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Biomedical Applications of Acridines

Derivatives, Syntheses, Properties and Biological Activities with a Focus on Neurodegenerative Diseases

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To memory of the late Dr. Dušan Drahoňovský, who contributed to the concept of human friendly science. He served as a very kind and good teacher of organic chemistry at Charles University, Prague. When we had started to study organic synthesis at University of Pardubice, Dušan's nickname was Acridine, because he had been devoted to carry out acridine synthesis since high school age.

Acknowledgements

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Contents

Acronyms

The three-letter code used for amino acids follows the rules of the IUPAC-IUB Commission on Biochemical Nomenclature. [1] The one-letter code used for amino acids follows the rules of the IUPAC-IUB Joint Commission on Biochemical Nomenclature. [2] These articles show also how to read formulas of peptides used in this work. [3] Selected abbreviations are commonly used especially for description of acridines in peptide conjugates and neurodegenerative disorders:

AAAcr	9-(p-aminomethylphenylamino)acridine-4-carboxylic acid
Aβ	amyloid β
AC04	5-(acridin-9-yl-methylidene)-3-(4-methyl-benzyl)-thiazolidine-2,4-dione
Acd	β-(9,10H-acridon-2-yl)alanine
AChE	Acetylcholinesterase
ACMA	9-amino-6-chloro-2-methoxyacridine
Acr	Acridin-9-yl
ACRAMTU	1-[2-(acridin-9-yl-amino)ethyl]-1,3-dimethylthiourea
Acr4CA	4-(2-methylaminoethylaminocarbonyl)-acridin-9-yl
AD	Alzheimer's disease
ADE	Amyloid degrading enzymes
AHMA	3-(9-acridinylamino)-5-hydroxy-methylaniline
ALS	Amyotrophic lateral sclerosis
AO	Acridine orange
Aol	3,6-bis-(dimethylamino)acridin-9-yl
APP	Amyloid precursor protein
BACE	β-secretase
BBB	Blood–brain barrier
BChE	Butyrylcholinesterase
BRACO19	*N*,*N'*-(9-(4-(dimethylamino)phenylamino)acridine-3,6-diyl) bis (3-(pyrrolidin-1-yl)propan-amide) trihydrochloride
BSE	Bovine spongiform encephalopathy

C-1305	5-dimethylaminopropylamino-8-hydroxytriazolo-acridinone
C-1748	9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine
CJD	Creutzfeldt–Jacob disease
Cl-DACA	*N*-(2-(dimethylamino)ethyl)-7-chloro-acridine-4-carboxamide
CMA	10-carboxymethyl-9-acridone
CTAC	cetyltrimethylammonium chloride
CWD	Chronic wasting disease
CYP3A4	Cytochrome P450 3A4
DABPA	9-(4-(1,2-diamine)benzene-*N*-1-phenyl)acridine
DACA	*N*-(2-(dimethylamino)ethyl)-acridine-4-carboxamide
DAPA	9-[3-(dimethylamino)propylamino]acridine
DFT	Density functional theory
DLB	Dementia with Lewy bodies
DPEPhos	bis[(2-diphenylphosphino)phenyl] ether
dppf	1,1'-ferrocenediyl-bis(diphenylphosphine)
FITU	Fluorescein 5-((amino)thiocarbonyl)
FFI	Familial fatal insomnia
FRDA	Friedreich's ataxia
FTLD	Fronto-temporal lobar degeneration
GPI	Glycophosphatidylinositol
GSS	Gerstmann–Sträussler–Scheinker syndrome
HD	Huntington's disease
hAChE	Human acetylcholinesterase
hBChE	Human butyrylcholinesterase
hMAO-B	Human monoamine oxidase B
HpβCD	hydroxypropyl-β -cyclodextrin
HuPrP106-126	Human prion peptide 106-126
hSIRT	Human sirtuin
Hsp90	Heat shock protein 90
IP	*Intraperitoneal*
IV	*Intravenous*
mAChE	Mouse acetylcholinesterase
MBAA	6-chloro-2-methoxy-*N*-(2-methoxybenzyl)acridin-9-amine
MCI	Mild cognitive impairment
MDR	Multidrug resistance
7-MEOTA	7-methoxy-9-amino-1,2,3,4-tetrahydroacridine
MSA	Multiple system atrophy
NEP	Neprilysin
NIID	Neuronal intranuclear inclusion disease
OHA	1,2,3,4,5,6,7,8-octahydroacridin-9-amine
PCB	Polychlorinated biphenyls
PCET	Proton-coupled electron transfer
PD	Parkinson's disease
P-gp	P-glycoproteine
Phthac	4,5-bis-(phenylthiomethyl)acridine

PPA	Polyphosphoric acid
PPAB	Poly(propargyl acridiniumbromide)
PPQB	Poly(propargyl quinoliniumbromide)
PrP	Prion protein
PrP^{C}	Cellular PrP
PrP^{res}	Proteainase K resistant PrP
PrP^{Sc}	Scrapie PrP
PrP^{sen}	Proteainase K sensitive PrP
PZA	Pyrazoloacridine
QSAR	Quantitative structure-activity relationships
Qui	6-chloro-2-methoxyacridin-9-yl
RAFT	Regioselectively addressable functional template
RCSB PDB	Research Collaboratory for Structural Bioinformatics Protein Databank
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$rPrP^{Sc}$	Proteainase K resistant PrP^{Sc}
RT-QuIC	Real time-quaking-induced conversion
SBMA	Spinal and bulbar muscular atrophy
SCA	Spinocerebellar ataxias
$sPrP^{Sc}$	Proteainase K sensitive PrP^{Sc}
Tac	1,2,3,4-tetrahydroacridin-9-yl
TEOF	Triethyl orthoformate
TSE	Transmissible spongiform encephalopathy
vCJD	Variant Creutzfeldt–Jakob disease

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Chapter 1
Introduction

Abstract Acridines interact with both nucleic acids and proteins. The targeting of these biopolymers is broadly applied in cancer therapy and gene delivery. Interestingly, due to direct interactions of acridines with various enzymes, acridines can be suitable drugs for treatment of neurodegenerative diseases, inflammation, immunological disorders, protozoal diseases, etc. General introduction to the field of acridines is provided including scope and limitations of the field. Some important structures, which are used as drugs, are highlighted.

