the studied subject, such as autoradiography or planar scintigraphy, but the tomographic methods introduced in the second half of twentieth century allowed three-dimensional imaging. Single-photon emission computed tomography (SPECT) was developed in 1960s, which employed soft gamma emitters. Positron emission tomography (PET) developed in 1970s makes use of positron-emitting radionuclides (Table 12.1). Both techniques are quantitative and are widely employed in the clinic as well as used for preclinical research. Thanks to the coincidence detection of two antiparallel 511 keV photons created during positron annihilation, PET does not require detector collimation

Table 12.1 Properties of selected positron-emitting radionuclides, FW20M: full width at 20% of maximum.

Nuclide	β^+ Branch (%)	Half-life	$E\beta^+$ max (keV)	Predicted resolution (FW20M) in soft tis- sue (mm)	Predicted resolution (FW20M) in lung tissue (mm)	Source of radionuclide
^{11}C	99.8	20 min	960.2	0.96	2.69	$^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$
^{13}N	99.8	10 min	1198.5	1.26	3.50	$^{16}\text{O}(\text{p},\alpha)^{13}\text{N}$
^{15}O	99.9	2 min	1732.0	1.87	5.30	$^{14}\text{N}(\text{d},\text{n})^{15}\text{O}$
^{18}F	96.7	110 min	633.5	0.54	1.52	$^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$
^{64}Cu	17.4	12.7 h	653.1	0.57	1.59	$^{64}\text{Ni}(\text{p},\text{n})^{64}\text{Cu}$
^{68}Ga	89.1	68 min	1899.1	2.07	5.83	$^{68}\text{Ga}/^{68}\text{Ge}$ generator
^{89}Zr	22.7	3.3 days	901.5	0.87	2.44	$^{89}\text{Y}(\text{p},\text{n})^{89}\text{Zr}$
^{124}I	22.8	4.2 days	2137.6	2.36	6.64	$^{124}\text{Te}(\text{p},\text{n})^{124}\text{I}$

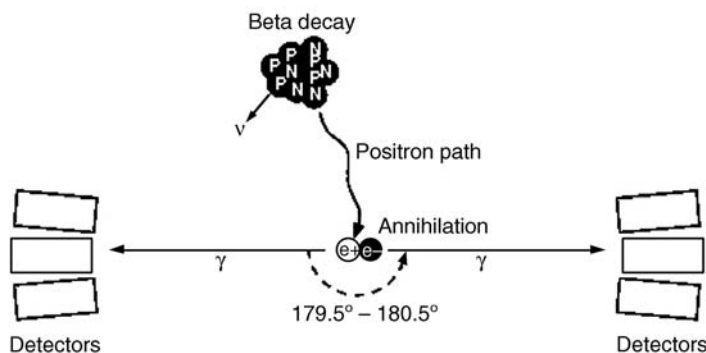


Figure 12.1 Coincidence detection of two 511 keV photons by ring of detectors in PET camera.

and background shielding; hence PET is more sensitive and yields quantitative images with better spatial resolution than SPECT. The theoretical limit of PET's spatial resolution is determined by two factors: (i) a distance a positron travels from the site of β^+ decay to the site of annihilation (the distance is proportional to the kinetic energy of the emitted positron and inversely proportional to the stopping power of the tissue (Table 12.1)) [4], and (ii) a deviation of emitted 511 keV photons from antiparallel orientation (180°). Due to the conservation of the residual momentum of annihilated positron, the angle varies between 180.5° and 179.5° (Figure 12.1). By combination of these factors with the physical size of the detectors, the resolution of PET cameras reach 5–7 mm for clinical full-body scanners, 3–4 mm for dedicated head cameras, and 1–2 mm for preclinical small animal PET scanners.

There are many positron-emitting radionuclides that are available from cyclotron products or from radionuclide generators. The most widely available ones, together with their physicochemical properties, are summarized in Table 12.1. The choice of radionuclide is largely determined by the properties of the chemical entity that is being labeled. For example, imaging of radiolabeled antibodies, which have long biological half-lives and slow accumulation in the target tissue, requires isotopes with long half-life either of the radiometals (copper-64, ^{64}Cu , and zirconium-89, ^{89}Zr), or ^{124}I [5]. Conversely, ^{18}F or ^{68}Ga are widely used for radiolabeling peptides with short biological half-life. Carbon, nitrogen, oxygen, and fluorine are present in many biologically active molecules and pharmaceuticals, and replacing these atoms with radioactive isotopes will not change the biological properties of the substance. The very short half-life of ^{15}O and ^{13}N limits the number of available synthetic transformations to bare minimum; hence only simple molecules such as $^{13}\text{N}[\text{NH}_3]$, $^{15}\text{O}_2$, and $^{15}\text{O}[\text{H}_2\text{O}]$ are routinely prepared for *in vivo* imaging studies. It should be noted that $[6\text{-}^{15}\text{O}]\text{-2-deoxy-D-deoxyglucose}$ was recently successfully synthesized and evaluated in animal models [6], and $^{13}\text{NH}_3$ was used to prepare $^{13}\text{N}[\text{cisplatin}]$ [7] and $^{13}\text{N}[p\text{-nitrophenyl carbamate}]$ [8].

The half-life of ^{11}C or ^{18}F is sufficient to allow multistep syntheses of complex organic molecules; the synthetic approaches to ^{11}C and ^{18}F radiolabeled compounds [9] and examples of their application in drug development will be discussed in this chapter.

12.2

General Considerations for Development of ^{11}C - and ^{18}F -labeled PET Tracers

For small molecules, the choice ^{11}C or ^{18}F is determined by a number of factors. The long half-life of ^{18}F allows transport of the isotope from the cyclotron site to the radiochemistry facility and then distribution of the product to remote imaging centers. Carbon-11 radiolabeled compounds, on the other hand, require on-site cyclotron, and the transport of ^{11}C -labeled tracer is possible only to a limited distance. Besides the logistical issues, the properties of the synthesized molecule have to be considered. All organic molecules contain carbon and are theoretically amenable for ^{11}C incorporation, but only a limited number of biologically active molecules contain fluorine atom suitable for isotopic substitution. The development of the PET tracer starts with the selection of the position to attach the radioactive isotope. In some cases the biologically active molecule contains a position or functional group where the radionuclide can be incorporated by replacing the carbon or fluorine already present with ^{11}C or ^{18}F ; however, in most cases, the molecule has to be modified to accommodate efficient radiolabeling, leading to a new molecular entity whose biological properties have to be re-evaluated. In general, the radiotracers should be as specific as possible to the target of interest. Their pharmacokinetics should provide plasma half-life short enough to result in low nonspecific background at later time points, but the tracer should have enough time to allow sufficient specific uptake at target to provide adequate target-to-background ratio. Renal clearance of the radiotracers is desired, rather than hepatobiliary excretion, since it provides low background in the abdominal cavity. The radiotracers should be metabolically stable for the duration of the imaging experiment; if metabolites are formed, they must not interfere with the studied process. The radioactive metabolites should not exhibit specific binding and should be quickly eliminated.

For neuroimaging, PET tracers require adequate distribution across the blood–brain barrier (BBB) to reach the targets [10]. The passive transport through BBB is possible for compounds with molecular weight below 500 Da and octanol/water partitioning coefficient ($\log P$) in the range 1.5–3 [11]. The tight junctions between endothelial cells of BBB limit diffusion through the interstitial space, and lipophilic cellular membranes effectively prevent polar compounds ($\log P < 1.5$) to enter the brain compartment by diffusion. Highly lipophilic molecules ($\log P > 3$) will likely exhibit significant nonspecific binding

to lipids and membrane-associated proteins. Nevertheless, even molecules with ideal properties to cross BBB are often intercepted by efflux transporters and pumped back to the blood compartment. The most active efflux transporters are P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The resulting transport through BBB can be estimated for a given molecule by measuring the transport through a monolayer of cells transfected with efflux transporters [12], in addition to calculation or determination of logP and other physicochemical properties (polar surface, number of H-bond donors). Metabolites formed from systemic catabolism of the tracers should be polar and unable to cross BBB by diffusion or substrates for efflux transporters, to minimize their interference with the imaged target in brain [10].

The *in vitro* binding affinity of the radiotracer to its target receptor, measured as dissociation constant (K_D), is usually in low nanomolar or subnanomolar range (10 nM–10 pM) [13]. The binding affinity required to obtain an ideal target-to-nontarget ratio is closely related to the amount of target available for binding (B_{avail}). The desired binding affinity can be estimated using the concept of binding potential (BP) defined *in vitro* as the ratio of the bound ligand concentration (B) and the free ligand concentration (F) at equilibrium or $\text{BP} = B/F$. The bound tracer concentration is proportional to amount of the target (B_{max}) and $1/K_D$ by equation $B = B_{\text{max}}F/(K_D + F)$. Assuming the chemical amount of the tracer is negligible ($F \ll K_D$), the two equations combine to $\text{BP} = B/F = B_{\text{max}}/K_D$. The desired *in vitro* BP values for imaging tracers are usually above 5.

The *in vivo* BP is related to, but does not equal, the *in vitro* measurements of B_{max}/K_D . The *in vivo* BP is obtained from the ratio of the specific binding concentration in tissue at equilibrium to an approximate measure of the free tracer concentration (Section 12.6) [14]. The *in vivo* BP is often lower than the *in vitro* estimated BP due in part to the presence of endogenous ligands occupying the target and decreasing the amount of target available for binding or B_{avail} (i.e., $B_{\text{max}} > B_{\text{avail}}$). Discrepancies between *in vivo* and *in vitro* measurements can also be attributed to the fact that the assay conditions for the *in vitro* measurements are not fully comparable to the *in vivo* conditions.

The radiotracers for interrogation of metabolic processes are frequently enzyme substrates and are expected to undergo the studied metabolic transformations; the products should be trapped on the site of the transformation to provide readout of the studied enzymatic activity. Examples of such transformations leading to trapped polar metabolites not capable of leaving the cytosol by passive diffusion through cell membrane are [^{18}F]-2-fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) to study glucose utilization [15], ^{18}F -fluorodopa ([^{18}F]-FDOPA) used to image dopaminergic neurons [16], tracers for imaging acetylcholinesterase activity in brain [17], and tracers for imaging hypoxia based on 2-nitroimidazole [18]. [^{18}F]-FDG is transported to a cell by glucose transporters, undergoes phosphorylation by hexokinase to form [^{18}F]-FDG-6-phosphate, and the highly polar [^{18}F]-FDG-6-phosphate is trapped in cytosol unless dephosphorylated by phosphatases. Neutral

[^{18}F]-FDOPA is transported to cells and decarboxylated by aromatic amino acid decarboxylase to form charged [^{18}F]-fluorodopamine that is sequestered in secretory vesicles. A number of acetylcholinesterase tracers are based on N -[^{11}C]methylpiperidin-4-yl acetate or N -[^{18}F]-2-fluoroethylpiperidin-4-yl acetate. The molecules are lipophilic enough to cross the BBB and are deacetylated by acetylcholinesterase in brain, forming polar metabolites unable to diffuse back to the blood pool. The 2-nitro group in hypoxia tracers [^{18}F]-FMISO [19] or [^{18}F]-FAZA [20] is reduced in hypoxic intracellular environment to the charged amino group and the resulting metabolites are cytosolically trapped.

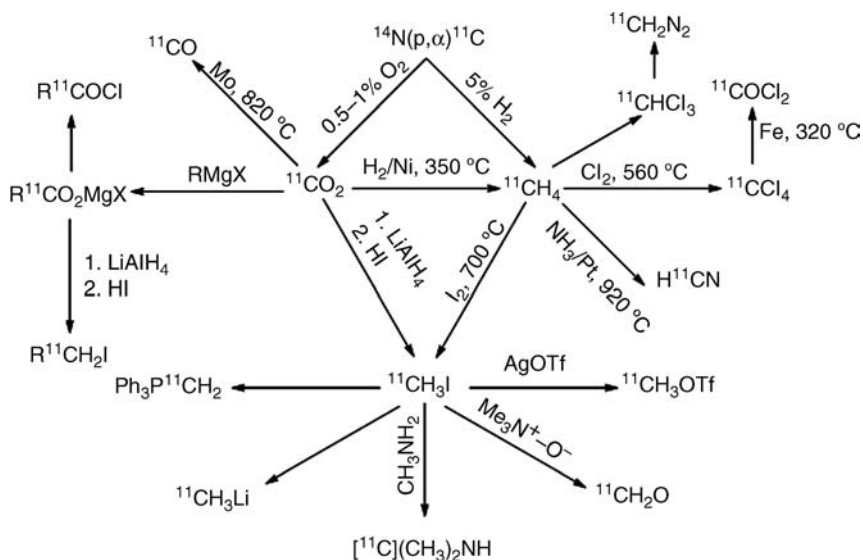
12.3

Radiolabeling Compounds with ^{11}C

12.3.1

Preparation of ^{11}C and Basic Reactive Intermediates

Carbon-11 is prepared in a cyclotron by $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction. The target material is gaseous N_2 bombarded with protons with energy 5–22 MeV [21]. In presence of trace amounts (0.5–1%) of oxygen the product is $^{11}\text{CO}_2$ and in presence of trace amounts (5%) of hydrogen the product is $^{11}\text{CH}_4$ (Scheme 12.1). The conversions of $^{11}\text{CO}_2$ and $^{11}\text{CH}_4$ to reactive synthetic building blocks are depicted in Scheme 12.1.



Scheme 12.1 Preparation ^{11}C and basic ^{11}C -containing reagents.

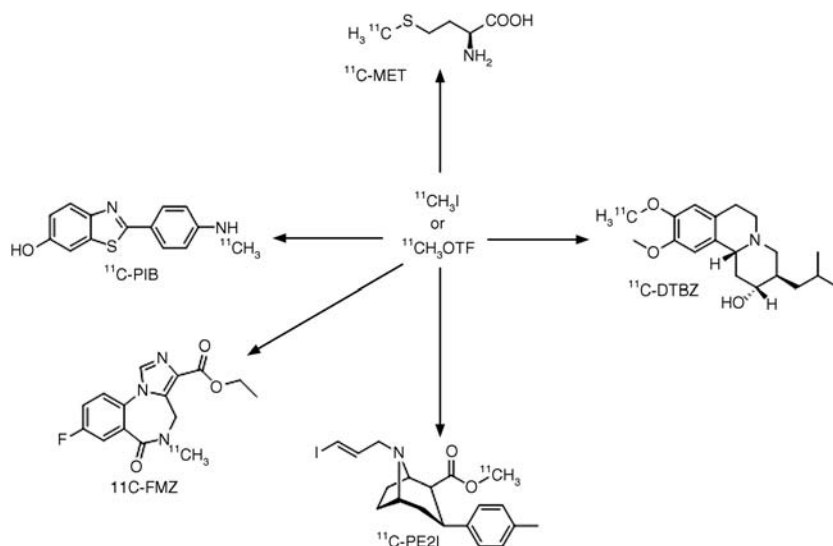
Most frequently employed ^{11}C -reagents are ^{11}C -methyl iodide ($^{11}\text{CH}_3\text{I}$) and ^{11}C -methyl triflate ($^{11}\text{CH}_3\text{OTf}$). $^{11}\text{CH}_3\text{I}$ can be prepared by reduction of $^{11}\text{CO}_2$ to lithium methanolate using LiAlH_4 and subsequent substitution of the hydroxyl group with iodide by treatment with HI . As an alternative to the “wet” method described earlier, $^{11}\text{CH}_3\text{I}$ can be prepared from ^{11}C -methane by a “gas-phase” method. The starting material ^{11}C -methane can be prepared directly in a cyclotron target or by catalytic hydrogenation of $^{11}\text{CO}_2$. The in-target-produced $^{11}\text{CH}_4$ is obtained in higher specific activity than $^{11}\text{CH}_4$ prepared from $^{11}\text{CO}_2$, avoiding the contamination with CO_2 during off-target reduction to $^{11}\text{CH}_4$. Radical iodination of $^{11}\text{CH}_4$ at high temperature also provides $^{11}\text{CH}_3\text{I}$ [22]. Optionally, $^{11}\text{CH}_3\text{I}$ can be converted to increase the reactive $^{11}\text{CH}_3\text{OTf}$ by passing through a heated cartridge filled with AgOTf . The gas-phase synthesis of $^{11}\text{CH}_3\text{I}$ has gained more popularity than the “wet” method due to its amenability to automation and the availability of synthesizers capable of producing both $^{11}\text{CH}_3\text{I}$ and $^{11}\text{CHOTf}$ [23]. The methylating reagents are starting materials used in a wide spectrum of syntheses of PET tracers labeled with ^{11}C by alkylation of heteroatoms or by formation of ^{11}C —C bonds.

12.3.2

^{11}C -Methylations, Formation of ^{11}C —X Bond (X = O, N, S)

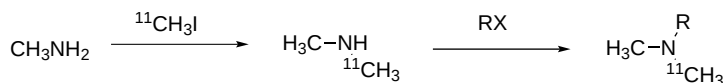
^{11}C -Methylation of heteroatoms in hydroxyl, amino, or thiol groups is the most common and straightforward way to incorporate ^{11}C into a molecule in a single step in high yield [24]. Primary or secondary amines in free base form or deprotonated phenols and thiols can be alkylated with $^{11}\text{CH}_3\text{I}$ or $^{11}\text{CH}_3\text{OTf}$ in high yields (Scheme 12.2). ^{11}C -PIB for imaging amyloid beta (A β) plaque burden in Alzheimer’s disease (AD) patients was synthesized using $^{11}\text{CH}_3\text{I}$ and 6-methoxymethyl (MOM)-protected precursor [25]; higher reactivity of $^{11}\text{CH}_3\text{OTf}$ allowed use of a nonprotected precursor and accomplished the synthesis in a single step [26]. Synthesis of ^{11}C -DTBZ for imaging vesicular monoamine transporter (VMAT2) to assess integrity of dopaminergic signaling system in Parkinson’s disease patients was accomplished by alkylation of 9-O-desmethyldihydrotrabenazine with $^{11}\text{CH}_3\text{I}$ [27]. Finally, ^{11}C -MET used for imaging glioblastomas was synthesized by alkylation of L-homocysteine [28] or L-homocysteine thiolactone [29] with $^{11}\text{CH}_3\text{I}$ or $^{11}\text{CH}_3\text{OTf}$ [30].