Acridines are heteroaromatic compounds (Figs. 1.1 and 2.1) that were applied for treatment of many diseases since the beginning of 20th century [1]. Namely, aminoacridines possessing higher solubility in physiological liquids were used as antiprotozoal and antibacterial drugs. The growth of transplanted tumor was suppressed by acriflavine (mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine) in the thirties [2]. In the past, anti-tumor activity of acridines was attributed to their interactions with nucleic acids; however, the more recent studies favor an explanation based on direct interaction of acridines with biologically important proteins [3–10]. According to search in RCSB PDB database conduced in August 2015, there were 48 structures containing acridine skeleton and 30 structures containing 1,2,3,4-tetrahydroacridine, respectively. From these 78 structures only 32 correspond to acridine binding to nucleic acids including one complex between amsacrine-nucleic acids and enzyme topoisomerase II. Thus, more than half of the structures represents direct binding of acridines to proteins.

The development of human society has incorporated acridine drugs to daily life of millions of individuals worldwide [6, 10–28]. For selected acridine drugs see Fig. 1.1. Due to high toxicity, compounds **1**–**4** are applied only as local antiseptic agents. *m*-Amsacrine (**6**) is very active against malignant lymphomas and acute leukemia. Asulacrine (**7**), which combines the substructures of *m*-amsacrine (**6**) and DACA (**5**), has improved pharmacokinetic profile and broader scope of applications against leukemia, Lewis lung tumors, and many solid tumors. Nitracrine (**8**) serves for treatment of mammary and ovarian tumors. BRACO19 (**9**) is powerful binder of quadruplexes. Pt-ACRAMTU (**10**) is mimicking anticancer cisplatin; however, it has different mode of action. Quinacrine (**11**) can be viewed as a universal medicine for

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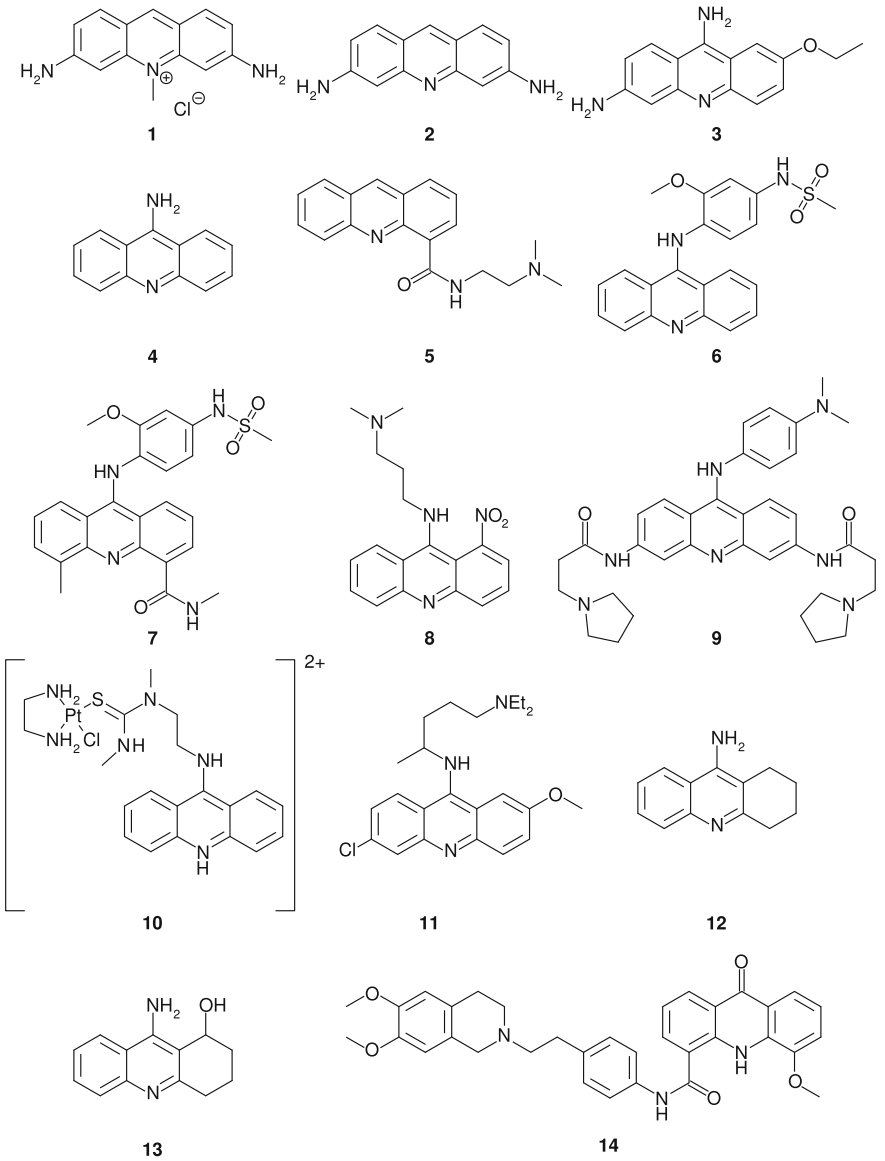