Amides can be deprotonated and alkylated with $^{11}\text{CH}_3\text{OTf}$ or $^{11}\text{CH}_3\text{I}$ as exemplified by synthesis of benzodiazepine receptor ligand ^{11}C -flumazenil (^{11}C -FMZ) [30]. Salts of carboxylic acids react with $^{11}\text{CH}_3\text{I}$ and $^{11}\text{CH}_3\text{OTf}$ to provide corresponding ^{11}C -methyl esters. A dopamine transporter (DAT) ligand (E)-N-(3-iodoprop-2-enyl)-2 β -(4'-tolyl) nortropane (PE2I) was radiolabeled by alkylation of its sodium salt precursor using $^{11}\text{CH}_3\text{OTf}$ in decay corrected yields of over 50% [31]. The reactions with $^{11}\text{CH}_3\text{I}$ or $^{11}\text{CH}_3\text{OTf}$ are performed by distillation of the methylating reagent into a vial containing the nucleophilic substrate or by passing gaseous $^{11}\text{CH}_3\text{I}$ or $^{11}\text{CH}_3\text{OTf}$ through the loop or cartridge containing the immobilized substrate [32].



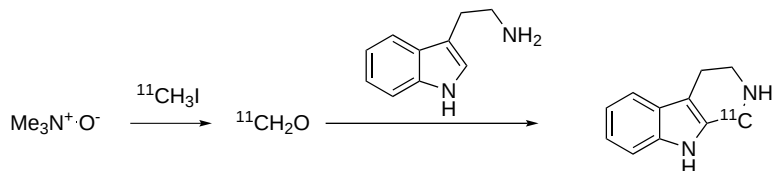
Scheme 12.2 Examples of compounds radiolabeled by heteroatom methylation using $^{11}\text{C}\text{H}_3\text{I}$ or $^{11}\text{C}\text{H}_3\text{OTf}$.

An intriguing reversal of chemistry can be achieved by reaction of $^{11}\text{C}\text{H}_3\text{I}$ or $^{11}\text{C}\text{H}_3\text{OTf}$ with methylamine (Scheme 12.3). The formed [^{11}C]dimethylamine can be subsequently alkylated to afford various compounds containing a [^{11}C]dimethylamine moiety [33].



Scheme 12.3 Preparation of [^{11}C]dimethylamine and its alkylation.

Although ^{11}C -formaldehyde was prepared from $^{11}\text{C}\text{H}_3\text{OH}$ in 1972 [34], the widespread use of this potentially useful ^{11}C -labeling reagent was hindered by numerous technical drawbacks. A more practical synthesis of ^{11}C -formaldehyde starting from $^{11}\text{C}\text{H}_3\text{I}$ was recently reported [35]. The reaction starts with *O*-alkylation of trimethylamine *N*-oxide followed by decomposition to ^{11}CHO . The ^{11}CHO was then reacted with tryptamine to yield ^{11}C -labeled 2,3,4,9-tetrahydrocarboline by Pictet–Spengler reaction in excellent radiochemical yield (Scheme 12.4).

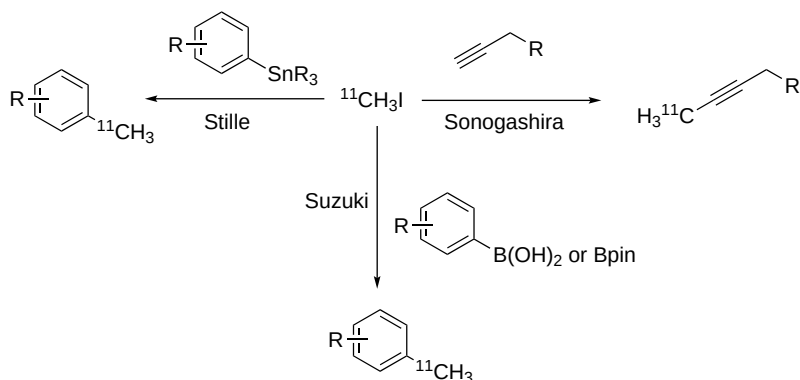


Scheme 12.4 Preparation of $^{11}\text{C}\text{H}\text{O}$ from $^{11}\text{C}\text{H}_3\text{I}$ and its use in Pictet–Spengler reaction.

12.3.3

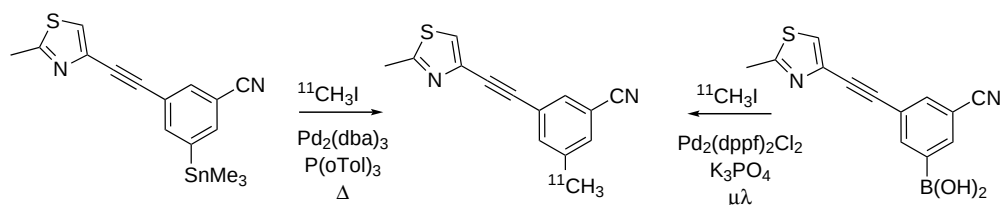
 ^{11}C -Methylations, Formation of ^{11}C —C Bond

Despite the vast majority of ^{11}C -labeled PET tracers in current clinical use are radiolabeled by heteroatom methylation, the diversity of biologically active molecules generates a constant need to expand the repertoire of chemical transformation for the incorporation of ^{11}C . Not surprisingly, transition metal-catalyzed reactions employing alkyl halides have been extensively investigated for synthesis of ^{11}C -labeled compounds (Scheme 12.5). Since the convenient starting materials, no-carrier-added $^{11}\text{CH}_3\text{I}$ and $^{11}\text{CH}_3\text{OTf}$, are used in dearth compared to the catalyst, the reaction should be termed transition metal mediated rather than catalyzed. The Stille coupling, a Pd-catalyzed cross-coupling of stannanes with alkyl halides, was explored for preparation of ^{11}C -labeled compounds. Alkenyl stannanes, aryl stannanes, and heteroaryl stannanes were successfully coupled with $^{11}\text{CH}_3\text{I}$ [36]. As an alternative to Stille coupling, Suzuki reaction of boronic acids or esters with alkyl halides was used to attach $^{11}\text{CH}_3\text{I}$ to various aromatic compounds, avoiding potentially toxic stannanes [37]. The model compounds (^{11}C -toulenes) were obtained in 56–92% yield and with high radiochemical purity. The Sonogashira coupling of alkyl halides and terminal alkynes was investigated for the synthesis of ^{11}C -labeled steroids [38]. Using classical conditions with CuI as cocatalyst, the Sonogashira coupling of $^{11}\text{CH}_3\text{I}$ provided only low yields, but the use AsPh_3 as a co-catalyst led to yields in the range of 49–64%.



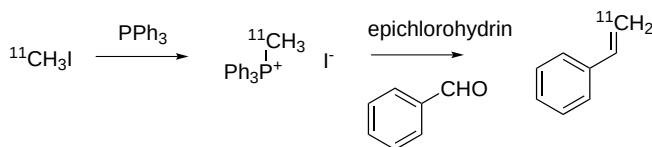
Scheme 12.5 Pd-mediated ^{11}C -methylations.

As an example to demonstrate the versatility of Pd-mediated ^{11}C -methylations, the ligand for metabotropic glutamate receptor type-5 (MTEB) could be prepared by Stille or Suzuki coupling, starting from the corresponding aryl stannane or aryl boronic acid (Scheme 12.6) [39]. Stille coupling of $^{11}\text{CH}_3\text{I}$ to a trimethyltin precursor was accomplished in DMF using $\text{Pd}_2(\text{dba})_3/\text{P}(\text{oTol})_3$ catalytic system under thermal heating. The preferred Suzuki coupling provided the same product using more a stable catalyst ($\text{Pd}_2(\text{dppf})\text{Cl}_2$) and microwave heating in higher yields (29%) within 20–23 min.



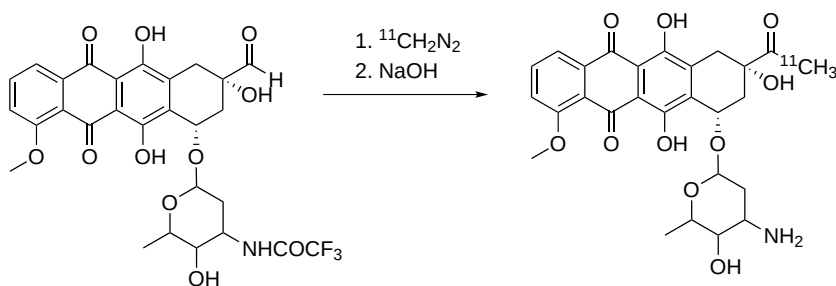
Scheme 12.6 Synthesis of ^{11}C -MTEB by Suzuki and Stille cross-coupling.

One-pot Wittig synthesis of ^{11}C -styrene as a model compound was reported by Kihlberg *et al.* [40]. Alkylation of triphenylphosphine with $^{11}\text{CH}_3\text{I}$ followed by treatment of the formed ^{11}C -methyltriphenylphosphonium iodide with a strong base provided the ^{11}C -triphenylphosphonium ylide. The optimized conditions used alkoxide generated from epichlorohydrin *in situ* as a base to form the ylide that reacted with benzaldehyde to give $[\beta\text{-}^{11}\text{C}]$ styrene in one pot in 86% radiochemical yield (Scheme 12.7).



Scheme 12.7 Wittig synthesis of $[\beta\text{-}^{11}\text{C}]$ styrene from $^{11}\text{CH}_3\text{I}$.

Partial radical chlorination of $^{11}\text{CH}_4$ to $^{11}\text{CHCl}_3$ followed by a reaction with hydrazine afforded another potentially useful ^{11}C -methylating reagent ^{11}C -diazomethane [41]. The procedure was recently optimized to provide $^{11}\text{CH}_2\text{N}_2$ in 20% radiochemical yield [42]. Carbon-11-labeled daunorubicin was proposed as a PET tracer for imaging activity of P-gp efflux pumps and synthesized by reaction of the corresponding aldehyde with $^{11}\text{CH}_2\text{N}_2$ followed by deprotection of the primary amine in 3% overall radiochemical yield within 53 min (Scheme 12.8) [43].



Scheme 12.8 Synthesis of ^{11}C -daunorubicin using $^{11}\text{CH}_2\text{N}_2$.

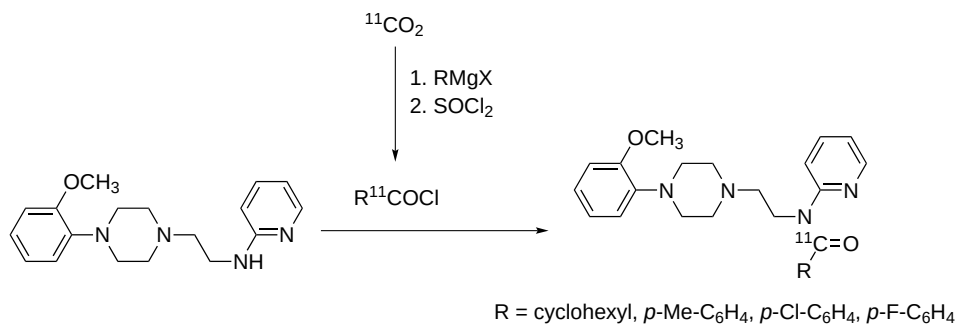
12.3.4

Reactions with $^{11}\text{CO}_2$

The cyclotron product $^{11}\text{CO}_2$ can be incorporated directly into organic molecules by Grignard reaction with alkylmagnesium halides. After quenching the reaction mixture with acid, the transformation provides $[1-^{11}\text{C}]$ carboxylic acids. The reduction of the carboxylate with LiAlH_4 followed by iodination leads to $[1-^{11}\text{C}]$ -labeled alkyl iodides ($\text{R}^{11}\text{CH}_2\text{I}$) useful for alkylation reactions analogous to transformations described earlier in this chapter. The reaction of the $[1-^{11}\text{C}]$ carboxylates with thionyl chloride gives reactive $1-[^{11}\text{C}]$ acyl chlorides, frequently used in acylation reactions (Scheme 12.1).

Several methods for preparation of a clinically used PET tracer ^{11}C -acetate (ACE) for imaging of metabolism in tumors, myocardium, and glia were reported. All procedures utilize quenching of methylmagnesium halides with $^{11}\text{CO}_2$, but differ in the isolation of the final product from the organic phase. As an example, the fully automated synthesis of ^{11}C -ACE was accomplished by passing of $^{11}\text{CO}_2$ through a narrow Teflon tube with immobilized methylmagnesium bromide. The trapped product is then flushed with aqueous hydrochloric acid, affording ^{11}C -ACE in 72% yield [44].

The synthesis of serotonin receptor $5\text{HT}_{1\text{A}}$ tracer [carbonyl- ^{11}C]WAY-100635 and its analogs was accomplished by $[1-^{11}\text{C}]$ -acylation of the secondary amino group of the precursor (Scheme 12.9) [45]. For [carbonyl- ^{11}C]WAY-100635, the $[1-^{11}\text{C}]$ cyclohexanoyl chloride was prepared by reacting $^{11}\text{CO}_2$ with cyclohexylmagnesium chloride coated on the inner surface of polypropylene tubing followed by flush with thionyl chloride into the vial containing the secondary amine; the procedure provided the desired amide [carbonyl- ^{11}C]WAY-100635 in 50–70% yield [46].

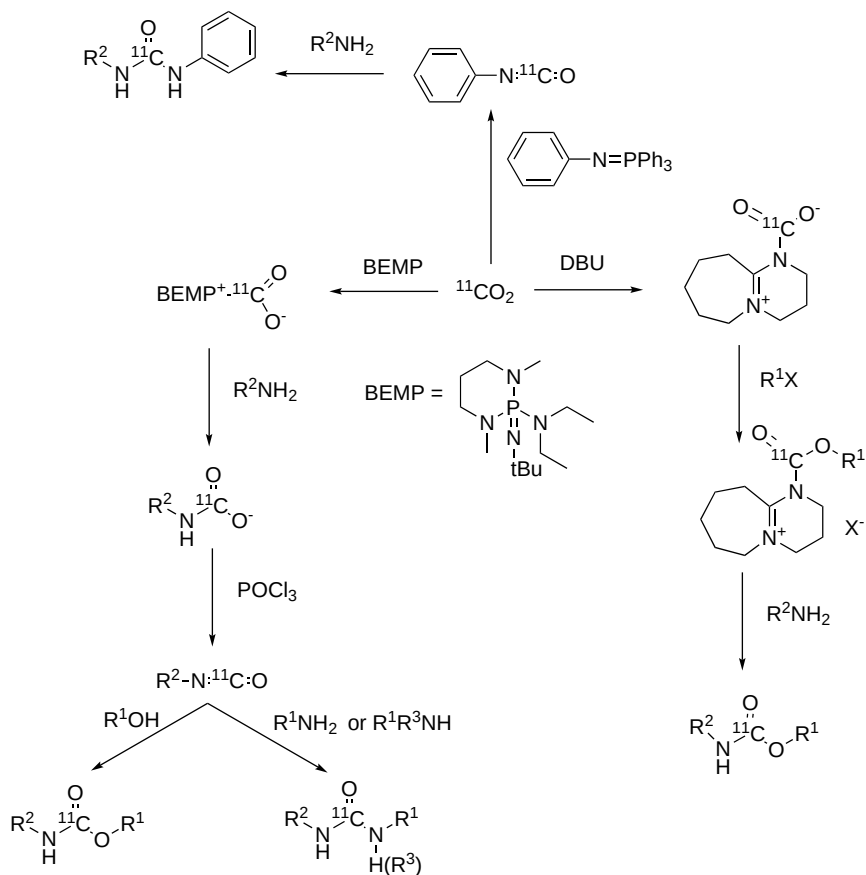


Scheme 12.9 Synthesis of [carbonyl- ^{11}C]WAY-100635 and its analogs.

The serotonin transporter tracer WAY-100635 was also radiolabeled by methylation of the corresponding phenol to provide $[O\text{-methyl-}^{11}\text{C}]$ WAY-100635. Since WAY-100635 is metabolized in periphery by hydrolysis of the amide bond, the [carbonyl- ^{11}C]WAY-100635 and $[O\text{-methyl-}^{11}\text{C}]$ WAY-100635 form different radioactive metabolites. $[O\text{-methyl-}^{11}\text{C}]$ WAY-100635 provides ^{11}C -labeled secondary

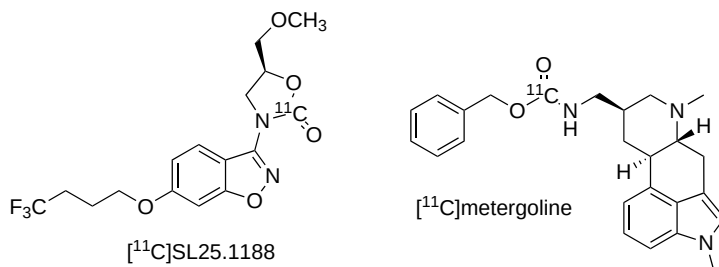
amine, and [carbonyl- ^{11}C]WAY-100635 metabolism leads to ^{11}C -labeled cyclohexanoic acid. The secondary amine is capable of crossing the BBB and binding nonspecifically and specifically to several targets, thus effectively hindering quantitative image analysis. Conversely, the radioactive metabolite of [carbonyl- ^{11}C]WAY-100635, [1- ^{11}C]cyclohexyl carboxylic acid, is not able to cross BBB, hence [carbonyl- ^{11}C]WAY-100635 is preferred over [O-methyl- ^{11}C]WAY-100635 for imaging 5-HT $_A$ receptors [10].