Fig. 1.1 Selected acridines used as antiseptic (**1**–**4**), anticancer (**5**–**10**), trypanocidal (**11**), neuroprotective (**11**–**13**), and transport-enhancing (**14**) drugs, respectively [4, 6, 11]. Acriflavine is a mixture of **1** and proflavine (**2**). Ethacrine (**3**). Aminacrine (**4**). DACA (**5**). *m*-Amsacrine (**6**). Asulacrine (**7**). Nitracrine (**8**). BRACO19 (**9**). Pt-ACRAMTU (**10**). Quinacrine (**11**). Tacrine (**12**). Velnacrine (**13**). Elacridar (**14**)

many diseases such as rheumatic arthritis, lupus erythrematosus, malaria resistant to chloroquine, the tapeworm infections (*Taenia saginata*), Chagas disease, epilepsy (refractory petit mal), and prion infections. Another acridine useful for treatment of neurodegenerative diseases, which has partially hydrogenated ring, is tacrine (**12**). It penetrates blood–brain barrier (BBB) and serves for treatment of Alzheimer's disease (AD). Velnacrine (**13**) served as less toxic analogue of tacrine with almost the same activity. Elacridar (**14**) is a strong inhibitor of the ABC transporters MDR-1 (P-gp) and BCRP. The bioavailability of cytotoxic antitumor drugs is enhanced by elacridar. Moreover, the levels of anti-HIV drugs in the brain and CNS are much higher after administration of elacridar. Rational drug design based on the concept of privileged structures was applied on acridine and quinoline scaffolds including dendrimers in study of neurodegenerative and protozoan diseases [8].

The aim of this book is an update and extension of the previous review [4] with focus to acridine–protein interactions and use of acridines for treatment of neurodegenerative diseases. However, since acridines are potent binders of nucleic acids including quadruplexes, chapter describing this topic cannot be omitted.

Owing to immense amount of published references, we are apologizing to all authors, whose papers are not included.

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Chapter 2
Nomenclature

Abstract Acridines have been known since 1870. Thus, their nomenclature has developed for long time. There are many numbering systems from which we have selected two most important ones. Moreover, plenty of trivial names exist and one has to be very careful when searching in the information databases.

This chapter provides a brief introduction for diving into the deep sea of acridine compounds without drowning, but will not show all possible names of acridines, which is far beyond the scope of the book.

Acridine was discovered and named by Graebe and Caro in 1870, when they investigated aromatic fractions of coal tar and found some basic impurity accompanying anthracene [1]: They named the new substance as "Acridin because of the sharp and biting effect that it exerts on the skin" [1] (from Latin acer = sharp, pungent, or acrid). This strong physiological effect is observable almost immediately after a drop of chloroform solutions of acridine touches a skin and persists for several minutes. The name "Acridin" (German) was imported to many languages with variations of c-k (Czech) and in some with suffixes -e (English, French) or -a (Spanish, Portuguese, Italian).

In past, the acridine ring was numbered differently than it is nowadays (Fig. 2.1) (cf. one of formers [2–5] and current [5–8] numbering). The current numbering reflects the locant order the same as was coined by Graebe [9], who applied it during understanding of 9-acridone structure. However, the numbers for carbon shared by more cycles were introduced later [5]. Thus, careful inspection of formerly published chemical structures is necessary in order to avoid confusion. For example, the most important 9-aminoacridines were called 5-aminoacridines in former works.

Concerning the numbering of acridine substituents, sometimes wrong labels are also used in literature. *m*-Amsacrine (**6**) can be named 4'-(9-acridinylamino) methanesulfon-*m*-anisidide [10], but when 1'-methanesulfonamide group is removed the priority of substituents is altered and 1' locant becomes 4' [11]. Sometimes priority of substituents is not followed and e.g. 5-hydroxymethyl is wrongly named 4-hydroxymethyl despite higher priority of carboxamide group [12].

Since the acridines are in use for more than a century, many of them have more than one trivial name [4, 6, 7, 13]: e.g. quinacrine (**11**) can be also named mepacrine,

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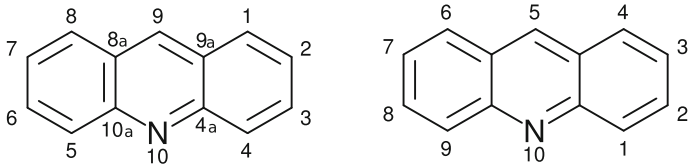

Fig. 2.1 Current (**15**) and former (**16**) numbering of acridine ring

and atabrine or atebrine. Other names for tacrine (**12**) are THA, cognex, tenakrin, or romotal [14–16]. Velnacrine (**13**) can be named 1-acridinol, 1-hydroxytacrine, and HP029 [17, 18]. Ledakrin can serve as a name for nitracrine (**8**) [19]. Thus, one has to be very careful conducing database-based search of acridines.

Some biologically active acridines are usually available not only under their trivial names but also under code numbers. Many examples can be given: GF120918 or GG918 can be named elacridar (**14**) [20] (PubChem CID: 119373); CI-921 stands for asulacrine (**7**) [12, 21, 22] (PubChem CID: 107924) ; NSC 601316 or XR 5000 is DACA (**5**) [23, 24] (PubChem CID: 107805); aminacrine (**4**) (PubChem CID: 7019); amsacrine (**6**) (PubMed CID: 2179); velnacrine (**13**) has number HP029 (PubChem CID: 3655); etc. Moreover different pharmaceutical forms have different numbers such as free proflavine (**2**) (PubChem CID: 7099); its salts: proflavine sulfate – isoflav (PubChem CID: 11111); proflavine hydrochloride (PubChem CID: 197873); proflavine hemisulfate (PubChem CID: 15741); etc.

Sometimes trivial name contained "acridine" despite the structure lacks the acridine ring i.e. acridine red is derivative of xanthene.

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Chapter 3
Syntheses

Abstract Modern syntheses of acridine scaffolds together with the conventional ones are described, thus the reader can easily select the appropriate one for a desired compound. For preparation of acridines, precursors with more aromatic/alicyclic rings are usually assembled using various catalysis. Then, electrophilic condensation led to formation of acridine ring. Modern syntheses are based on Buchwald–Hartwig amination, hypervalent iodine chemistry, iodocyclization, Rh(III)-catalyzed tandem reactions, and recyclable polymeric acido-basic catalysis. Usually, substituents are introduced by selection of appropriate precursors during the ring formation. Because the acridines readily undergo aromatic nucleophilic substitution at *C9* position, various derivatives are prepared by reaction with nucleophiles from *C9*-activated acridines. At positions 3 and 6, the aromatic electrophilic substitutions occurred; however, overreactions are possible. Many building blocks for peptide synthesis bearing acridine unit are available. The synthesized peptide conjugates can serve as drugs, gene delivery systems, imaging agents, etc. Furthermore, acridines with chelating groups can be used as rigid ligands for organometallic catalysis.