Recent reports showed that strong nitrogen bases can trap $^{11}\text{CO}_2$ and the formed carbonate can be transformed into unsymmetrical carbamates or ureas (Scheme 12.10). Unsymmetrical phenylureas were prepared by trapping $^{11}\text{CO}_2$ with phenyltriphenylphosphinimine at low temperature followed by conversion to ^{11}C -phenylisocyanate by heating and subsequently reacting with primary amine to yield the desired ^{11}C -phenylurea [47]. Hooker *et al.* reported trapping $^{11}\text{CO}_2$ with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) followed by O-alkylation with alkyl halides, and subsequent treatment with secondary amine gave rise to various



Scheme 12.10 Synthesis of ^{11}C -labeled ureas and carbamates from $^{11}\text{CO}_2$.

carbamates [48]. The DBU-mediated fixation of CO_2 was used for synthesis of the ^{11}C -labeled serotonin receptor antagonist metergoline in 35% yield within 45 min (Scheme 12.11) [49]. Vasdev and coworkers used 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine (BEMP) to trap cyclotron-produced $^{11}\text{CO}_2$; the carbonate was first treated with amine to form carbamate salt that was dehydrated with POCl_3 to provide the ^{11}C -isocyanate [50]. The addition of alcohols to the ^{11}C -isocyanate led to formation of ^{11}C -carbamates [51], and the addition of primary or secondary amines to the ^{11}C -isocyanate provided unsymmetrical ureas [52]. Experimental monoamine oxidase B imaging tracer ^{11}C -SL25.1188 was prepared via the BEMP-mediated $^{11}\text{CO}_2$ fixation in 12% uncorrected radiochemical yield within 30 min (Scheme 12.11) [53].



Scheme 12.11 ^{11}C -SL25.1188 and ^{11}C -metergoline were prepared by $^{11}\text{CO}_2$ -fixating reaction.

The strong base-mediated $^{11}\text{CO}_2$ -fixating reactions are attractive because the cyclotron-produced $^{11}\text{CO}_2$ is used directly without the need to prepare reactive intermediates. Moreover, $^{11}\text{CO}_2$ is used as a reagent corresponding to the same synthon as ^{11}C -phosgene, which is significantly less stable and significantly more difficult to prepare by radical chlorination of $^{11}\text{CH}_4$ [54].

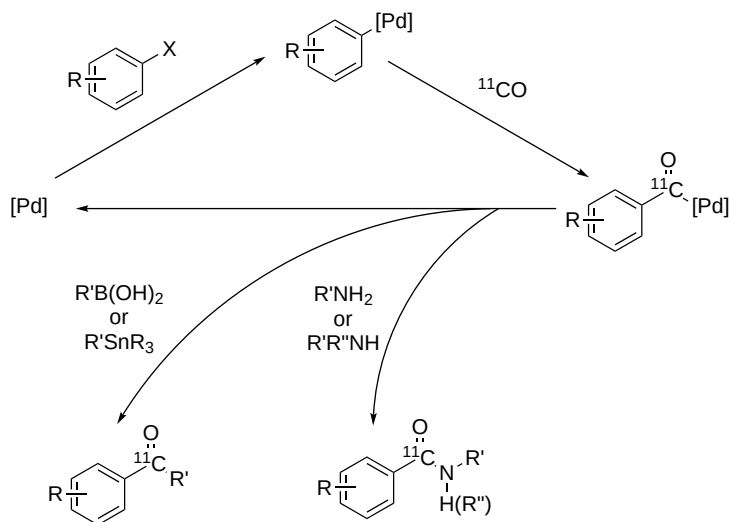
12.3.5

Reactions with ^{11}CO

Catalytic carbonylation is a versatile reaction, providing an access to diverse biologically interesting molecules containing carbonyl group. The reaction utilizes CO , transition metal catalyst, and it is often performed at elevated temperature under high pressure. For the synthesis of ^{11}C -labeled compounds, ^{11}C -CO is readily available by reduction of cyclotron-produced $^{11}\text{CO}_2$ by zinc or molybdenum at high temperature (Scheme 12.1) [55]. Automated high-pressure reactor systems and microautoclaves have been also developed for catalytic ^{11}C -carbonylations [56]. The use of ^{11}CO for a preparation of ^{11}C -labeled molecules was pioneered by Långström and coworkers, who reported syntheses of ^{11}C -labeled ketones, amides, imides.

The widely used palladium-mediated ^{11}C -carbonylation has been successfully applied to synthesis of arylketones and amides. The reaction proceeds through three-step catalytic cycle: (i) oxidative addition of aryl halide or vinyl halide to $\text{Pd}(0)$,

(ii) formation of Pd–carbon monoxide complex and insertion of carbon monoxide into Pd–C bond, and (iii) nucleophilic attack at carbonyl and reductive elimination of the product. The starting aryl or vinyl derivatives are usually iodides, bromides, or triflates. The electrophiles attacking the inserted ^{11}C -carbonyl group leading to [carbonyl- ^{11}C]-arylketones are aryl boronic acids or esters [55–57] or aryl stannanes [58]. The nucleophilic attack with primary or secondary amines leads to formation of [carbonyl- ^{11}C]-amides (Scheme 12.12).

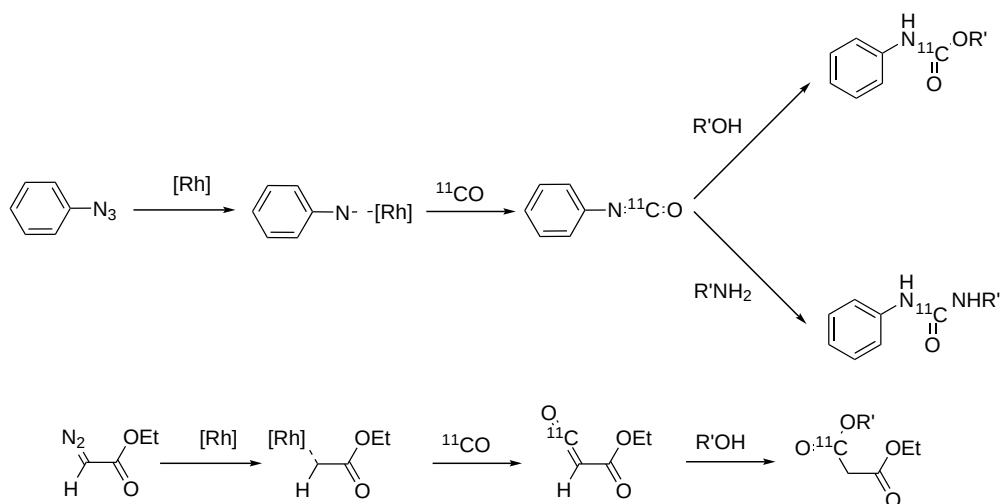


Scheme 12.12 Pd-Catalyzed ^{11}C -carbonylation. Synthesis of [carbonyl- ^{11}C]amides and [carbonyl- ^{11}C]ketones.

The rate-limiting step is the insertion of ^{11}CO , hence higher pressure is required for the reaction to overcome the low partial pressure of carbon monoxide and its low solubility in organic solvents. Nonetheless, atmospheric pressure synthesis of [^{11}C]benzylbenzamide and [^{11}C]phthalide was accomplished by trapping and concentrating ^{11}CO in the form of $\text{BH}_3\text{-}^{11}\text{CO}$ adduct [59].

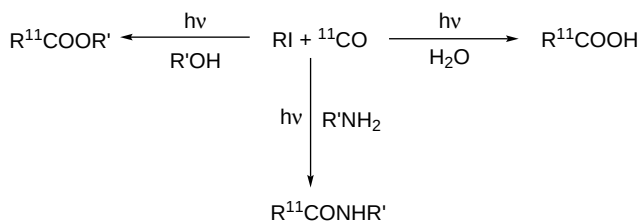
Rhodium-mediated ^{11}C -carbonylation was studied by synthesis of malonates [60] and urea derivatives [61]. The synthesis of ^{11}C -arylureas started with the corresponding arylazide forming Rh(I) complex and the release of N_2 . The preparation of [carbonyl- ^{11}C]diethylmalonate started with 2-diazoacetate forming a complex with Rh-catalyst. Both reactions continued through insertion of ^{11}CO , presumably followed by reductive elimination of ^{11}C -isocyanate or ^{11}C -ketene intermediates, which undergo a nucleophilic attack to provide the final products (Scheme 12.13). Alternatively, the nucleophilic attack takes place on the Rh complex prior to reductive elimination of the isocyanate or ketene.

The radical ^{11}C -carbonylation (Scheme 12.14) was explored for the synthesis of ^{11}C -labeled carboxylic acids, esters, and amides [62]. The transformation utilizes alkyl iodides as a starting material and the presence of water, alcohols, or amines



Scheme 12.13 Rhodium-catalyzed ^{11}C -carbonylation.

defines the desired product. The radical carbonylation is promoted by UV light (280–400 nm) and various photosensitizers, high ^{11}CO pressure is also required. The reaction tolerates a variety of functional groups, providing an interesting alternative to the earlier discussed Grignard synthesis of ^{11}C -carboxylic acids and their derivatives.



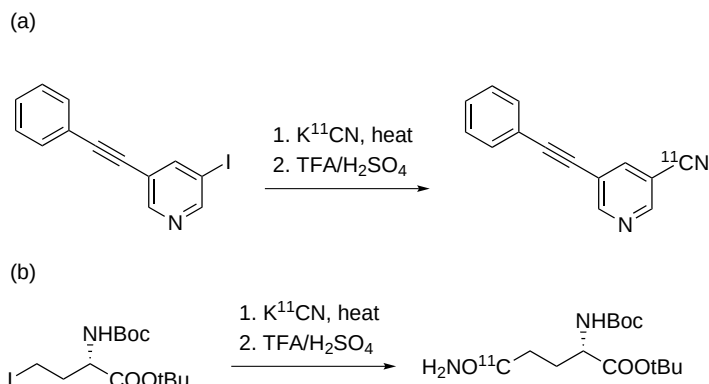
Scheme 12.14 Radical insertion of ^{11}CO .

12.3.6

Reactions with H^{11}CN

Hydrogen cyanide labeled with ^{11}C was evaluated for the synthesis of various compounds containing the nitrile group. H^{11}CN is prepared by reaction of $^{11}\text{CH}_4$ with NH_3 over platinum at high temperature (Scheme 12.1) [63]. The H^{11}CN has been incorporated into aromatic compounds using $[^{11}\text{C}]\text{CuCN}$ -mediated Rosenmund–von Braun reaction or palladium-catalyzed ^{11}C -cyanation [64]. A synthesis of mGluR5 tracer $[^{11}\text{C}]\text{LY2232645}$ was carried out with $[^{11}\text{C}]\text{CuCN}$, providing the final product in 2.5% yield (Scheme 12.15) [65]. The ^{11}CN group can also be incorporated into aliphatic systems by

nucleophilic substitution. A preparation and preclinical evaluation of L-[5- ^{11}C] glutamine as an imaging tracer for tumor metabolism was recently reported [66]. The synthesis was accomplished by coupling H^{11}CN to protected 4-iodohomoalanine followed by simultaneous hydrolysis of nitrile to amide and removal of protecting groups. The product [5- ^{11}C]glutamine was obtained in 25% radiochemical yield within 60 min (Scheme 12.15).



Scheme 12.15 The use of H^{11}CN for preparation of [^{11}C]LY2232645 (a) and [5- ^{11}C]glutamine (b).

12.4

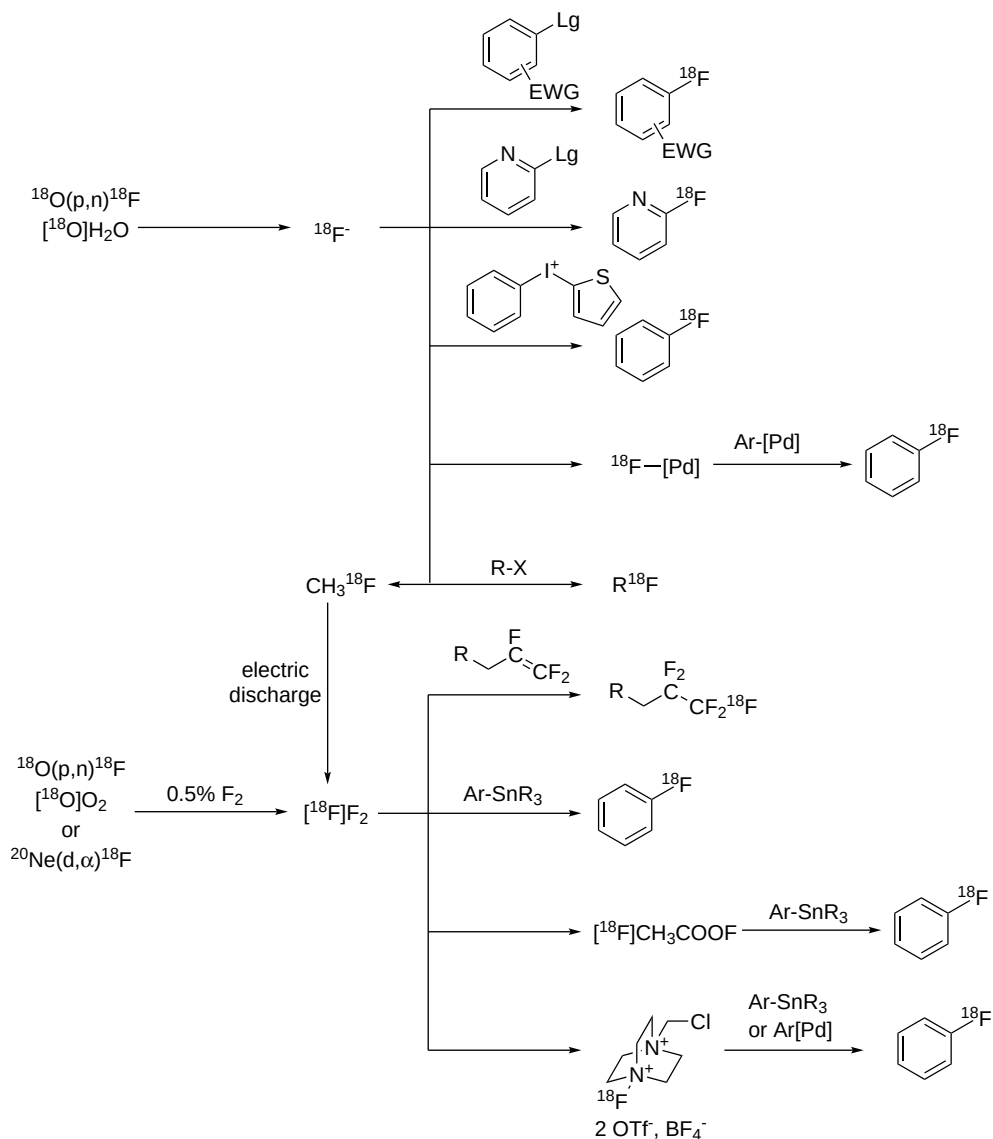
Radiolabeling Compounds with ^{18}F

Fluorine-18 is currently the most frequently used radionuclide for PET. The half-life of the isotope (Table 12.1) allows multistep radiochemical syntheses and transport of the final product from the site of the cyclotron and radiochemistry facility to the imaging center. Fluorine-18 is produced in cyclotron by the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ nuclear reaction. The target material is [^{18}O]H $_2$ O with 98% enrichment for ^{18}O in the case where the desired product is ^{18}F -fluoride. In case the desired product is $^{18}\text{F}_2$, the target material is $^{18}\text{O}_2$ gas containing trace amounts (0.5%) of carrier F_2 gas. Alternatively, $^{18}\text{F}_2$ can be prepared by $^{20}\text{Ne}(\text{d},\alpha)^{18}\text{F}$ reaction with neon, containing 0.5% carrier F_2 as target material (Scheme 12.16). The strategies to incorporate ^{18}F into biologically active molecules are discussed in this section.

12.4.1

Formation of C— ^{18}F Bond, Nucleophilic Substitutions

^{18}F -fluoride is produced in an aqueous solution that is an inferior solvent for nucleophilic substitutions. The small size of fluoride ion and its high electronegativity lead to formation of a tightly packed solvation sphere surrounding



Scheme 12.16 Preparation of ^{18}F and transformations leading to formation of $\text{C}-^{18}\text{F}$ bonds.

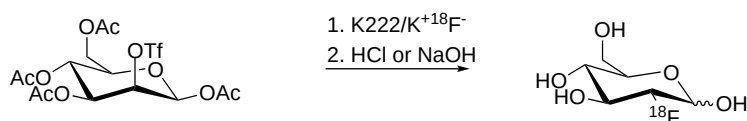
the fluoride anion. Fluoride also tends to form tight ion pairs with metal cations, decreasing its nucleophilicity. Using dipolar aprotic solvents, and exchanging the small counteranion to bulky organic cations such as tetrabutylammonium cation or potassium cation coordinated with kryptands (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (Kryptofix 2.2.2, K222)) or crown ethers (18-crown-6), the solvation envelope is disrupted and the fluoride anion

becomes exposed (“naked”), resulting in a significant increase of its nucleophilicity. Large alkali metal cations like rubidium and cesium have also been reported in nucleophilic ^{18}F -fluorinations. Additionally, the bulky cation improves solubility in the dipolar, aprotic solvents required to dissolve both the ^{18}F -fluoride and the precursor. The bulk of target material (H_2^{18}O) is usually removed using anion exchange resin followed by elution of ^{18}F -fluoride with acetonitrile, containing the phase transfer catalyst. The residual water is removed by several rounds of azeotropic distillation with acetonitrile. The ^{18}F -fluoride is then resolubilized in a dipolar, aprotic solvent such as dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), or acetonitrile. A modification of the procedure involving drying of a cartridge-trapped ^{18}F -fluoride followed by elution in anhydrous solvent containing phase transfer catalyst was developed to save time normally needed for multiple rounds of azeotropic water removal [67]. Bulky protic *tert*-alcohols were also investigated and successfully used for nucleophilic ^{18}F -fluorinations, with alkali metal fluorides and tetrabutylammonium fluoride (TBAF) [68].

12.4.2

Aliphatic Nucleophilic ^{18}F -Fluorination

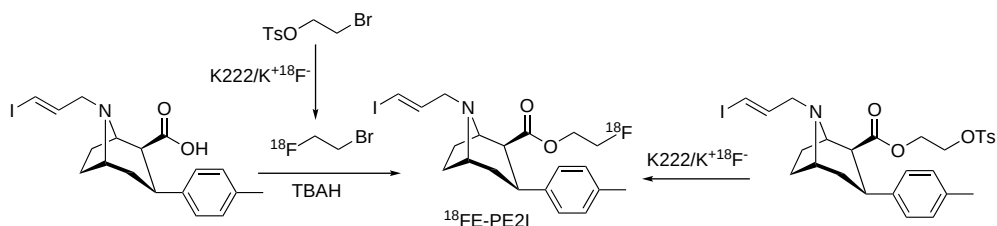
The common leaving groups required for successful nucleophilic aliphatic ^{18}F -fluorination by the $\text{S}_{\text{N}}2$ mechanism with inversion of stereochemistry at the attacked carbon are halides, and hydroxyl groups converted to triflates, tosylates, or mesylates. The substrate reactivity decreases with the sterical hindrance imposed by groups attached to the attacked center with primary carbons reacting the fastest. The first Food and Drug Administration (FDA)-approved PET tracer 2-deoxy-2- ^{18}F fluoroglucose (^{18}F -FDG) is prepared by fluorination of protected mannose triflate, using $[\text{K}222]\text{K}^{+18}\text{F}^{-}$ followed by acid- or base-mediated deprotection of acetylated hydroxyl groups in radiochemical yields above 50% in less than 1 h (Scheme 12.17) [69].



Scheme 12.17 Synthesis of ^{18}F -FDG.

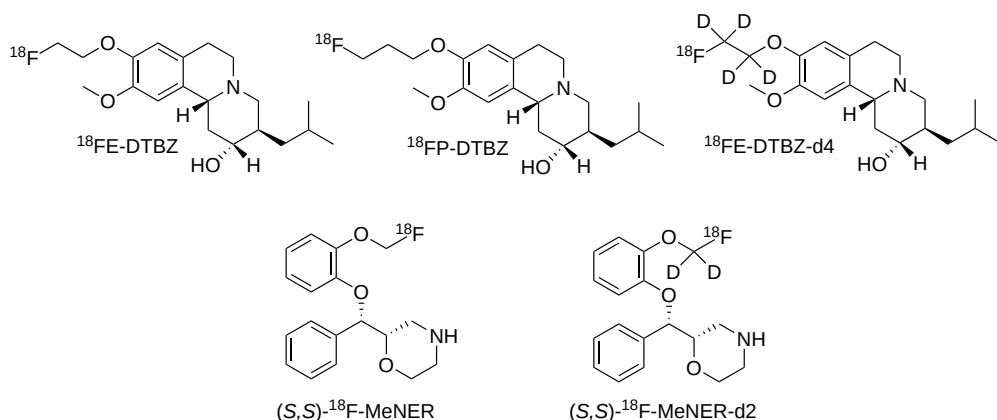
^{18}F -Fluoroalkyl groups are frequently incorporated into PET tracers in place of a ^{11}C -methyl group attached to heteroatoms. The synthesis can be carried in one or two steps, assuming no further transformation such as functional group deprotection is required. In the one-step method, the precursor is directly fluorinated and, in the two-step procedure, the heteroatom alkylation is accomplished with ^{18}F -fluoroalkyl halide, triflate, tosylate, and so on. The two-step and single-step approaches can be exemplified by synthesis of dopamine transporter tracer $[\text{F}^{18}]\text{-(E)-N-(3-iodoprop-2-enyl)-2}\beta\text{-carbofluoroethoxy-3}\beta\text{-(4'-methyl-phenyl)-}$

nortropine (^{18}F -PE2I) (Scheme 12.18). The original two-step approach employed the same precursor as was used for the synthesis of ^{11}C -PE2I (Scheme 12.2). In the first step, the $[1\text{-}^{18}\text{F}]$ fluoro-2-ethylbromide was prepared by nucleophilic fluorination, and coupled with the free acid precursor in the presence of tetrabutylammonium hydroxide (TBAH) in the second step. The product was obtained in 7% uncorrected yield within 90 min [70]. The single-step procedure was carried out using the preformed tosylate precursor and provided the desired ^{18}F -PE2I in 20% yield within 70 min [71].



Scheme 12.18 Single-step and two-step synthesis of ^{18}F -PE2I.

^{18}F -Fluoroalkylated compounds could be metabolically defluorinated, providing ^{18}F -fluoride ion that rapidly accumulates in bone. ^{18}F -Alkyl derivatives of ^{11}C -DTBZ (Scheme 12.2), a PET tracer used for imaging VMAT2, were prepared by ^{18}F -fluoroalkylation of desmethyl-DTBZ or from the corresponding mesylate precursors and evaluated in preclinical imaging experiments (Scheme 12.19) [72]. Although both ^{18}F -ethyl and ^{18}F -propyl derivatives of DTBZ bind VMAT2 with high affinity, the ^{18}F -ethyl analog (^{18}F -DTBZ) was found to be metabolically unstable, and a significant accumulation of ^{18}F in bone was observed [73]. Because of its superior metabolic stability, the ^{18}F -DTBZ is currently being evaluated in clinical trials for imaging integrity of the dopaminergic system in Parkinson's disease patients and imaging β -cell mass in type-2 diabetes mellitus patients. Further

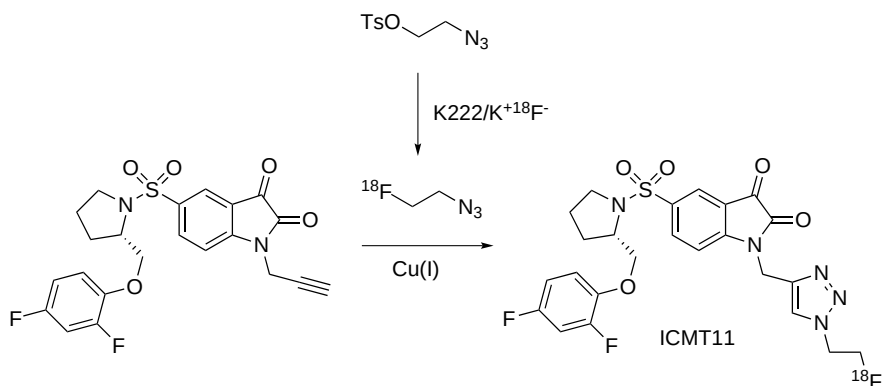


Scheme 12.19 ^{18}F -Derivatives of VMAT2 tracer DTBZ and radioligands for NET.

experiments with ^{18}F -DTBZ led to a synthesis of tetra-deuterated derivative ^{18}F -DTBZ-d4 that was sixfold more metabolically stable than ^{18}F -DTBZ [74].

The ^{18}F -fluoromethyl group is the closest surrogate of the ^{11}C -methyl group and it is frequently used to prepare ^{18}F -analogs of successful ^{11}C -tracers, but it is also prone to enzymatic defluorination [75]. The PET tracer for the norepinephrine transporter (NET) (*S,S*)-FMeNER was prepared by *O*-fluoromethylation with ^{18}F fluoromethylbromide or ^{18}F fluoromethyltriflate. *In vivo* studies in cynomolgus monkey resulted in a skull uptake of ^{18}F -fluoride, interfering with the quantification of NET-specific cortical uptake of (*S,S*)-FMeNER, but defluorination was diminished with the use of the dideuterated analog (*S,S*)-FMeNER-d2 (Scheme 12.19) [76]. Tracers ^{18}F -fluoroethylated at the heteroatom such as DAT tracer ^{18}F -FECNT could also be metabolized by oxidative dealkylation leading to another troublesome metabolite ^{18}F -fluoroacetate (^{18}F -FACE) [77]. ^{18}F -FACE is further metabolized to ^{18}F -fluorocitrate and trapped intracellularly. It can also cross BBB and evenly distribute in brain tissue.

^{18}F -Fluoroalkylation can be also accomplished by formation of a 1,4-disubstituted triazole by copper(I)-mediated 1,3-dipolar cycloaddition of ^{18}F -alkylazides and alkynes or ^{18}F -alkynes and azides, respectively. Both approaches were used to radiolabel peptides without the need of protecting groups in amino acid side chains [78], small proteins [79], and small molecules [80]. For example, the selective caspase-3/7 tracer ICMT-11 was prepared by coupling ^{18}F fluoroethylazide to an alkyne precursor in 65% radiochemical yield (Scheme 12.20) [81].

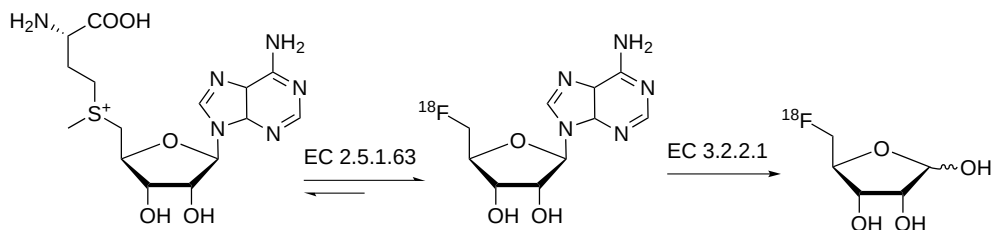


Scheme 12.20 Synthesis of ^{18}F -ICMT11 by Cu(I)-mediated 1,3-dipolar cycloaddition.

Recently, a transition metal-catalyzed allylic substitution was investigated for preparation of ^{18}F -labeled compounds. As a model reaction, the cinnamyl methyl carbonate was treated with ^{18}F TBAF in the presence of $\text{Pd}(\text{dba})_2$ and PPh_3 , providing the cinnamyl ^{18}F fluoride in 10–51% yield within 30 min. For comparison, the corresponding allyl halides provided cinnamyl fluoride in yields around 40% [82].

The formation of a ^{18}F —C bond was also accomplished by the enzyme fluorinase isolated from the bacterium *Streptomyces cattleya* (EC 2.5.1.63) [83]. The substrate

(*S*)-adenosyl-L-methionine was converted to 5'-[^{18}F]fluoro-5'-deoxyadenosine using fluorinase at a concentration of 36 mg/ml and at 37 °C in 97% yield within 1 h, starting from ^{18}F -fluoride (Scheme 12.21). The product was subsequently enzymatically converted to 5-deoxy-5-[^{18}F]fluoro-D-ribose.



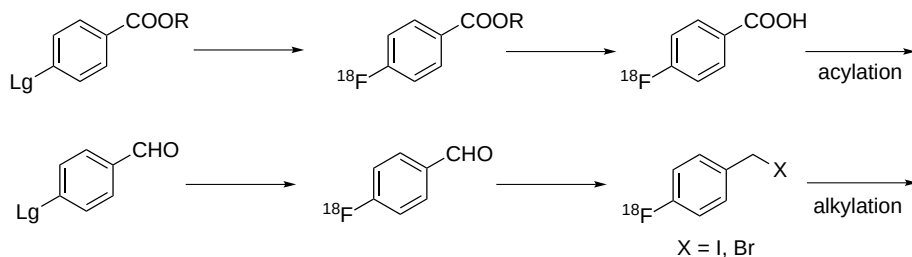
Scheme 12.21 Enzymatic ^{18}F -fluorination and synthesis of 5-deoxy-5-[^{18}F]fluoro-D-ribose.

12.4.3

Aromatic Nucleophilic ^{18}F -Fluorination

Unlike aliphatic alkyl fluorides, aryl fluorides are metabolically stable and not susceptible to an enzymatic defluorination. Aromatic hydrocarbons activated with electron-withdrawing groups can be ^{18}F -fluorinated by nucleophilic substitution. The decreased electron density at *ortho*- and *para*-positions relative to electron-withdrawing group such as carbonyl, nitrile, or nitro group allows nucleophilic attack with “naked” ^{18}F -fluoride. Various leaving groups could be replaced with ^{18}F -fluoride; the most commonly employed are trimethylammonium triflates [84], nitro groups, and also certain halides. The reaction conditions are identical to the aliphatic nucleophilic substitutions described in Section 12.4.2. The ideal precursors of radiolabeling require only a single step to obtain the final product (optionally followed by deprotection), but ^{18}F -aromatics are frequently employed for complex multistep syntheses by converting the activating electron-withdrawing groups to reactive intermediates (Scheme 12.22).

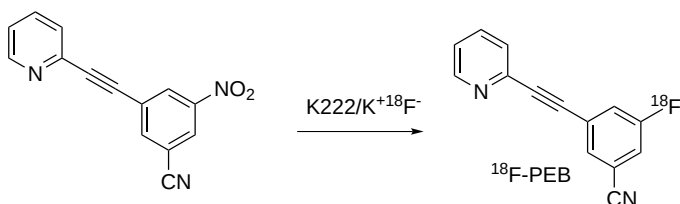
Lg: $\text{N}^+\text{Me}_3\text{TfO}^-$, NO_2 , halogen



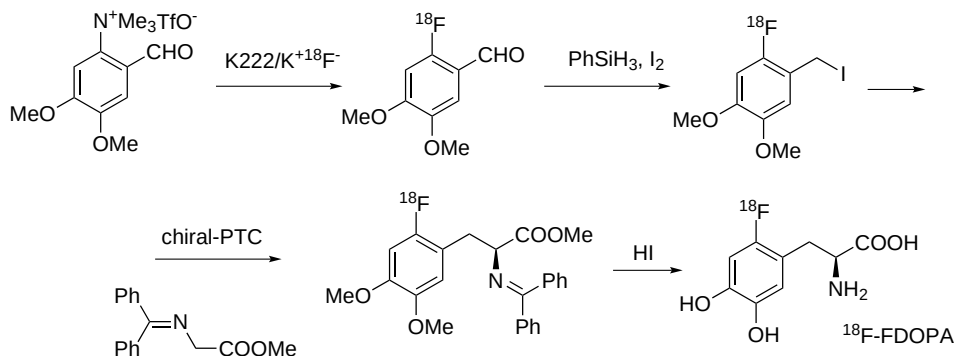
Scheme 12.22 Preparation and conversion of ^{18}F -fluorobenzoic acid and ^{18}F -fluorobenzaldehyde into reactive ^{18}F -reagents.

The mGluR5 radioligand ^{18}F -PEB was prepared in a single step from a 3-nitro precursor, but the yield was only 5% due to unfavorable position (*meta*) of the activating electron-withdrawing group [85]. The optimized four-step synthesis of ^{18}F -FDOPA for imaging dopaminergic neurons starts with ^{18}F -fluorination of a protected aldehyde containing a trimethylammonium triflate or nitro leaving group in the *ortho*-position. The $[2\text{-}^{18}\text{F}]$ -fluoro-4,5-dimethoxyaldehyde was subsequently reduced and converted to benzyl iodide in a single step. The formed benzyl iodide was used for alkylation of protected glycine in the presence of chiral phase transfer catalyst, and final deprotection provided the desired product in 20% overall yield within 120 min (Scheme 12.23).

(a)



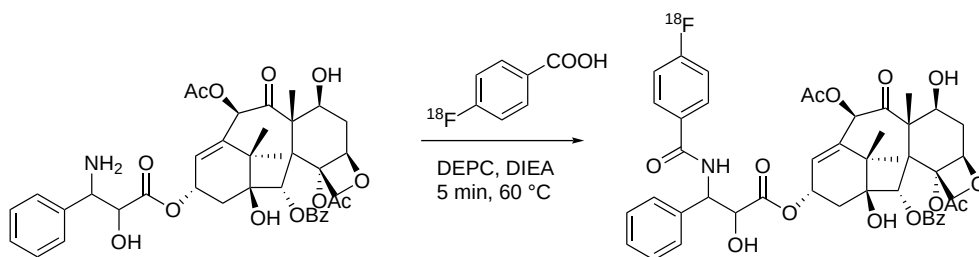
(b)



Scheme 12.23 Synthesis of mGluR5 tracer $[^{18}\text{F}]$ -PEB in a single step (a) and synthesis of $[^{18}\text{F}]$ -FDOPA in four steps, employing nucleophilic aromatic ^{18}F -fluorination (b).

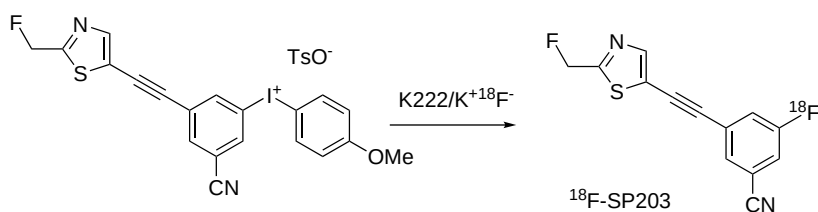
^{18}F -Fluorobenzoic acid can be prepared starting from (4-trimethylammoniumtriflate) benzoate ester followed by deprotection of the carboxyl group (Scheme 12.22). Activated 4- ^{18}F -fluorobenzoic acid was successfully used to radiolabel peptides in solution or on solid phase [86]. A radiolabeled analog of the potent chemotherapeutic paclitaxel, ^{18}F -fluoropaclitaxel, was evaluated as a tracer for assessment of multidrug resistance transporters (P-gp). ^{18}F -Fluoropaclitaxel was prepared by diethyl cyanophosphonate (DECP)-mediated coupling of ^{18}F -fluorobenzoic acid to *N*-debenzoylpaclitaxel

or by conversion 18F-fluorobenzoic acid to ^{18}F -fluorobenzoyl chloride and subsequent reaction with a *N*-debenzoyl precursor (Scheme 12.24) [87]. The DECP-mediated coupling provided lower but more consistent yields by avoiding work with the volatile acyl chloride. The overall yield of ^{18}F -paclitaxel prepared using DECP was 18%.



Scheme 12.24 Synthesis of ^{18}F -fluoropaclitaxel.

Diaryliodonium salts were explored as substrates for ^{18}F -fluorinations of electron-neutral and electron-rich aromatics. The reaction proceeds with fluorination of the electron deficient ring; hence electron-rich arenes like 2-thienyliodonium or 4-methoxyphenyliodonium were employed as leaving groups to control the regioselectivity. In a recent study comparing the reactivity of various substrates, the 2-, 3-, and 4-methoxyphenyl-(2-thienyl)iodonium salts were treated with K222/ $\text{K}^{+18}\text{F}^{-}$, providing corresponding 2-, 3-, or 4-methoxy ^{18}F fluorobenzenes in 61, 20, or 29% yield [88]. The ^{18}F -fluorinations of an mGluR5 tracer in *meta*-position relative to electron-withdrawing nitrile group provided only 5% yield when the 3-nitro precursor was used, but the use of iodonium salt provided a 40% yield of the *meta*- ^{18}F -fluorinated product ^{18}F -SP203 (Scheme 12.25) [89].

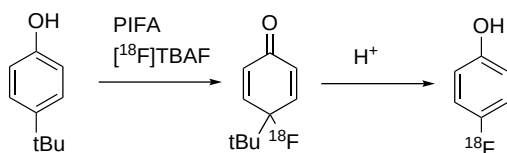


Scheme 12.25 Synthesis of SP203 by substitution of *p*-methoxybenzyliodonium moiety.