3.1 Syntheses of Acridine Ring and Its Precursors

Retrosynthetic scheme describes ways towards basic precursors of substituted acridines derivatized at position 9 (Fig. 3.1). Acridines substituted at position 9 are readily available by aromatic nucleophilic substitutions (Fig. 3.1, transformation i. and vi.). The acridine derivative (**17**) is obtained by direct reaction of nucleophile with activated acridine (**18**) [1] or reaction of strong nucleophile with *C9* unsubstituted acridine (**21**) in the presence or absence of hydride ion scavenger – Chichibabin like reaction [1–5]. However, the use of very strong bases makes the Chichibabin reaction harsh for a wide spectrum of functional groups. When X is chlorine, the 9-chloroacridines (**18**) are the most common precursors of 9-alkoxy-, 9-alkylthio-, 9-amino-, and 9-cyanoacridines [6–14]. Recently, the reaction of chloroacridines with sulfur nucleophiles was investigated by combination of computational and kinetic approaches [15]. Other important precursors of 9-substituted acridines are 9-alkoxyacridines, where the leaving group is very mild acid compatible with many

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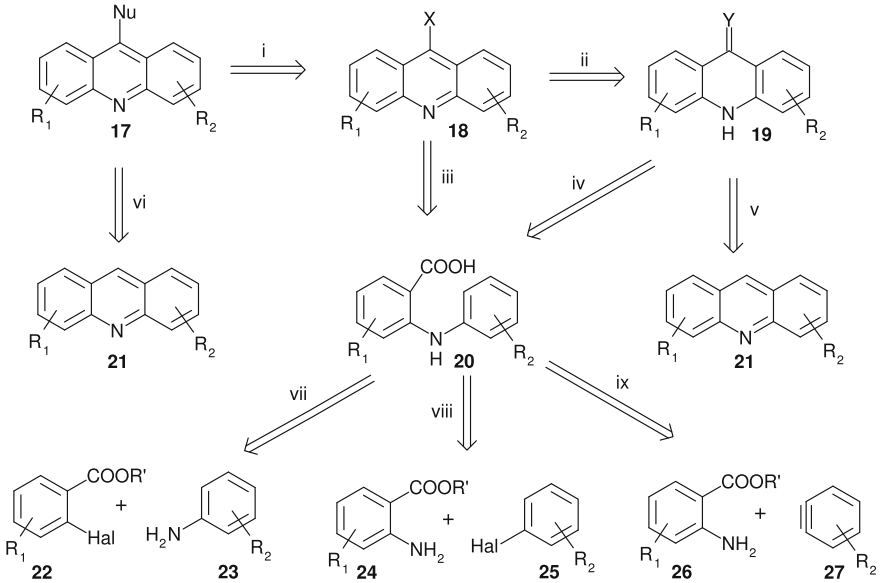

Fig. 3.1 Retrosynthetic scheme providing basic precursors of 9-substituted acridines. X stands for F, Cl, Br, I, NO_2, N_2^+, OR, SR, and NHR, respectively. Y represents O and S. Hal is F, Cl, Br, I. R_1 and R_2 are desired substituents sometimes with appropriate protection. Descriptions of individual transformations are only illustrative for more details see also text: *i*. HNu. *ii*., *iii*. POX_3, reflux. X = Cl, Br. *iv*. H_2SO_4 or PPA 100 °C, or P_4S_{10} dehydration. *v*. S_8, 220 − 230 °C. *vi*. NaNu, $K_3[Fe(CN)_6]$. *vii*, *viii*. 1. Ullmann–Jourdan reaction (Cu powder, K_2CO_3, pyridine, amyl alcohol, reflux) or Buchwald–Hartwig amination ($Pd(OAc)_2$, DPEPhos), 2. NaOH (optional). *ix* DCE, 80 °C

acid sensitive linkers [16]. 9-Heterocyclic substituted acridines can serve as activated acridines, as well [17–19]. Nucleophilic substitution at *C9* position is sometimes reversible, thus groups such as alkoxy, alkylthio, alkylamino are more or less interchangeable [20–27].

Alternative way to syntheses of *N9*-alkyl derivatives of acridines is a reductive amination of commercially available 9-aminoacridine [28, 29] using $NaBH_3CN$ with 1% AcOH/MeOH. Another approach is based on reaction of 9-aminoacridine with arenes activated for S_NAr such as *o* and *p*-nitrofluorobenzenes [28], *o* and *p*-halopyridines, and *o*-halopyrimidines [29] using Cs_2CO_3 as a base and DMF as a solvent at 90 °C. In some cases, the 9-aminoacridine can react with haloquinone electrophiles under the same conditions. However, when the reaction with quinones is carried out in refluxing EtOH in the absence of base, it provides more general route towards acridino-quinone hybrids [29].

N9-acylacridines can be prepared by reaction of acyl chlorides with 9-aminoacridine [30]. The prepared acridin-9-yl acrylate was used for syntheses of fluorescent polymers. Other acylations of 9-aminoacridine were described with DIC/HOBt/NMM [31, 32] and DCC/HOBt [33]. Aminoacridines can be also derivatized with isothiocyanates to corresponding thioureas [34].

Treatment of *N*-phenylanthranilic acids (**20**) or (thio)acridones (**19**) with $POCl_3$ led to activated 9-chloroacridines (**18**) [6–11]. During the reaction of the acid, firstly, the corresponding acridone is formed which is subsequently chlorinated. Activated 9-methylthioaciridines (**18**) can be prepared from corresponding thioacridones (**19**) with methyliodide [35]. Other activated compounds (**18**) are prepared by nucleophilic substitution. In some cases, the activation with $POCl_3$ can be carried out "one pot" with subsequent nucleophilic substitution, e.g. with 1,2,4-triazole [18]. The synthesis of chloroacridines as well as their conjugation with amines can be accelerated by microwave irradiation [36].