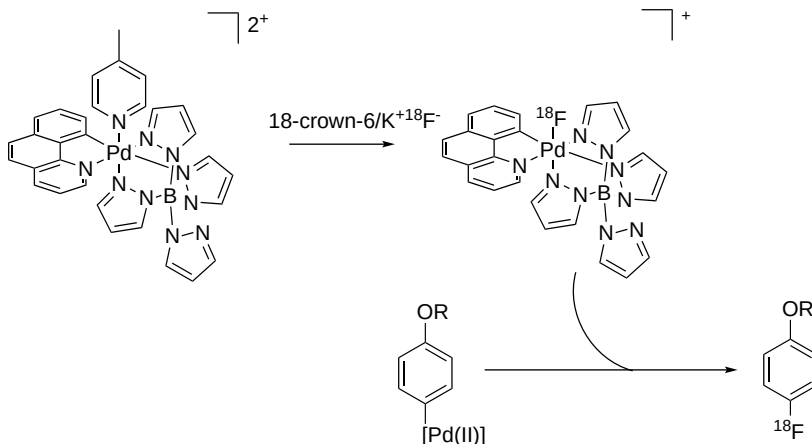
Pyridine rings are susceptible to nucleophilic ^{18}F -fluorination in the 2-position without additional activating groups. Due to its high electron density, the pyridine 3-position is unfavorable to nucleophilic ^{18}F -fluorination by halogen exchange. However, the use of 4-methoxyphenyliodonium as a leaving group provided 3- ^{18}F fluoropyridines in 55–63% radiochemical yields, and 3- ^{18}F fluoroquinolines were obtained in 22–25% yields [90].

Nucleophilic ^{18}F -fluorination of many electron-rich aromatic substrates is not possible by procedures described earlier. Nevertheless, a procedure for nucleophilic ^{18}F -fluorinations of unprotected phenols in *para*-position was recently described. The *para*-position of phenols is unfavorable for nucleophilic attack, but the polarity of the substrate can be reversed (“umpolung”) by oxidation, causing a transient loss of aromaticity. The optimal substrates are 4-*tert*-butyl phenols, which by oxidation provide 4-*tert*-butyl-2,5-cyclohexadien-1-on-4-ium cation. The cation is attacked by fluoride in 4-position and the formed 4- ^{18}F fluoro-2,5-cyclohexadien-1-one decomposes to 4- ^{18}F fluorophenol in acidic environment (Scheme 12.26a). As an example, a series of 4-*tert*-butylphenols was converted to 4- ^{18}F fluorophenols via oxidative fluorination by treatment with ^{18}F tetrabutylammonium fluoride (^{18}F TBAF) and phenyliodine diacetate (PIFA) as oxidation agent in yields around 20% [91].

(a)



(b)



Scheme 12.26 ^{18}F -Fluorination of electron-rich aromatic substrates by substrate umpolung (a) and conversion of fluoride to electrophilic reagent via Pd(IV) complex (b).

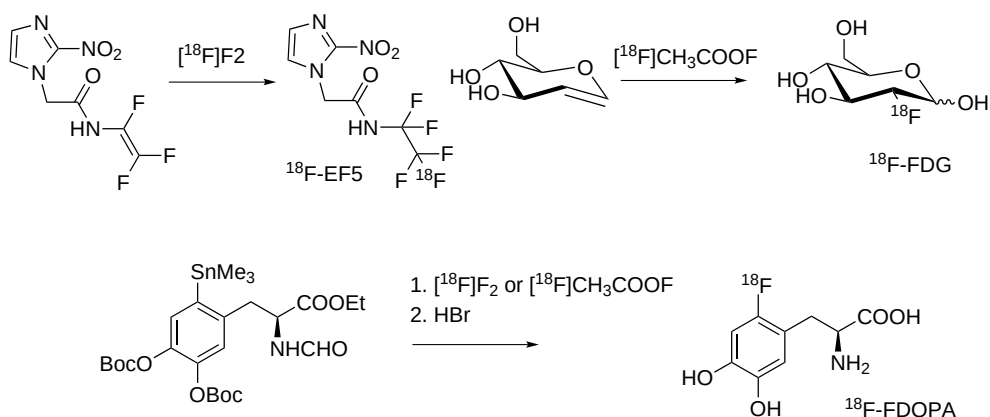
Complementary to the substrate umpolung, the ^{18}F -fluorination of electron-rich aromatic systems could be also achieved by reversing the polarity of the ^{18}F -fluorinating reagent. Since the reagents for electrophilic fluorination described in Section 12.4.4 are unstable and require the preparation of the reactive ^{18}F F₂,

the methods for conversion of ^{18}F -fluoride to an electrophilic reagent were sought. Ritter and coworkers recently reported a preparation of a high oxidation state palladium–fluoride complex ^{18}F -Pd(IV) stabilized with multidentate ligands that prevent reductive elimination. The ^{18}F -Pd(IV) bond can be attacked at its antibonding orbital by the aryl-Pd(II) reagent, causing oxidation of aryl-Pd(II) and ^{18}F -fluorine transfer followed by reductive elimination of ^{18}F -alkyl fluoride (Scheme 12.26b). The Pd(IV)-Pd(II) technique was used to prepare three model compounds in 10–30% yields [92].

12.4.4

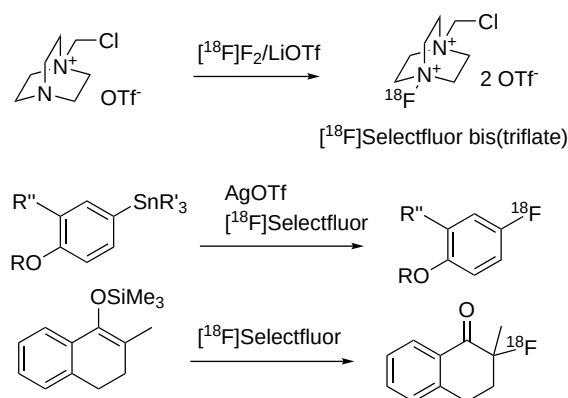
Electrophilic ^{18}F -Fluorination

The reactive electrophilic ^{18}F -fluorination reagent $[^{18}\text{F}]\text{F}_2$ is usually prepared directly in a cyclotron. However, the requirement of F_2 as carrier gas leads to a product with low specific activity (1 GBq/ μmol). High specific activity $[^{18}\text{F}]\text{F}_2$ (up to 55 GBq/ μmol) is obtained by decomposition of $[^{18}\text{F}]\text{CH}_3\text{F}$ in an electrical discharge chamber (Scheme 12.16) [93]. The obtained $[^{18}\text{F}]\text{F}_2$ gas can be used directly or converted to less-reactive ^{18}F -fluorinating reagents. Addition of F_2 to a double bond was used to prepare a hypoxia imaging agent $[^{18}\text{F}]\text{EF5}$ [94]. Also, the first radiosynthesis of $[^{18}\text{F}]\text{-FDG}$ was accomplished by reaction of unsaturated precursor D-glucal with $[^{18}\text{F}]\text{acetylhypofluorite}$ (Scheme 12.27) [95]. Demetalation of aromatic precursors is the most common technique for preparation of electron-rich alkyl fluorides inaccessible via nucleophilic fluorination. A tracer for imaging the dopamine system $[^{18}\text{F}]\text{-FDOPA}$ was prepared in two steps by demetalation of protected aryl trimethyltin or aryl mercury precursors with $[^{18}\text{F}]\text{F}_2$ gas or $[^{18}\text{F}]\text{acetylhypofluorite}$, followed by deprotection. The organotin precursor is preferred due to formation of less toxic byproducts, providing desired $[^{18}\text{F}]\text{-FDOPA}$ in 25% yield (Scheme 12.27) [96].



Scheme 12.27 Synthesis of ^{18}F -EF5, ^{18}F -FDG, and ^{18}F -FDOPA via electrophilic ^{18}F -fluorination.

Since the high reactivity of $[^{18}\text{F}]\text{F}_2$ and $[^{18}\text{F}]\text{acetylhypofluorite}$ can sometimes lead to mixtures of undesired radioactive products, several groups investigated synthesis and use of mild and selective ^{18}F -fluorinating reagents. Recently, N - ^{18}F - ^{18}F -fluorinating reagents such as ^{18}F - N -fluorobenzenesulfonimide (NFSi) and $[^{18}\text{F}]\text{Selectfluor}$ were prepared. Selectfluor (1-chloromethyl-4-fluorodiazonia-bicyclo[2.2.2]octane bis(tetrafluoroborate)) is a mild and easy to handle reagent for electrophilic fluorinations, with an exceptional synthetic scope [97]. ^{18}F -Labeled $[^{18}\text{F}]\text{Selectfluor}$ was prepared by treatment of 1-chloromethyl-4-aza-1-azoniabicyclo[2.2.2]octane triflate with $[^{18}\text{F}]\text{F}_2$ at low temperature (Scheme 12.28). The reagent was employed for electrophilic silver-catalyzed ^{18}F -fluorodestannylation of several electron-rich substrates in radiochemical yield around 15–20% and for the synthesis of α -methyl- α - $[^{18}\text{F}]\text{fluorotetralone}$ (Scheme 12.28) [98].



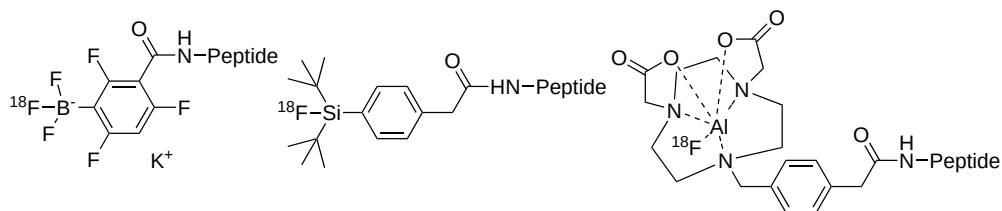
Scheme 12.28 Preparation of $[^{18}\text{F}]\text{Selectfluor}$ bis(triflate) and its use for electrophilic fluorination of various substrates.

12.4.5

Formation of ^{18}F -Al, Si, B Bond

Recent reports on ^{18}F -fluorination of peptides, small proteins, and nucleic acids have explored the possibility of attaching ^{18}F to molecules through a fluorine–metal bond. The metals successfully used to form strong bonds with fluorine were aluminum, boron, and silicon. It has been demonstrated that electron-deficient aryl boronic acids and esters react with ^{18}F -fluoride in aqueous solution to form aryl $[^{18}\text{F}]\text{trifluoroborates}$ [99]. The method was employed for preparation of the matrix metalloproteinase inhibitor $[^{18}\text{F}]\text{marimastat}$, and this tracer was evaluated in animal models of human cancer [100]. Fluorescent dye BODIPY was labeled directly by treatment of the hydroxyl precursor with an aqueous solution of $[^{18}\text{F}]\text{KHF}_2$ [101]. Various biomolecules have been labeled using silicon-based fluoride acceptors (SiFA) as amino or thiol group-reactive prosthetic groups [102]. For example, bombesin analogs labeled with ^{18}F -SiFA prosthetic groups were evaluated *in vivo* for targeting gastrin-releasing peptide receptor positive tumors

[103]. Another intriguing approach employs the capture of ^{18}F -fluoride from aqueous media in complex with aluminum. The resulting cation, presumably AlF^{2+} , could be chelated with macrocyclic ligand 1,4,7-triazacyclononane- N,N' -diacetic acid (NOTA) preconjugated to the peptide (Scheme 12.29) [104].



Scheme 12.29 Labeling peptides using prosthetic groups containing ^{18}F -metal bond.

Sections 12.3 and 12.4 described established strategies and selected promising modern approaches to compounds labeled with ^{11}C and ^{18}F for PET. Section 12.5 will illustrate their use in preclinical and clinical drug development as well as in routine clinical use.

12.5

PET Imaging in the Clinic, Research, and Drug Development

The inclusion of PET in nuclear medicine has greatly impacted patient management, mainly in oncology, but also in neurology and cardiology. PET imaging provides insight into the underlying processes in normal and pathological states, which is important for drug discovery and development in addition to diagnostic and treatment management [105].

12.5.1

PET in Oncology

The glucose analog ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F -FDG) is the most extensively used PET radiotracer in oncology. ^{18}F -FDG is recognized by the glucose transport proteins (GLUT) on cell membranes and is phosphorylated by cytoplasmic hexokinase. The lack of a 2'-hydroxyl group prevents FDG from further metabolism and it effectively becomes trapped intracellularly as ^{18}F -FDG-6-phosphate and thus accumulates over time. The relatively high uptake of ^{18}F -FDG by tumors can be explained by the increased rate of glycolysis in cancer cells, which can be facilitated by an overexpression of GLUT on the cell membrane [106] or overexpression of intracellular hexokinases [107].

^{18}F -FDG-PET imaging is used for diagnosis, staging, recurrence detection, and progression of several types of cancer [108]. Furthermore, several studies suggest that the FDG-PET response [109] correlates well with clinical outcome and survival and may be an early predictor of tumor response to therapy in

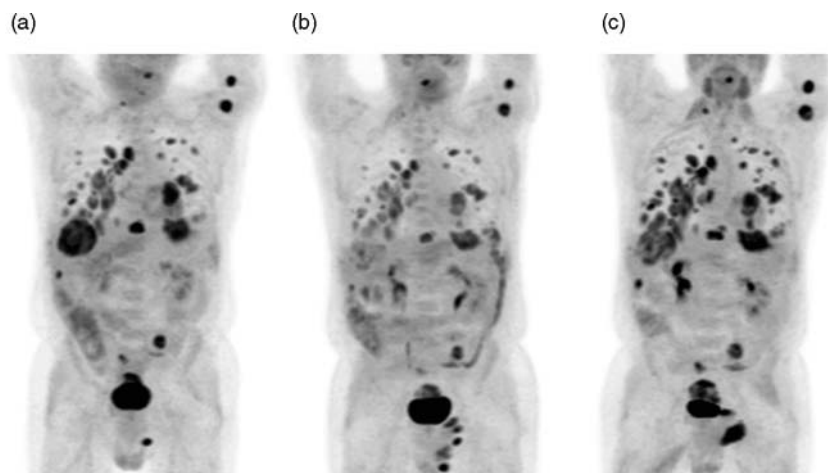


Figure 12.2 FDG-PET MIP images from a colorectal cancer patient with a KRAS G12V mutation, participating in a phase I trial of the MEK pathway inhibitor GDC-0973. The patient received daily 60 mg doses of GDC-0973 on dosing schedule of 21 days on/7 days off. (a) Shows the screening scan performed prior to cycle 1 day 1. A partial metabolic

response was observed at cycle 1 day 12 (b) in target lesions (FDG uptake decrease of 40%). At the end of cycle 1 (off-drug), the FDG uptake returned to baseline values (c). This patient demonstrated stable disease by RECIST CT assessment at the end of cycle 2 and remained on study for 112 days (Courtesy of Williams *et al.* [112]).

lymphoma, lung, head and neck, and other cancers. The clinical experience to date clearly suggests FDG-PET can provide extremely valuable information for drug development [108,110e,108,110]. Many of the new targeted cancer therapies are cytostatic instead of cytotoxic, thus functional readings of tumor metabolic activity with FDG-PET may provide additional information on tumor response and drug pharmacodynamics [111]. An example of the use of FDG-PET to assess drug PD is shown in Figure 12.2 for the MEK inhibitor GDC-0973 in a colorectal cancer patient [112].

FDG-PET in oncology imaging also has some disadvantages. FDG uptake is normally seen in the kidneys and the urinary tract, and activity accumulates in the urine, hindering tumor detection and uptake quantification in these areas. Additionally, some cancer lesions may have low glycolysis rate or poor ^{18}F -FDG avidity, in which cases, the negative findings cannot exclude the presence of the disease and FDG-PET cannot be used to stage or monitor the disease. Finally, ^{18}F -FDG uptake can also be a result of inflammation and infection. Although this can be of clinical value for other applications [113], it can confound the interpretation of the images within the frame of the oncological evaluation. There are several available molecular imaging PET probes that can potentially be used when FDG-PET is not indicated or informative. These ligands have been used to address research questions in clinical settings and can provide further insight on specific aspects of the biochemistry of cancer, such as, proliferation, apoptosis, receptor expression, metabolism, and hypoxia [114].

Advances in molecular and cellular biology have made possible the identification of markers associated with prognosis and likely treatment response by histological examination of tumor specimens from individual patients. This has had a dramatic impact on the development of cutting edge pharmaceuticals, in particular monoclonal antibodies (mAb) [115] and tyrosine kinase inhibitors (TKIs) [116].

The current state of the art of PET imaging and its relatively easy translation into the clinic opens the opportunity to assess biodistribution and expression of a particular target in individual patients. In particular, immunoPET technology based on PET imaging with radiolabeled monoclonal antibodies has matured over the past 20 years into a viable clinical technology that could provide comprehensive noninvasive immunohistochemistry *in vivo* [5,117]. The use of ^{89}Zr , a positron-emitting radionuclide with a half-life of 3.3 days and residualizing properties, has significantly contributed to the recent success of immunoPET [118]. In addition, the robustness of the technology has been demonstrated by proof-of-concept studies performed with head and neck squamous cell carcinoma [119] and breast cancer patients [120]. With the advent of new personalized therapies based on antibody drug conjugates (ADCs) [121], immunoPET imaging with residualizing radionuclide could become an even more valuable tool assisting in the selection of patients expressing the target antigen for treatment and in clinical trials, providing insight into the delivery and retention of the cytotoxic agent in the tumors.

12.5.2

PET Neuroimaging

The radiolabeling of a drug itself can provide information on its brain penetration, distribution, and tissue kinetics. However, one of the most valuable approaches is the optimization of a radioligand that can provide information on its target distribution, and in many instances it can be used to provide a measure of target density changes (occupancy studies) induced by drug candidates and the duration of the target engagement (time-on-target studies). As such, PET can be used to confirm if a drug reaches its target and assist in the dose selection and prediction for efficacy studies. This is particularly important in trials where PET could help differentiate between underdosing (thus lack of target engagement) and lack of activity (mechanism of action). For example, it has been shown that full saturation of the central neuropeptide Y5 receptor (linked to the stimulation of food intake) can be achieved in humans without clinically significant weight loss [122]. PET imaging can also assist in avoiding overdosing and the appearance of unwanted adverse effects. For example, [^{11}C]raclopride imaging can be used to determine the dose of antipsychotics required to produce sufficient occupancy of dopamine D2 receptors for enough duration of time to achieve efficacy without inducing extrapyramidal adverse effects [123].