9-Acridones (**19**) are prepared by dehydration of *N*-phenylanthranilic acids (**20**) with H_2SO_4 or polyphosphoric acid [7, 37]. If *in-situ* prepared 9-chloroacridines are hydrolyzed during workup, dehydration can be carried out with $POCl_3$, as well [38]. Thioacridones are available by dehydration with P_4S_{10} via route iv (Fig. 3.1). However, when some acridine precursor is available, e.g. acridine orange (AO) (see also Fig. 3.21, compound **101**), it can be oxidized by elemental sulfur to thioacridone (**19**) via route v [35]. Similarly to pyridine chemistry, acridine-*N*-oxides underwent rearrangement to 9-acridinyl (thio)acetate upon treatment with acetyl anhydride or acetyl thioanhydride [39, 40]. These esters decomposed to corresponding acridones or thioacridones. Other syntheses of acridones and thioacridones have been recently reviewed [41, 42].

Acridones may be reduced with Al amalgam and subsequent reoxidation with $FeCl_3$ [43–45] provides acridines unsubstituted at *C9* position. This reaction tolerates various groups such as carboxylic acid.

The Lehmstedt–Tanasescu reaction enables the synthesis of acridone **30** and its derivatives from 2-nitrobenzaldehyde (**28**) and aromatic components **29** [46–48] (Fig. 3.2) or from benzo[c]isoxazoles (anthranil derivatives) [49].

Acridines and phenanthrolines have been synthesized from β-chlorovinyl aldehydes and various aniline derivatives. Many valuable substituents including ketone, nitro or amino groups at the heterocyclic core were incorporated into the acridine ring [50].

Acridine reacts with aliphatic carboxylic acids to 9-alkylacridans under photochemical decarboxylative conditions [51]. Illumination of δ-(9-acridyl)valeric acid or ε-(9-acridyl)caproic acid by UV light affords spiro compounds, 9,9-tetramethylene- and 9,9-pentamethyleneacridan, respectively. Broadening of the reaction to quinoline as a substrate yields 2- and 4-alkylquinolines and 4-alkyl-1,2,3,4-tetrahydroquinolines. Analogous reaction of isoquinoline gives 1-alkylisoquinolines.

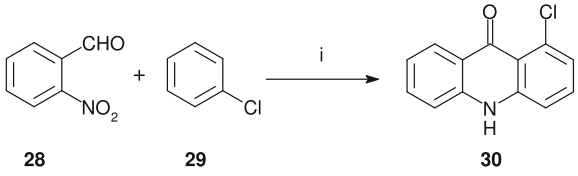

Fig. 3.2 Lehmstedt–Tanasescu acridone synthesis [46–48]. *i*. $NaNO_2$, H_2SO_4

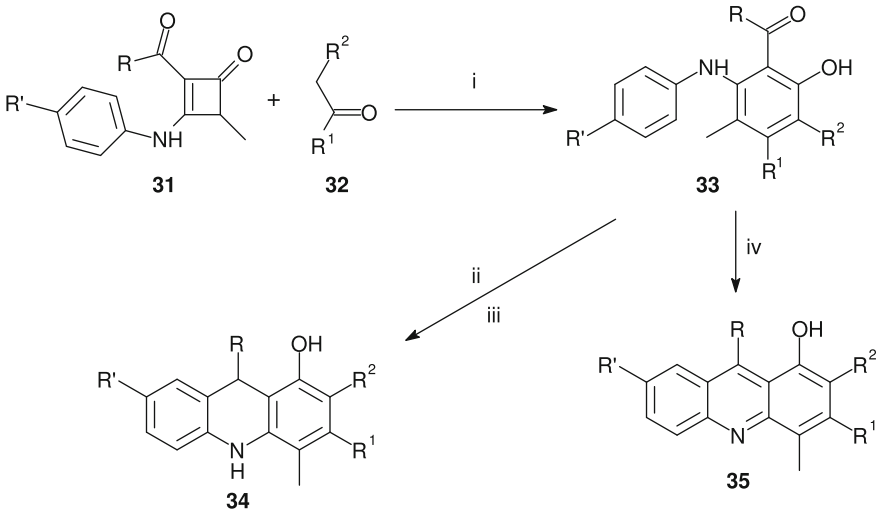

Fig. 3.3 Regiospecific [4 + 2] cycloaddition as an alternative route towards polysubstituted *N*-phenylanthranilic acids and acridines [64]. *i*. LDA/THF 80–140 °C. *ii*. $NaBH_4$/MeOH. *iii*. $BF_3.Et_2O$. *iv*. $In(OTf)_3$/DCE reflux

N-Phenylanthranilic acids (**20**) are available via three main routes by Ullmann–Jourdan reaction (routes vii and viii) [37, 38, 52–56], by Buchwald–Hartwig amination (routes vii and viii) [57, 58], and by reaction with thermochemically generated benzyne (route ix (Fig. 3.1)) [59]. The Ullmann–Jourdan reaction is limited to electron rich anilines and electron poor benzoic acids, otherwise Buchwald–Hartwig amination is recommended [60]. The Ullmann–Goldberg reaction can be accelerated by ultrasonication of the reaction mixture [61]. Recently, direct transformation of aromatic amines to anthranilic acids was described using Pd-catalysis [62]. Another example of Pd-catalysis is direct amination of benzoic acids [63].

A benzannulation approach for synthesis of *N*-phenylanthranilic acids proceeding via a regiospecific [4 + 2] cycloaddition of easily accessible cyclobutenones and active methylene ketones was developed [64] (Fig. 3.3). Applying this strategy, persubstituted anilines and phenols having up to six different functional groups on the benzene ring were obtained. This base-accelerated benzannulation reaction is completely regiocontrolled. In one step, many functional groups are introduced into the benzene ring. These polysubstituted *N*-phenylanthranilic acids (**33**) served as building blocks for syntheses of highly substituted acridine derivatives (**34** and **35**).