Positron-emitting probes have been developed for the study of various neurotransmitter receptors and transporters [105a] that are essential components of the chemical synaptic transmission process. Changes in neuroreceptor function are observed in both normal and pathological states. For example, during the early stages of development, a synaptogenesis process and a subsequent phase of synaptic elimination is observed in the primate cerebral cortex [124]. These synaptic changes are accompanied by changes in several neurotransmitter receptor densities (dopaminergic, adrenergic, serotonergic, cholinergic, and GABAergic receptors) [125]. Changes in neuroreceptor and enzymatic systems have been observed not only in normal aging [126] but also in neurological and psychiatric disorders, including epilepsy [127], Parkinson's disease [128], schizophrenia [129], depression [130], narcolepsy [131], autism [132], dementia [133], and neuroinflammation [134].

Recently, the National Institute on Aging and the Alzheimer's Association incorporated imaging biomarkers into Alzheimer's disease (AD) and mild cognitive impairment (MCI) diagnostic criteria [135]. In particular, two types of PET markers are of great interest: (i) amyloid PET imaging that can provide information on the accumulation of A β , and (ii) cerebral glucose metabolism measured using ^{18}F -FDG. Changes in the FDG brain uptake pattern (especially, decreases in the temporoparietal cortex) can be used as a marker of neural degeneration or injury [136].

In the past decade, several amyloid PET ligands labeled with ^{11}C or ^{18}F have been developed as potential biomarkers of amyloid load [133b,137]. The Pittsburgh compound B (PiB), a thioflavin analog labeled with ^{11}C [25], has been used in thousands of research studies in numerous imaging sites, investigating amyloid deposition in different types of AD as well as other dementias. In addition, a recent phase II clinical trial with the anti-A β antibody bapineuzumab included ^{11}C -PiB imaging to study the effect of the antibody. A decrease in ^{11}C -PiB binding was observed in the treatment group in comparison to the placebo group, suggesting amyloid PET imaging can be used for the *in vivo* assessment of treatment effect on A β load [138]. In April 2012, the FDA approved AmyvidTM (florbetapir or AV-45) for human clinical A β imaging [139]. In addition, the development of several other ^{18}F -labeled radiotracers is moving into phase II and III clinical trials [140]. The development of ^{18}F -labeled radiotracers has made amyloid PET imaging more widely available for research and routine clinical purposes. It is expected that amyloid PET imaging will be able to measure *in vivo* changes and inform the efficacy of novel anti-amyloid therapies. Figure 12.3 shows florbetapir images of two subjects with low and high amyloid loads (a) and (b), respectively. The displayed images were normalized by tracer uptake in the cerebellum gray matter. The cerebellar cortex has negligible levels of amyloid, therefore the tracer uptake is assumed to reflect the nondisplaceable component in the brain gray matter. The normalized tracer uptake can be used to quantify tracer retention and perform within – and between – subject analyses of amyloid burden.

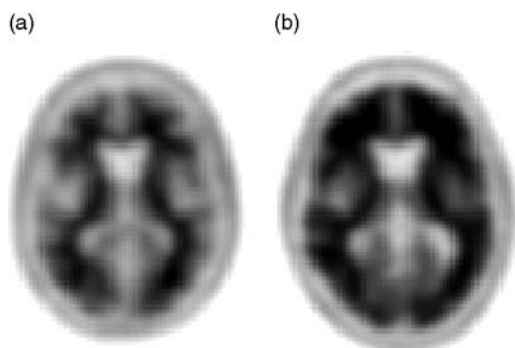


Figure 12.3 Visualization of amyloid burden using florbetapir PET. The images illustrate negative (a) and positive (b) amyloid scans. The images correspond to the tracer uptake approximately 50 min after injection, normalized by the uptake in the cerebellum gray matter.

Another hallmark of AD, is the presence of tau neurofibrillary tangles (NFT) made up of tau protein. Postmortem studies in AD showed that the minimal state examination (MMSE) score correlates with the presence of NFT but not with amyloid load in the hippocampal formation and frontal cortex [141]. Furthermore, the combination of amyloid and tau PET imaging could help identify and determine the most appropriate clinical management of AD and other dementias such as dementia with Lewy bodies (DLB), which has been associated with amyloid pathology, or other tauopathies such as frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). However, the development of tau PET imaging is still at an early stage and most candidates have not moved beyond the *in vitro* and small *in vivo* animal imaging evaluation [142]. The only clinical data reported to date (PubMed and Google searches) was presented at the Society of Nuclear Medicine in 2012 by Villemagne *et al.* using ^{18}F -THK523 [143]. Although the preclinical evaluation of the tracer demonstrated high affinity and selectivity for tau fibrils [144], the ^{18}F -THK523 imaging was unable to distinguish AD patients from healthy controls [143].

PET brain imaging biomarkers hold great promise as multitracer imaging, assessing several targets. Furthermore, the combination with other endpoints obtained from anatomical and functional magnetic resonance imaging and fluid markers can increase the understanding of the pathophysiological process of neurological diseases with direct clinical utility, supporting the development of new therapeutics.

12.5.3

PET in Cardiology

PET myocardial perfusion imaging (MPI) is widely used for diagnosis, staging, and management of patients with known or suspected coronary artery disease (CAD), using rubidium-82 (^{82}Rb) or ^{13}N -ammonia ($^{13}\text{NH}_3$) [145]. The decision of using either radiopharmaceutical is mostly dependent on its accessibility.

Although the relatively large tissue positron range of ^{82}Rb (2.6 mm versus 0.7 mm for ^{13}N) and the low count rate due to its short half-life (78 s versus 10 min for $^{13}\text{NH}_3$) impact the image resolution and quality of the acquired images, ^{82}Rb is still often used because it is produced using $^{82}\text{Sr}/^{82}\text{Rb}$ in commercially available generators, in contrast to the need of a local cyclotron required for ^{13}N -ammonia production.

Metabolic imaging with ^{18}F -FDG-PET is used for imaging myocardial viability for evaluating patients with partial loss of heart muscle, to distinguish between dysfunctional, but viable, myocardial tissue and scar tissue, where revascularization is not recommended. The combination of metabolic and perfusion imaging provide further insight on determining tissue viability and may help predict functional recovery after revascularization [146].

Atherosclerosis is a chronic inflammatory disease considered to be the most common underlying pathology responsible for many adverse cardiovascular events (e.g., myocardial infarction, ischemic attacks, strokes, and acute coronary syndromes). FDG-PET has provided a noninvasive means for assessing plaque inflammation. However, further studies are necessary to fully validate and characterize the technique for its application in the optimization of patient management and to determine its validity as a biomarker of the disease. Furthermore, the combination of FDG-PET and/or other molecular PET agents and other imaging techniques will most probably be able to provide further insights and understanding of the pathology of atherosclerosis and support the development of new therapeutics [147].

12.6

PET Tracer Kinetic Modeling for Quantification of Tracer Uptake

One of the most important properties of PET is that it permits assessment of biochemical and physiological processes in a fully quantitative, yet noninvasive or minimally invasive manner. This technology provides not only *images* of the local radioactivity in tissue but also accurate information on the time course of the tissue distribution (tracer tissue kinetics) of the radiotracers. The rate of accumulation and clearance of the radiotracer from tissue and the relationship between the tracer kinetics in tissue and plasma using mathematical models permit characterization of the underlying dynamic processes. These models incorporate known biochemical and physiological information about the tracer and the studied biological process. For example, information about the tracer metabolism and the membranes (e.g., blood–brain barrier) that must be crossed to reach the target helps to accurately describe the transport of a given tracer and its interactions in tissue.

The most common mathematical models used for tracer kinetics are compartmental models that include two or more rate constants to describe the transport of radioligand between the different model compartments [148]. A typical compartmental model for neuroreceptor ligands is shown in Figure 12.4. The boxes

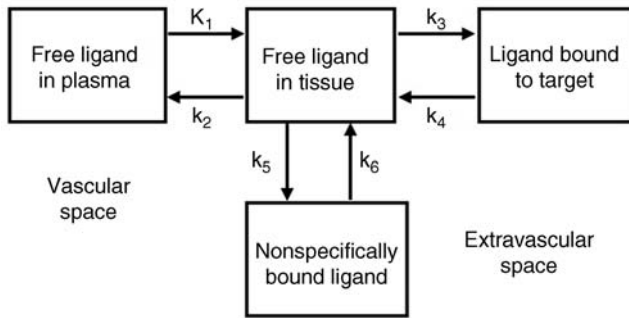


Figure 12.4 Full compartmental model describing the kinetics of a ligand in tissue.

represent pools in which the tracer is assumed to be uniformly distributed, and the arrows indicate the pathways followed by the tracer. The amount of tracer leaving a compartment per unit of time is proportional to the total amount in the compartment. The proportional factor is the rate constant and is usually denoted by k_i , where the index i refers to the pathways shown in Figure 12.4 (K_1 – k_6). Although the following description is based on the interaction of a ligand and its target in the brain, the models can be adapted to describe other types of interactions; for example, tracer binding to surface receptors in tumor cells [149].

In a typical dynamic PET study, a given radioligand is introduced into the vascular space by intravenous injection. Within the vascular space, a fraction of the tracer in plasma can bind to nonspecific sites (protein binding), whereas the unbound fraction of plasma tracer is free to cross the blood–brain barrier and enter the extravascular space in the brain. In the case of neuroreceptor ligands, it is assumed that the transport into the tissue occurs by passive diffusion with the transport rate constant K_1 . The plasma tracer concentration in the supplying blood can be measured from peripheral arterial blood samples and is considered as the *input function* to the tracer kinetic system. The injected tracer may be metabolized in the body, resulting in the appearance of metabolites in the blood circulation. In the case of brain imaging, it is desirable that labeled tracer metabolites do not cross the blood–brain barrier because their interactions with the target receptors or other receptors may complicate the interpretation of the PET data. If the tracer metabolites are radioactive and do not enter the brain, the radioactivity concentration measured in the arterial blood samples needs to be metabolite corrected in order to accurately estimate the model input function.

The tracer extracted from the vascular space into the brain (free ligand in tissue compartment) can escape back to the vascular space with a rate constant k_2 or diffuse to the neuronal synapses. The tracer in tissue can bind specifically to its target receptors or bind nonspecifically to other receptors and/or protein and lipid components of the tissue in the interstitial and cellular spaces. The rate of binding of the free ligand k_3 depends on the association rate constant k_{on} and on the receptor density available for binding. If B_{max} is the total receptor density and C_S is

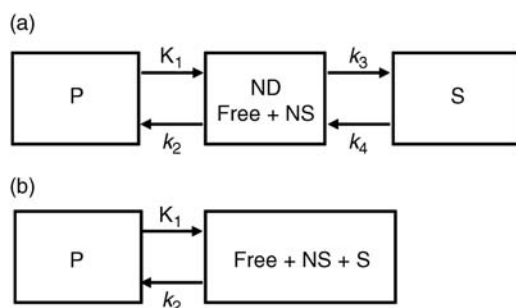


Figure 12.5 Simplified two-tissue and one-tissue compartmental models. (a) The free and nonspecific compartments (F + NS) have been combined into one nondisplaceable (ND) compartment. k_2 and k_3 are the new rate constants for transfer out of the combined ND compartment back to the circulation (P)

or to the specifically bound ligand compartment (S), respectively. (b) All extravascular compartments are combined into one single tissue compartment $T = \text{Free} + \text{NS} + \text{S}$. k_2 is the new rate constant for transfer out of the combined total tissue compartment back to the circulation (P).

the labeled tracer concentration specifically bound to the receptors, $k_3 = k_{\text{on}}(B_{\text{max}} - C_S)$. The rate of dissociation of bound tracer from the receptor sites back to the free ligand compartment is k_4 , assumed to be equal to the *in vitro* k_{off} .

The parameters k_5 and k_6 denote the rate of tracer binding and dissociation from the nonspecific sites, respectively. However, in most PET studies, the free and nonspecific bound tracer compartments cannot be kinetically differentiated because equilibrium between them is quickly reached, thus both compartments are lumped together. Figure 12.5a shows the simplified two-tissue compartmental model. Though the contribution from the nonspecific compartment to the PET signal can be neglected in high receptor concentration regions, this might not be the case in low receptor regions. Information about the free and nonspecific compartments may be obtained by performing additional PET experiments, where the target receptors are preblocked by introducing unlabeled tracer or any other drug binding to the receptors under study. The two-tissue model is the most complex model configuration in which parameters can be estimated when using a single bolus injection of the tracer. Solution to the full compartmental model requires at least three tracer injections with different specific activities of the tracer [150].

The volume of distribution of the compartments equals the ratio at equilibrium of the tracer concentration in each compartment to the concentration of the tracer (excluding any labeled metabolite) in plasma (C_P). The volume of distribution of the free tracer compartment is $V_F = C_F/C_P$, of the nonspecifically bound tracer compartment is $V_{\text{NS}} = C_{\text{NS}}/C_P$, and that of the specifically bound tracer compartment is $V_S = C_S/C_P$. The total volume of distribution of the tracer in tissue equals the sum of the volumes of distribution of all tissue compartments and is equal to the ratio of total tracer concentration in tissue to C_P : $V_T = [V_F + V_{\text{NS}}] + V_S = V_{\text{ND}} + V_S$.

In many instances, the tracer kinetics does not allow differentiation of any tissue compartment in the extravascular space. Therefore, the model is reduced to the one-tissue compartmental model illustrated in Figure 12.5b.

Tracer-specific binding may be reversible or irreversible in the time frame of the kinetic PET study. Examples of reversible binding in human brain are the amyloid PET ligands mentioned before and ^{11}C -raclopride binding to dopamine type 2 receptors [129e]. All kinetic constants for reversible tracers are greater than zero. In contrast, $k_4 = 0$ for irreversible tracers. A well-known example of apparent irreversible binding is the intracellular trapping of ^{18}F -FDG-6-phosphate after administration of ^{18}F -FDG [151]. Although some dephosphorylation can be observed in the brain after approximately 1 h [152], FDG uptake in tumors continues to increase during 2.5 h following tracer injection and others reach uptake plateaus (even though the tracer plasma concentration decreases over time) as illustrated by Lowe *et al.* [153].

Compartmental models are described using first order differential equations, assuming that the amount of tracer leaving a compartment is proportional to the total amount in the compartment. The number of equations used to describe a system is equal to the number of tissue compartments. The physiological process and molecular interactions being studied are considered to be in *steady state* during the PET measurements. This means that the transport rates between compartments are not changing with time during the experiment. Note that, in general, the tracer introduced into the system is not in steady state and its kinetics is used to extract information about a given process. Given the small amounts of tracer used in PET experiments, the steady state of the system is not expected to be altered.

In PET studies, dynamic data is acquired in a sequence of time intervals or frames. Initial frames following a fast bolus tracer administration are short (~ 10 – 15 s) in order to follow the initial rapid kinetics of the tracer in tissue. The length of the frames increases gradually during the study to up to 5 or 10 min, depending on the duration of the experiment and the tracer kinetics. Figure 12.6 shows a simulated time–activity curve (TAC). Each experimental point corresponds to the number of counts in the corresponding frame divided by the frame length. Calibration procedures of the scanner allow expression of the activity concentration in activity units (mCi/ml or Bq/ml).

The aim of tracer kinetic analysis is to estimate the set of rate constants of the compartmental model, which best describe the observed measurements. This is achieved by adjusting the model parameters step by step to minimize the difference between the model response and the experimental TAC measured with PET. The estimated rate constants are then combined to determine the *in vivo* tracer BP or volume of distribution (V) as defined by Innis *et al.* [14]. The *in vivo* BP (Section 12.2) is obtained from the ratio of the specific binding concentration in tissue at equilibrium to an approximate measure of the free tracer concentration:

- $\text{BP}_F = (V_T - V_{ND})/f_p = K_1 k_3 / f_p k_2 k_4$ (where f_p corresponds to the tracer plasma-free fraction) is obtained using the free concentration in plasma assuming that

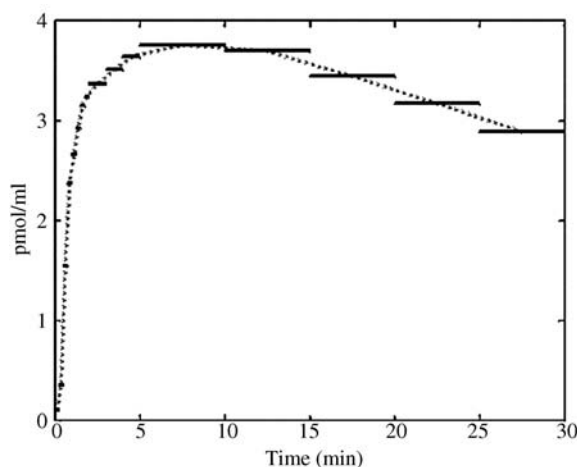


Figure 12.6 Time activity curve (TAC) measured after bolus tracer administration. Each horizontal bar corresponds to the average number of counts in the

corresponding frame. The dotted line shows the linear interpolation of the mean frame activity as function of the mean frame time.

the ligand crosses the BBB only by diffusion. In this case, the free tissue and plasma concentrations are assumed to be equal.

- $BP_P = V_T - V_{ND} = K_1 k_3 / k_2 k_4$ uses the total parent radioligand (excluding labeled metabolites) in plasma not corrected for the fraction of tracer bound to plasma proteins.
- $BP_{ND} = (V_T - V_{ND}) / V_{ND} = k_3 / k_4$ uses the nondisplaceable radioligand in tissue, usually measured in a receptor-free tissue (reference region) as a surrogate for the free tracer concentration.

To a large extent, the *in vivo* BP that can be measured depends on the ability to obtain arterial samples to assess the tracer plasma concentration and the concentration of its labeled metabolites, in addition to the plasma protein binding fraction.