Formal [3 + 3] annulations of aromatic azides and aromatic imines or azobenzenes provided acridines and phenazines, respectively [65]. This is a cascade process, which is Rh(III)-catalyzed, consisting of amination, intramolecular electrophilic aromatic substitution and aromatization. Acridines are directly synthesized from aromatic aldehydes using in situ generated imines with benzylamine as catalyst.

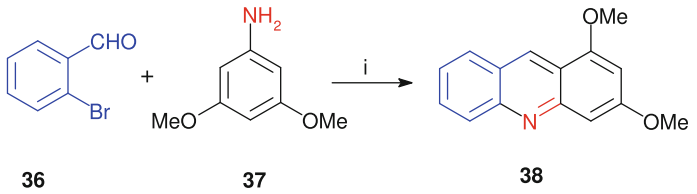

Fig. 3.4 Tandem Buchwald–Hartwig coupling and cyclization reaction [66]. *i*. $Pd_2(dba)_3$ (2.5 mol %), dppf (5 mol %), NaOtBu (2 eq), toluene, reflux, 12 h

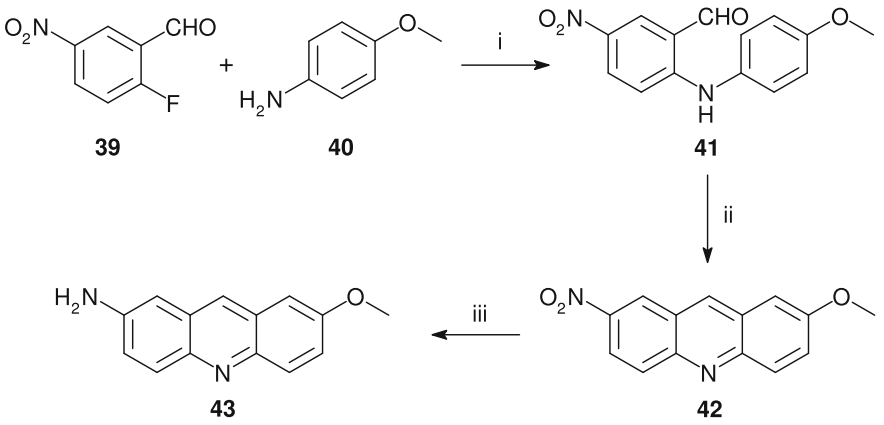

Fig. 3.5 Synthesis of 2-aminoacridines [67]. *i*. Et_3N, DMSO, 120 °C. *ii*. TFA, 80 °C, 24h (83% over two steps). *iii*. Pd/C, H_2, MeOH, DCM, 0 °C → 20 °C

Synthesis of series of acridines via a facile and efficient approach by the tandem coupling/cyclization of substituted 2-bromobenzaldehydes (**36**) and anilines (**37**) was described [66] (Fig. 3.4). The reaction can be easily completed by a catalytic amount of $Pd_2(dba)_3$ and diphosphine ligand dppf, giving a broad variety of substituted symmetrical and unsymmetrical acridines carrying multifunctional substituents as well as the unusual 1,2,3,4-tetrahydroacridine in good to excellent yields (up to 99%). The cyclization of less electron-rich anilines requires the promotion by Lewis acid like $AlCl_3$. Antileukemic agent DACA can be easily obtained in just two simple steps using this method.

Anilines react with 2-fluoro-4-nitrobenzaldehyde (**39**) affording suitable precursors **41** for acridine formation (Fig. 3.5). The acridine ring **42** is closed with TFA at elevated temperature. Careful hydrogenolysis of nitro group provides 2-aminoacridines **43** [67].

Derivatives of anthranilic acid can be constructed from arynes, where three component reactions among aryne, amine, and CO_2 occur [68]. Very reactive arynes, generated by elimination of TMS-OTf, can convert hydrazones to acridones or acridinium salts [69]. Generated arynes reacted with electron-deficient acridines in presence of dimethyl phosphite via 9,10-addition to heterocyclic system (Fig. 3.6) [70].

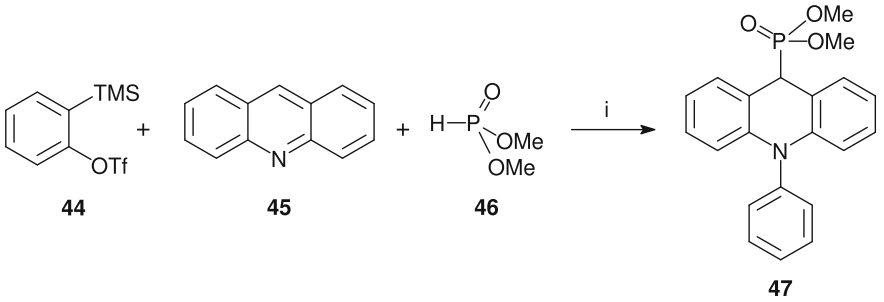

Fig. 3.6 9,10-Addition of arynes to electron-deficient acridines [70]. *i*. KF (1.5 eq), 18-crown-6 (1.5 eq), THF, r.t., 16 h

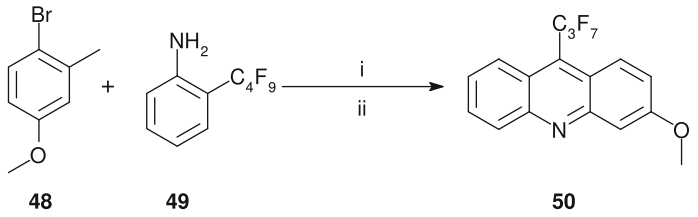

Fig. 3.7 Grignard reaction as a tool for synthesis of 9-perfluoroalkylated acridines [72]. *i*. Mg/THF reflux. *ii*. O_2/H_2O

2-Oxo-substituted aryl azides react in the presence of $BF_3.OEt_2$ with benzene, affording mainly 9-substituted acridines via formal 2-anilino-oxobenzene-BF_3 complexes [71]. The reaction is rapidly followed by intramolecular cyclo-dehydration of the activated carbonyl groups. 2-Azidobenzoic acid gives under the same conditions mainly 2-anilinobenzoic acid and trace amounts of the 9-acridone. 2-Azidobenzenecarbonitrile gives on the other hand the 9-aminoacridine via a conjugated imine, which undergoes intramolecular cyclization. The $BF_3.OEt_2$ catalyzed dissociation of aryl azides to aryl nitrenium ions is comparable to those catalyzed by $AlCl_3$ or a strong protic acid (TFA/TFMSA mixture).

Reaction of 2-(perfluoroalkyl)aniline (**49**) with 2-tolylmagnesium bromide or chloride or their substituted analogs affords an acridine including a shorter perfluoroalkyl group at the 9-position and without the methyl group of the Grignard reagents [72] (Fig. 3.7). Surprisingly, no acridine is formed in a reaction with aryl magnesium bromide without the *ortho* methyl group. With 2-fluoro-6-methylphenylmagnesium bromide the methyl group is eliminated and the fluorine is retained in the acridine product. Mechanistic studies strongly indicate that loss of the methyl group occurs in the form of methanol during air oxidation and hydrolysis of the intermediate products during aqueous processing.

10-Aryl- or 10-(arylmethyl)acridin-9(*10H*)-ones were synthesized via the one-pot reaction of 2-fluorophenyl-2-halophenylmethanones [73], which can be obtained by an easy two-step reaction starting from easily available 1-bromo-2-fluorobenzenes and 2-halobenzaldehydes with benzenamines and arylmethanamines, respectively.

The coupling of 2-bromostyrene and 2-chloroaniline derivatives catalyzed by Pd affords stable diphenylamines, that are transformed to five-, six-, or seven-membered heterocycles (indoles, carbazoles, acridines, and dibenzazepines) [74, 75]. The selectivity of these conversions is partly ligand-controlled and provides efficient ways to important classes of heterocycles from a common precursor.

A Synthesis of 9-aminoalkyl and 9-aminoalkylidene derivatives of acridan was patented [76].

A short and effective synthesis of a wide spectrum of acridine derivatives was developed using a $ZnCl_2$-promoted cyclization reaction of easily accessible *o*-arylaminophenyl Schiff bases under quite mild conditions [77]. A reliable mechanism for the cyclization of *o*-arylaminophenyl Schiff bases was proposed. The imine *N* atom in the *o*-arylaminophenyl Schiff base forms a $ZnCl_2$ complex. Its Zn atom caused a polarization of the imine C=N bond generating positive charge on the C atom. The cyclization proceeds by an intramolecular electrophilic reaction of the C atom with the aromatic ring at an *ortho*-position to the amine group with a formation of zwitterionic intermediate. Deprotonation of the allylic carbocation and proton transfer to the nitrogen anion led to formation of 9,10-dihydroacridine. The unstable 9,10-dihydroacridine was aromatized and an aniline molecule was split off.

Another source of acridine ring is isatin (**51**). After *N*-arylation, isatin rearranges under basic conditions to acridine-9-carboxylates (**53**) [78–80] (Fig. 3.8), which is an application of Pfitzinger reaction [81]. The yield can be increased by use of microwaves [82]. Reaction of isatin with cyclic ketones affords 1,2,3,4-tetrahydroacridine-9-carboxylic acid (**54**) [83]. Substituted isatins can be obtained from corresponding anilines, and can serve also as sources of anthranilic acids [79, 84]. Tacrine can be prepared by combination of isatin method and Hofmann degradation [85].

The synthesis of chiral crown ethers having an acridine-9-carboxylic acid subunit has been accomplished. These macrocycles were used for preparation of the new

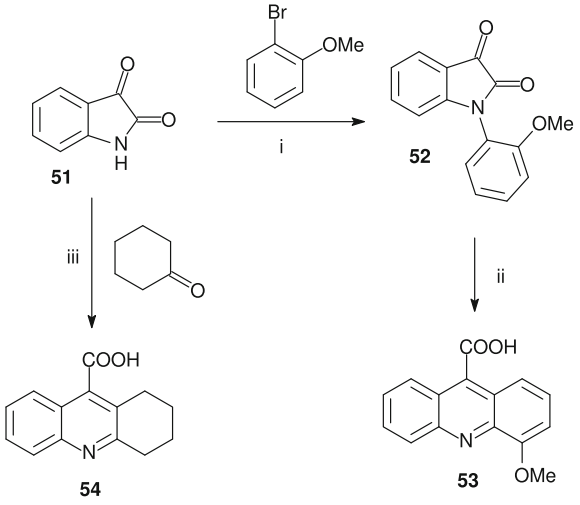

Fig. 3.8 Synthesis of acridine-9-carboxylic acids from isatin [78–80, 82, 83, 85]. *i*. CuO, DMF, 120 °C. *ii*. NaOH, H_2O, 100 °C. *iii*. KOH, EtOH/H_2O 1:1, reflux

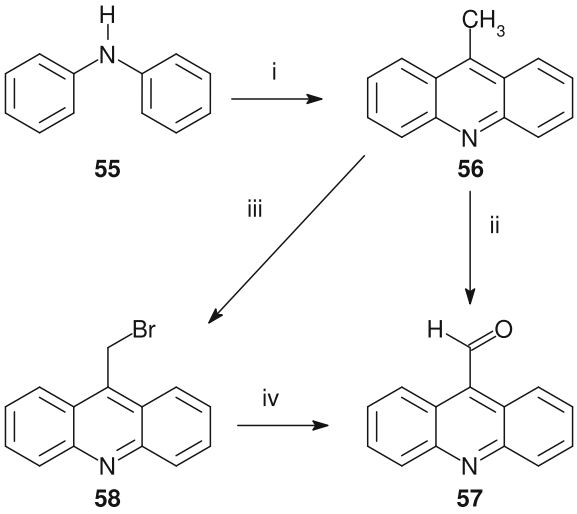

Fig. 3.9 Synthesis of acridine-9-carboxaldehyde [87–89, 91–93, 97]. *i.* AcOH, $ZnCl_2$, 210 °C, microwaves. *ii.* SeO_2, 1,4-dioxane/H_2O, reflux *iii.* NBS, $(PhCOO)_2$, $CHCl_3$. *iv.* sodium, MeOH, 2-nitropropane, DMF

acridino-18-crown-6 ether–based spherical HPLC silicagel [86]. These chiral stationary phases were able to efficiently separate mixtures of enantiomers of selected protonated primary arylalkylamines. Both chiral stationary phases demonstrated the best enantioseparation of the mixtures of enantiomers of 1-(*p*-nitrophenyl)-ethan-1-amine.