The clinical validation of a PET tracer using prolonged dynamic PET acquisitions and full kinetic modeling, supports simplified imaging protocols that provide reliable information on the imaged target and reduce the burden on patients, by reducing the scanning session duration and avoiding arterial blood sampling [151,154]. For example, the use of FDG-PET in dementia and oncology does not require dynamic PET imaging, starting with the tracer injection. In routine cancer FDG-PET, the PET acquisition starts sometime after tracer administration, when the contribution of the ^{18}F -FDG in the nondisplaceable compartment is expected to be relatively small and the measured activity is mostly due to the intracellular ^{18}F -FDG-6-phosphate [151,154a]. Amyloid PET imaging also uses static PET images acquired sometime after tracer administration, depending on the kinetics of the imaging probe [154c,155]. The measured activity is expected to reflect the

ligand bound to A β . Furthermore, since amyloid deposition in the cerebellar cortex of AD patients is negligible, the tracer uptake in this region is generally used to estimate the nondisplaceable component in gray matter areas. The ratio of tracer uptake in target regions to the cerebellum is used as a simplified endpoint to quantify tracer binding to plaque.

12.7

Concluding Remarks

The use of molecular imaging in drug development is now established. The acceptance by the FDA and other regulatory authorities of the use of microdoses, that is, PET probes administered at tracer doses far below pharmacological active doses, has enabled a streamlined and accelerated path to the clinic. Imaging-based biomarkers enable selection of the appropriate patient population and provide evidence for biological activity, as well as dose optimization. As such, they can be used to guide the design of larger clinical trials. In this way, it is envisaged, by increasing the chance of success in pivotal trials, that opportunity cost will be realized. Nevertheless, the development and verification of an imaging approach is a costly and time-consuming process. Often the investment in developing a novel PET ligand needs to parallel the development of the drug candidates, and resources cannot be gated on identifying a successful drug lead prior to PET tracer development and thus it incurs the associated risks. Despite the recognition of the role of molecular imaging in early drug development, both by the pharmaceutical industry and regulatory authorities, greater impact will only be realized when the imaging readout is accepted as a surrogate for clinical outcome. This is a high bar and requires clinical validation. To achieve this goal, an alternative approach needs to be considered – one in which, through collaborations between academia and the pharmaceutical industry consortia are used to advance the field. Indeed, several groups have now embraced this challenge and are addressing this issue (ACRIN, ADNI, HRP, OAI, and OBQI). In this way it is hoped that sufficient clinical data is obtained to establish the correlation and predictability between imaging biomarker and patient health. It is expected that the role of imaging in drug discovery and development will continue to increase.

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13

Medicinal Chemistry in the Context of the Human Genome

Andreas Brunschweiler and Jonathan Hall

13.1

Introduction

In the past 10 years, genome-wide sequencing has delivered the protein-coding sequences of all human genes, as well as those of many model and target organisms used in biomedical research. The value of this effort to pharmaceutical target discovery [1] and medicinal chemistry [2] is only now being appreciated.

Bioinformatic analyses of the genome sequence have provided classifications of proteins according to their predicted functions into families and subfamilies. Some of these belong to the “druggable genome,” a distinct group that is considered likely to be amenable to medicinal chemistry [3]. These developments have helped to drive a new model in medicinal chemistry in which candidate drug structures are no longer designed for binding to a unique protein, but rather are optimized in the broader context of an entire protein family [4,5]. In addition to the synergies won by working with proteins with similar structures and functions – protein production, assay development, scientific expertise, and so on, there are several beneficial features to be gained through this new paradigm. First, it aids the design of progressively more selective drugs, such that binding to “antitargets” – proteins homologous to the intended target whose modulation causes toxicity – can be minimized. Second, as compounds are characterized for their effects on families rather than on individuals, new ligands for other family members that are not of primary concern are also obtained [6]. Not only can these serve as valuable tools to help establish the function of all members of a family (including their potential as antitargets or targets for other diseases), but may also be applied in polypharmacological approaches, an increasingly used strategy to help circumvent redundancy pathways in complex drug–target networks [7].

A direct outcome of the sequencing of the human genome was the advent of structural genomics, representing an effort to solve the structures of all proteins of a given genome using combined high-throughput crystallography and molecular modeling. Hence, several structural genomics projects have been

launched in the last decade. These have resulted in filling of the Protein Data Bank (PDB) with thousands of protein structures. One initiative in particular, the Structural Genomics Consortium (SGC), was launched with the intention to determine the structures of the entire druggable proteome [8,9], and today there are more than 1000 high-resolution structures available [10]. These also include the recently deposited structures of several kinase mutants resistant to drug treatments [11]. Many of the proteins were cocrystallized with ligands to demonstrate modes of binding as well as conformational changes caused by induced fit of the ligands. This structural information is used in computer-assisted drug design to bring forth new ligands for putative targets from a wide array of protein families.

Advances in methods of medicinal chemistry catalyzed by the advent of genomics are impacting the development of personalized medicine, here defined as the means to diagnose and treat patients with a maximal therapeutic efficacy and minimal risk based on their genetic makeup [12]. Thanks to modern sequencing technologies, the diagnosis of mutations, for example, whether causative or acquired in response to treatment, allows the definition of subpopulations of patients likely to respond best to drug treatment [13]. Where mutations are sufficiently prevalent, chemists have designed and developed drugs for standard treatment-resistant proteins. There are also today increasing numbers of treatment prescriptions being guided by classification of genetic markers [14,15]: The dose setting of warfarin can be dictated by genotype variants of the drug-metabolizing enzyme CYP2C9 and the target VKORC [16]; the EGFR2-targeting antibody Herceptin is prescribed for breast cancer patients who show amplification of the epidermal growth factor receptor 2 gene [17]; the CCR5 antagonist maraviroc is prescribed for HIV patients who are infected with HIV1-strains that use only the host C–C chemokine type 5 (CCR5) receptor for entry into CD4 T cells [18]. These include, in particular, the first examples of where systematic sequencing of mutations in patient tumors guides the prescription of kinase inhibitors (see below). These examples of personalized approaches to therapies are aligned with, and will benefit from, the drive to develop selective small-molecule inhibitors for all members of the druggable proteome.

13.2

Drugs Targeting Kinases

The kinase family is one of the most important classes of drug targets for anticancer therapy. The human genome codes for 518 kinases (kinome) [19]. High-throughput sequencing and other genomics technologies have helped to demonstrate the pathophysiological roles of several kinases in the initiation and progression of certain types of cancer [20], and a handful of drugs targeting these kinases have proven to be effective in cancer patients. Genomics technologies also stand behind the efforts of pharmaceutical researchers to make progress with two

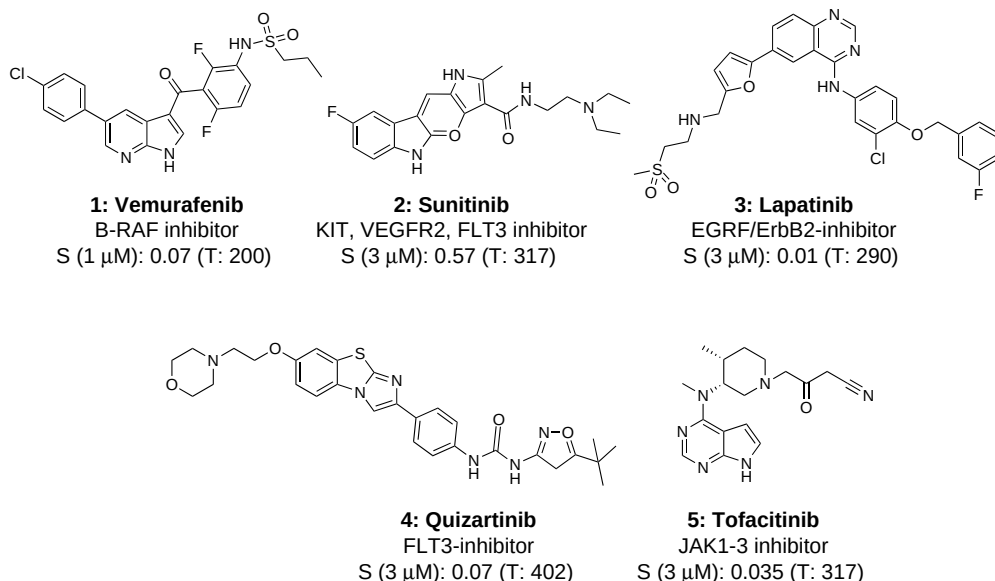


Figure 13.1 Kinase inhibitors tested recently in clinical trials.

current challenges in kinase drug discovery – contending with cardiotoxicity and the acquisition of treatment-resistant mutations, which has developed into an important element of personalized medicine.

In recent analyses of the impact of genomics on drug and target discovery, we highlighted the case of vemurafenib (1; PLX-4032) (Figure 13.1). Vemurafenib represents state of the art for the medicinal chemistry of anticancer kinase drugs. The drug targets selectively a mutated, oncogenic form of B-RAF kinase, which came to light after high-throughput sequencing of melanomas [21]. Development of the drug began with a small-molecule screening program against a diverse collection of kinase domains [22]. Dozens of compounds were then cocrystallized with selected kinase domains so as to identify selective ligands for the mutant B-RAF. From 200 tested kinases, vemurafenib was found to inhibit only 13 at submicromolar concentrations [23]. The drug was finally approved in the United States in 2011 for treatment of melanoma. It is prescribed to those patients if the presence of the kinase mutation is confirmed by a routine genetic test. This provides a prominent example for personalized medicine in this family. Unfortunately, drug resistance mutations have already been characterized and the drug provides for patients only a few months of respite from the disease [24].

Some of the first-generation kinase inhibitors, for example, sunitinib (2), inhibit a large fraction of the “kinome,” whereas others, for example, lapatinib (3) are highly selective [25]. There is growing evidence that a weak selectivity within a family may actually be beneficial to patients, particularly where targets are integrated in signaling networks comprising redundancy pathways: hence,

a drug that inhibits a subset of isoforms might be more efficacious than a specific drug [26]. Thus, a recent survey has shown that several old, as well as successful new drugs effectively inhibit multiple targets [27]. Imatinib, the first approved kinase inhibitor, targets a fusion protein BCR-ABL that is produced by a chromosomal translocation and causes chronic myelogenous leukemia (CML) [28]. Imatinib is an effective drug for CML, however it owes some of its success in the clinic to its inhibition of two other kinases (KIT and PDGF) for treatment of gastrointestinal stromal tumors [29]. On the other hand, one incentive for chemists to develop highly selective kinase drugs has been to help eliminate the cardiotoxicity thought to derive from the unintended inhibition of kinase antitargets [30,31]. Systematic studies using mouse models identified several kinases that guard against cardiac malfunction [30]. Thus, highly targeted inhibitors in general would likely provide safer anticancer drugs and possibly also create new therapeutic openings for their use in non-life-threatening therapeutic indications. Achieving specificity for kinase-targeting drugs is made difficult by the size of the family and the high homology shared by its members [32]. In this respect the large number of structures provided by structural genomics origins has been enormously valuable [33]. An empirical approach to determination of ligand selectivity, which was made possible by the sequencing of the human genome, and the subsequent identification of all kinases is provided by KINOMEScan, a technology in which more than 400 recombinantly expressed kinases are used to measure a complete profile for affinities for ligands of interest [34,35]. This technology was used during the lead optimization of a FLT3-inhibitor, quizartinib (4; AC-220), which is used to treat an especially aggressive acute myeloid leukemia (AML) caused by domain mutations that enhance the kinase activity. The drug is a potent inhibitor of FLT3. However, it also shows activity against other members of the type III RTK family of kinases, but has little effect on approximately 400 other kinases [36–38]. It is currently being tested in phase II clinical trials [39]. The search for selective inhibitors of Jak kinases, as well as spleen tyrosine kinase SYK, led to tofacitinib (5), which has been recommended for approval in the United States for treatment of rheumatoid arthritis [40–42]. The lack of side effects from this pan-Jak inhibitor in patients is probably at least partly due to its selectivity within the kinase family [25,43].

Kinase inhibitors are often cited as the earliest examples of small-molecule drugs used in personalized medicine [44]. This is also because the emergence of the resistance to drug treatment in cancer patients caused by one of several possible mutations in the kinase domains of the targets generates the need for individualized treatment. For example, mutations in the target that result in resistance to imatinib treatment have been characterized [45,46] and have served as new targets. Structural information has been the key to the design of new inhibitors [47]. Similarly, some of the recent medicinal chemistry of EGFR inhibitors has been necessitated by the development of mutations in the active site of the kinase [48]. Erlotinib and the related compound gefitinib both lose their efficacy in the treatment of non-small cell lung cancers through acquired

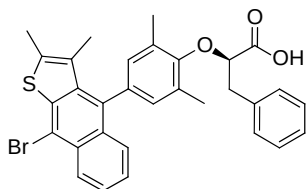
mutations in the target site [11] driving the search for mutant-selective EGFR inhibitors [48]. Therefore, the addition of clinically relevant mutants of BCR-ABL, B-RAF, and EGFR, as well as dozens of other disease-relevant mutated kinases, is a natural extension to the kinase platform and testifies to the growth of personalized medicine.

13.3

Drugs Targeting Phosphatases

Protein tyrosine phosphatases (PTPs) catalyze the dephosphorylation of proteins and shut down signal cascades [49]. In the human genome, the phosphatase superfamily (phosphatome) comprises 107 members of which 38 are tyrosine specific [50]. Several phosphatases have been investigated clinically as targets, including PTP1B, because of its role in diabetes [51]. Ertiprotafib (**6**) (Figure 13.2), a highly potent inhibitor of PTP1B, failed in clinical trials, reportedly because of toxicity and a lack of efficacy [52]. This might have been because PTP1B is highly similar to T-cell tyrosine protein phosphatase (TC-PTP), a genetic knockout of which caused mortality in mice [53].

As for the kinase family, the identification of isoform-selective inhibitors is a priority activity in the phosphatase field. In addition to likely making phosphatase inhibitors safer drugs, it will also deliver valuable tool compounds to help determine the functions of all phosphatase isoforms [54]. The phosphatases share a high homology at the catalytic center and therefore structural information is crucial for the design of selective ligands. Recently, more selective inhibitors have been identified for PTP1B, hematopoietic protein tyrosine phosphatase (HePTP), lymphoid PTP (LYP), and Src homology 2 domain-containing PTP (SHP2), all of which are bidentate and target the active center with a carboxylic acid moiety and an adjacent binding pocket with a lipophilic (aromatic) moiety [55–59]. Structures of the catalytic domains from all of the PTP subfamilies have been published by the SGC [60]. This has enabled a five-pronged classification of PTPs defined by the nature of secondary phosphotyrosine binding sites. For example, SHP1, SHP2, TCPTP, BDP1, LYP, PEST, PTPBAS, DEP1, MEG2, and GLEPP1 can be grouped into a single PTP1B-like class. Crystal structures have



6: Ertiprotafib

Figure 13.2 Ertiprotafib, a PTP1B inhibitor.

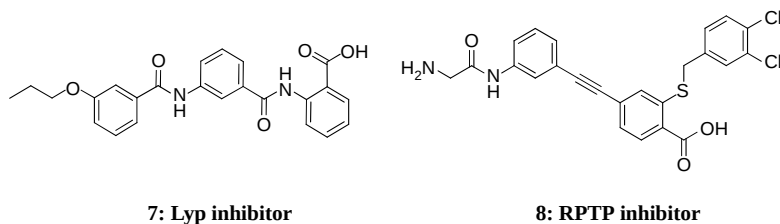


Figure 13.3 Examples of structure-guided design of phosphatase inhibitors.

shown that the catalytic domains of the various isozymes adopt open, closed, or intermediate-open states [60], in addition to an unusual open-inactive form typified by LYP, GLEPP1, and STEP. Recently, *in silico* screening based on the structure of LYP in the open-inactive conformation yielded an inhibitor of LYP with micromolar activity (7) (Figure 13.3) [61]. In a further example of this structure-based approach to ligands, binding of a molecule to the receptor-like protein tyrosine phosphatase γ (RPTP γ) stabilized this protein in a so-called superopen conformation. Lead optimization resulted in a micromolar inhibitor of RPTP γ showing good selectivity against PTP1B (8) [62]. Although selectivity of the compounds against all of the phosphatome has not been achieved in these examples, they do show how the structure-based, family-wide approach is at the center of efforts to exploit the different conformations of PTPs to achieve selectivity.

13.4

In silico-Based Lead Discovery in the GPCR Family

Since the beginning of genome-wide sequencing, a variety of *in silico*-based methods of lead discovery have evolved, each with their strengths and weaknesses. Using information from databases of protein structures, ligand-based, sequence-based, and structure-based methods of identifying leads have been introduced based on a paradigm of “similar receptors bind similar ligands” [63–67]. These techniques are particularly attractive when the structure of a target is not known, as is usual for members of the GPCR family. The human GPCR family is composed of more than 900 predicted members, which are classified into families [68]. Only a fraction of these likely represent valid drug targets and they include mutated GPCRs that are linked causatively to disease pathologies. For example, mutations in the human melanocortin 4 receptor (hMC4R) are responsible for 0.5–2.5% of severe obesity cases and there is a high interest in designing antiobesity drugs for these genetically afflicted patients [69].

The sequence-based “target hopping” approach to GPCR lead discovery first employs sequence alignment of members to reveal key amino acid residues in the transmembrane binding elements of the proteins [70]. GPCRs with binding site homologies close to that of the target protein are identified. If

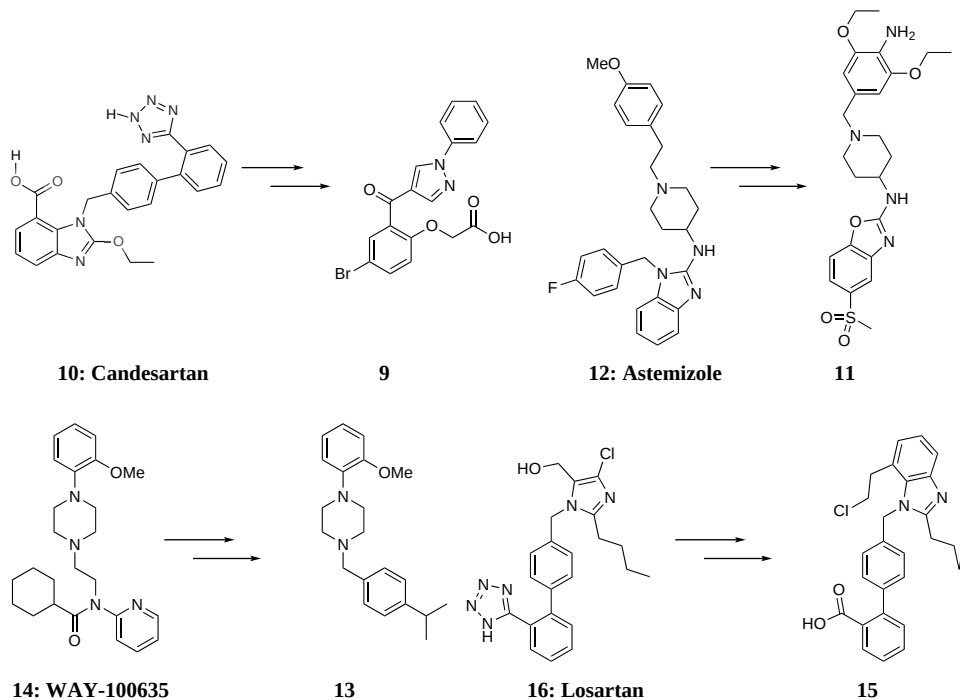


Figure 13.4 GPCR ligands derived from *in silico* screening methods.

ligands are available, then these may serve as lead structures for the candidate. A principal weakness of this approach is that selectivity against the original “seed” structure needs to be developed. Using this method, a CRTH2 receptor antagonist (**9**) (Figure 13.4) was elaborated from the angiotensin receptor antagonist candesartan (**10**) [71,72]. The somatostatin receptor subtype 5 (SSTR5) is grouped with the class A GPCRs, which include receptors for histamine, serotonin, dopamine, and the opioids. In the search for a SSTR5 antagonist, a focused screen of compounds that target receptors for these biogenic amines yielded structure **11** derived from the H1 receptor antagonist astemizole (**12**) [73,74].

A prominent example of application of the similar receptors bind similar ligands approach is provided by the development and use of the Novartis tertiary amine library (TAM). GPCRs were searched for members containing the sequence motif from the serotonin receptor 5-HT_{1A} that interacts with the ligand amino group. Fifty receptors were identified, the majority of which were not only receptors for biogenic amines and peptides but also included orphan receptors. For the 5-HT₇ receptor, a subgroup of the library composed of compounds with similarity to ligands (e.g., compound **14**) for a close homolog – the 5-HT_{1A} receptor – was screened. A potent nanomolar 5-HT₇ antagonist (**13**) emerged from the effort. Again, the principal difficulty with this method is that ligand selectivity often requires considerable optimization [67].

Finally, during a structure-based approach to lead structures, a composition of 13 three-dimensional pharmacophore models for class A GPCRs were assembled employing 10 GPCR–ligand homology models and three crystal structures of receptor–ligand complexes. The pharmacophores were then used to create “chemoprints” and subsequently searched among a group of 270 GPCRs. The models were used in an *in silico* screen and led to an agonist of the complement component 3a receptor 1 (C3AR1) (**15**) from losartan (**16**) [75].

13.5

Targeting Epigenetic Regulation: Histone Demethylases

A variety of posttranslational modifications of histone proteins lies behind a large part of epigenetic regulation of gene expression. These families have come to the interest of medicinal chemists in recent years because of the roles that some of them play in pathophysiological mechanisms.

The demethylation of histone lysines and histone arginines by the demethylases (HDM) occurs by discrete mechanisms [76]. These modifications are carried out by the flavin adenine-dinucleotide (FAD)-dependent, lysine-specific HDMs LSD1 and LSD2, and a superfamily of 30 proteins comprising 7 conserved subfamilies known as the Jumonji C domain-containing histone demethylases (JHDMs) [76–78]. LSD1 was identified in 2004 [79] and LSD2 was discovered in 2009 [80]. LSD1 demethylates the mono- and dimethylated lysines of histone H3K4. The first of the JHDMs was cloned in 2006. Database mining was then used to rapidly uncover the rest of this highly conserved family [76,81]. These enzymes are of interest to pharmaceutical researchers because some members (e.g., JMJD2C) have been shown to modulate proliferative mechanisms and therefore may be of value as targets for drugs treating proliferative diseases [77]. An ongoing task with this family is to define the function of the various isoforms with respect to their histone substrates, their sites of activity (i.e., arginines or lysines), the importance of their methylation status, and finally their druggability. Selective small-molecule ligands are expected to provide enabling tools for this effort. Structural characterization of the demethylases is leading the way to these ligands and indeed crystal structures of JMJD2A have already provided the first structural evidence of how JMJD2A may select for histone H3 and, in particular, how it is able to differentiate the degree of methyl substitution on lysines [82]. Crystal structures were important to the development of methylstat (**17**) (Figure 13.5), a micromolar inhibitor of JHDMs that uses a ferrous ion in its active site [83–86]. This compound is rather selective for the JHDM isoforms JMJD2A, JMJD2-C, and JMJD2-E over other histone-modifying enzymes such as LSD1 and the histone deacetylases, and also the more distantly related prolyl hydroxylases PHD1–PHD3. Crystal structures of the H3K27me3-specific lysine demethylase JMJD3 in complex with its substrate and cofactor were crucial for the design of the recently published JMJD3/UTX (KDM6 subfamily)-selective inhibitor

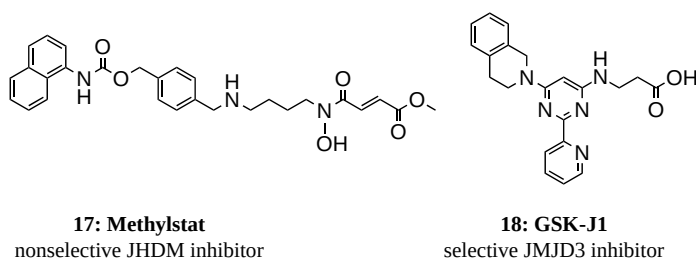


Figure 13.5 Inhibitors of lysine demethylases.

GSK-J1 (**18**) [87]. Expression of JMJD3 is induced in activated macrophages and participates in turn in the transcriptional induction of cytokines such as TNF- α . GSK-J1 inhibits lipopolysaccharide-induced TNF- α production in human macrophages. Thus, it was shown that inhibition of the epigenetic regulation of proinflammatory cytokines with a selective histone demethylase small-molecule inhibitor may represent a novel approach to treat diseases that are caused by exacerbated production of proinflammatory cytokines, for example, arthritis. GSK-J1 demonstrated the feasibility to design selective JHDM inhibitors to elucidate the potential of JHDM isoenzymes as drug targets [87].

13.6

Targeting Epigenetic Regulation: Histone Deacetylases

Histone deacetylases (HDACs) cleave selected *N*-acetyl groups of histone lysines and thereby regulate the activity of transcription factor/DNA complexes. The first member of the family HDAC1 was isolated from Jurkat cells during a chemogenomics effort that identified it as the target of the cyclic tetrapeptide trapoxin, a natural product with potent antitumor activity [88]. Subsequently, new members of the family (HDAC2 and HDAC7) were discovered using yeast two-hybrid screens in nuclear complexes [89,90]. The rest of the family, HDAC-3 [91], HDAC-4, HDAC-5, HDAC-6 [92], HDAC-8 [93], HDAC-9 [94], HDAC-10 [95], and HDAC-11 [96], emerged from database mining of genomic sequence, bringing the total number of HDACs in the human genome to 11. These are divided into three classes together with the related sirtuins.

Vorinostat (**19**) (Figure 13.6) is a pan-inhibitor of HDACs that has been approved for the treatment of cutaneous T cell lymphoma. It is an efficacious drug, but it causes severe hematologic side effects that may derive from mechanism-dependent and/or mechanism-independent off-target effects [97,98]. Mouse knockouts of individual HDACs demonstrate a variety of phenotypes, including mortality (class I HDACs, HDAC7) and cardiac deformations (class IIA). The principal targets for cancer from this family are currently from the class I HDACs 1–3, however, there is a high interest in identifying isoform-selective inhibitors. The class I-selective inhibitor mocetinostat (**20**) is

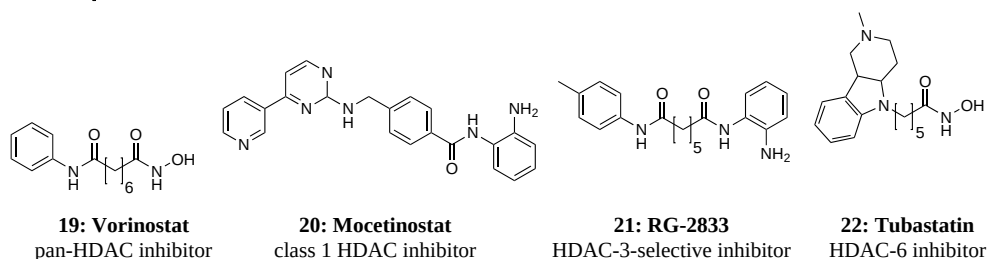


Figure 13.6 Pan- and isoenzyme-selective HDAC inhibitors.

highly potent *in vitro* and *in vivo* [99]. Although its use was also associated with numerous adverse effects in clinical trials, the hematologic side effects observed with vorinostat were not experienced with this drug [100]. None of the current HDAC inhibitors in clinical testing is isoform specific; however, there is a reasonable hope that isoform-selective drugs will lead to clinical efficacy without serious side effects [101]. Recent reports indicate that structural information can help the development of isoform-selective inhibitors [102–105]. For example, a crystal structure of class I HDAC8, a promising drug target in T cell lymphoma, was instrumental in the rational design of selective inhibitors [101]. The first isoform-selective sirtuin inhibitors have been designed, exploiting information from a crystal structure of the SIRT2 enzyme [106].

As with the kinase drugs, the development of selective HDAC inhibitors with improved safety profiles may open up opportunities for their use in alternative disease indications. RG-2833 (21) is an HDAC3-selective drug that has orphan drug status for Friedreich's ataxia [107,108]; Tubastatin A (22), an HDAC6 inhibitor, may be of value in the treatment of Charcot–Marie–Tooth disease [109,110].

13.7

A Family-Wide Approach to Poly(ADP-Ribose) Polymerases

Poly(ADP-ribose) polymerases (PARPs) are a family of enzymes that catalyze the transfer of linear or branched ADP-ribose chains to several classes of proteins, including nuclear proteins associated with DNA [111]. The first member of the family, PARP1, was cloned in 1987 and after sequencing of the genome, an additional 21 human proteins that possess a predicted ADP-ribosyl transferase catalytic domain were identified [112,113]. After the progress of PARP1/2 inhibitors veliparib (23) and olaparib (24) (Figure 13.7) to late-stage cancer clinical trials [114], a number of chemistry groups have sought to identify selective inhibitors so as to characterize all members of the family for their biology as well as their development as a new class of targets in cancer and other indications [115]. For example, PARP5a (tankyrase1) and PARP5b (tankyrase2) have recently come to the forefront after an inhibitor of tankyrase was demonstrated to suppress Wnt signaling, which can drive the growth of tumors when dysregulated [116].

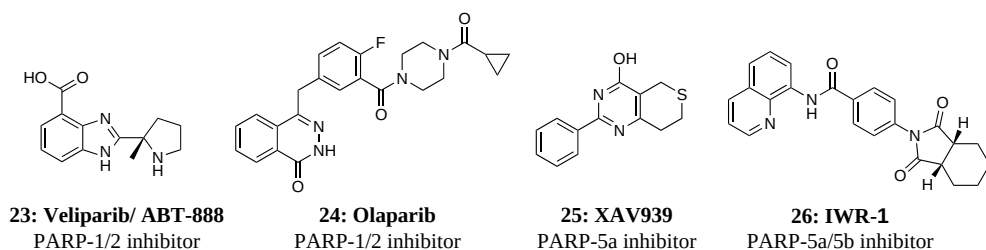


Figure 13.7 PARP and tankyrase inhibitors.

The push toward isoform-selective inhibitors of PARPs has been aided considerably by structural genomics activities that have resulted in the deposition of 26 new structures in the PDB, 17 of which are cocrystal structures (<http://www.thesgc.org/publications/>). Most of the ligands compete with the binding of the nicotinamide cofactor. Knowledge of how veliparib binds to PARP2 provided a solid basis for the design of selective PARP2 inhibitors. It revealed how the compound binds tightly to a glutamate residue, which is an aspartate in the homologous PARP1 [117]. Structures of both tankyrases are now in the public domain. The structure of XAV939 (25) bound to PARP5b suggests possible approaches to achieve some level of selectivity for the various isoforms [118,119]. Furthermore, the first report of a PARP5b fragment cocrystallized with a selective inhibitor (26) that does not bind to the nicotinamide binding site but forces the protein to adopt a novel conformation precluding binding of nicotinamide is of great interest [120].

13.8

Future Drug Target Superfamilies: Ubiquitination and Deubiquitination

The human genome expresses a variety of enzyme families that perform posttranslational conjugation and deconjugation of ubiquitin and a variety of ubiquitin-like peptides (Nedd8, SUMO, FAT10, ISG15) to proteins. Conjugation or cleavage provides signals important to several cellular processes, including degradation, mitosis, and cellular transport. The precise function of the modification depends upon the identity and the location of the conjugated peptide. The conjugation process is carried out by a complex that includes three enzymes: the ubiquitin – or ubiquitin-like – protein-activating enzyme (E1), the conjugating enzyme (E2) that transfers the ubiquitin or its variant to the substrate protein, and/or an E3 ligase that determines the substrate recognition. Sequencing of the human genome has revealed 10 E1 proteins, about 40 E2 enzymes, and approximately 600 E3 enzymes (Ubls). The reverse reaction, cleavage of the peptide from the protein, is performed by a family of 90 deubiquitinases (DUBs).

The ubiquitin–proteasome system has been of considerable interest to pharmaceutical researchers [121–123] since the approval of the proteasome

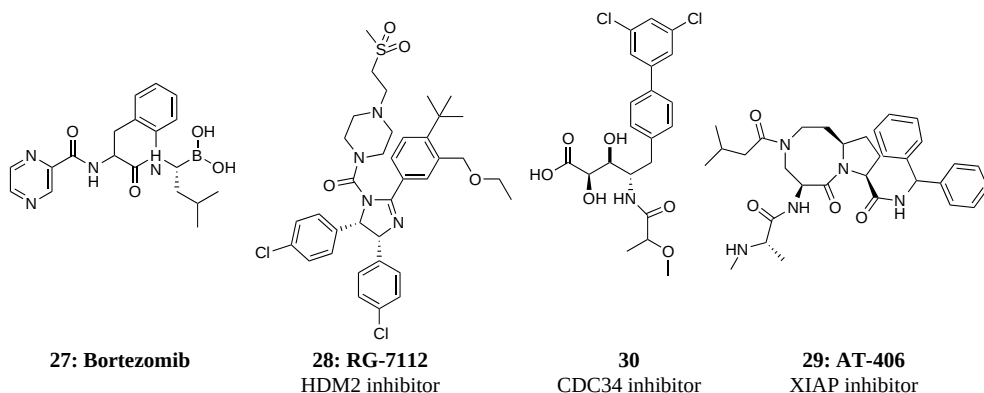


Figure 13.8 Inhibitors of E2 and E3 ubiquitin ligases.

inhibitor bortezomib (**27**) (Figure 13.8) used to treat a variety of cancers [121]. However, in analogy with the other families discussed above, most of the biology of the individual members remains to be clarified. The means to achieve a selective inhibition of a particular ubiquitination step would likely require the disruption of specific E2–E3 protein–protein interactions, and therefore progress has been slow. Nevertheless, some successes have indicated that proteins from these families may make good drug targets. For example, a derivative of nutlin (RG-7112, **28**) an inhibitor of the E3 HDM2, was recently promoted into phase I trials after showing impressive effects in murine models of cancer [124,125]. Inhibitors of the closely related E3 ligases XIAP, cIAP-1, and cIAP-2, have entered phase I clinical trials. One of these (**29**, AT-406) shows selective inhibition of XIAP and cIAP-1 over E3 ligases *in vitro* [126]. A screen for inhibitors of the E2 CDC34 yielded a selective allosteric inhibitor (**30**). The ligand prevents attachment of ubiquitin to its substrates, which include the tumor suppressor p27 [127]. Biomedical research in these families is in its infancy and can be compared with the early days of kinase research. The identification of selective small-molecule ligands will help to pinpoint individual family members that play dominating roles in disease pathways, and at the same time will determine the druggability of the family as a whole. Thus, methods to identify such ligands are of importance. Recently, new screening methods have been reported for a selection of E3 ligases and deubiquitinylases providing important first steps in this direction [128,129].

13.9

Summary and Outlook

The value of the sequence of the human genome and genomics technologies to medicine has crystallized in the last few years with the discovery and approval of drugs that otherwise likely would not exist [1]. The earliest impact

of genomics has been in the biological disciplines, on our understanding of disease leading to the selection of new drug targets [1]. However, knowledge of the human genome has also begun to impact significantly the activities of medicinal chemists [2]. Sequencing of the human genome yielded the protein coding sequence of all genes, including all drug targets. It also lifted many of the routine activities of modern medicinal chemistry to higher levels of throughput and detail. These include protein crystallization, genetic mouse models, mRNA and noncoding RNA expression profiling, proteomics, molecular modeling, and others. Collectively, these developments have obliged chemists to take into account multiple members of a family during the design of individual drug targets. This paradigm shift is delivering better characterized and therefore safer drugs through higher selectivity for their targets. In this chapter we have summarized some examples that illustrate these directions. The combined use of bioinformatics, structural genomics, and family-wide profiling assays has, for example, enabled identification of highly selective kinase inhibitors, primarily for cancer treatment but also in other disease indications. Other superfamilies are beginning to follow suit.

The growing collections of small-molecule ligands capable of binding selectively to ever larger numbers of individual proteins in the proteome represent an additional benefit of perhaps underestimated importance to this new paradigm of medicinal chemistry. These are being used as tools by chemical biologists to help delineate functions of less well-characterized proteins, in particular for their potential roles as targets or antitargets in disease pathways. They will also find use in polypharmacological approaches to addressing complex disease pathways, in which it is recognized that selective modulation of multiple targets simultaneously will bring greater efficacy. Finally, an access to selective modulators for all proteins is fully aligned with the first steps of personalized medicine, most prominently exemplified here with the kinase inhibitors.

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