Acridine-9-carboxaldehydes (**57**) can be prepared by oxidation of 9-methylacridines (**56**) with SeO_2 [87], pyridinium chlorochromate [88–90], or *p*-nitroso-*N*,*N*-dimethylaniline [91]. 9-Methylacridines (**56**) are available via Friedel-Crafts reaction from e.g. *N*,*N*-diphenylamines (**55**), acetic acid and $ZnCl_2$ [92, 93] (Fig. 3.9). The yields of Bernthsen reaction were increased by microwaves [94, 95]. Replacement of acetic acid with benzoic acid derivatives led to 9-phenyl substituted acridines in solvent free medium [96]. The synthesis proceeds with commercial chemicals using microwaves under heterogeneous conditions in the presence of $ZnCl_2$ and 2,2-dimethoxypropane as water scavenger. Great advantages of this approach are short reaction time, easy work-up, and high yields. Conversion of 9-bromomethylacridine (**58**) to acridine-9-carboxaldehyde has been described [97]. Various heterocycles such as thiazolidines can be anchored to acridine via aldehyde (**57**) [89]. Syntheses of 1-aryl-3-substituted-4-(9-acridinyl)azetidin-2-one derivatives and 2-(9-acridinyl)-3-aryl-1,3-thiazolidin-4-one derivatives were described [98]. The formation of 1-aryl-3-substituted-4-(9-acridinyl)azetidin-2-one proceeded through [2 + 2] cycloaddition between Schiff base and ketene. The Schiff base was obtained from acridine-9-carboxaldehyde and substituted anilines, whereas the ketene was generated from 2-chloroacyl chlorides in the presence of triethylamine *in situ*. Analogously cycloaddition of mercaptoacetic acid to the same Schiff base provided 2-(9-acridinyl)-3-aryl-1,3-thiazolidin-4-ones. 9-Bromomethylacridine (**58**) can be also converted to amine with urotropine and various spirocyclic compounds can be prepared e.g. spiro[dihydroacridine-9'(10'*H*),5-imidazolidine]-2-thiones have been obtained

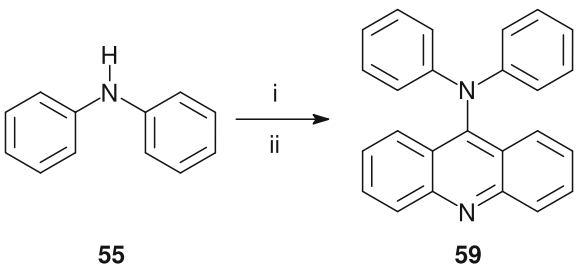

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Fig. 3.10 Photochemical synthesis of *N*,*N*'-diphenylaminoacridines [100]. *i*. CBr_4, hν, *n*-hexane. *ii*. $NaHCO_3$

by spontaneous cyclization of 1-substituted 3-(acridin-9-ylmethyl)thioureas, which were obtained from 1-(acridin-9-yl)methanamine, *N*-(acridin-9-ylmethyl)propan-1-amine, and *N*-(acridin-9-ylmethyl)benzylamine and alkyl/aryl isothiocyanates [99]. Imidazolidine-2-thiones acquired were transformed with mesitylnitrile oxide to imidazolidine-2-one analogues, some of them partly reopened to the appropriate (acridin-9-ylmethyl)ureas. 3-(Acridin-9-ylmethyl)-1-(acridin-9-yl)thioureas with two acridine rings undergo an unusual spirocyclization via a CH carbanion instead of the *N*-1 nitrogen. Structural alterations in positions 1, 3, and 4 of the spiro ring together with various NMR spectroscopies and X-ray crystallography were used to rationalize a general predisposition of various 9-substituted acridines to undergo easy spirocyclization.

An alternative to Friedel–Crafts reaction can be photochemical synthesis of 9-diarylamino-substituted acridines from various diphenylamines with tetrabromomethane [100]. The reaction afforded *N*,*N*'-diphenylacridin-9-amine (**59**), 2,7-dimethyl-*N*,*N*-bis(4-methylphenyl)acridin-9-amine, and 2,7-bis(1-methyl-1-phenylethyl)-*N*,*N*-bis[4-(1-methyl-1-phenylethyl)phenyl]acridin-9-amine, which are donor-acceptor type compounds (Fig. 3.10).

o-Nitrophenyl derivatives of phenylalkane-nitriles can after dissolution in 90% H_2SO_4 provide 9-alkylacridines (**63**) [101, 102] (Fig. 3.11).

A common acridine synthesis has been developed based on diphenylamine-2-carboxaldehydes [103]. Diphenylamine-2-carboxylic acids are transformed to their *p*-toluenesulfonylhydrazides. Their decomposition via a modified McFadyen–Stevens reaction yields an aldehyde derivative which affords the acridine by treatment with mineral acid. For more data see also [104].

Indazol-3-ylidene, the *N*-heterocyclic carbene of indazole is a convenient starting material for synthesis of heterocycles. Its rearrangement provides functionalized acridines [105]; however, in the presence of carbon disulfide quinazoline-2,4(*1H*,*3H*)-dithiones are formed.

Syntheses of 9-anilinoacridines have been recently reviewed [106].

The anthranilic acid precursor of amsacrine derivatives substituted at positions 4 and 5 could be prepared by Ullmann-type reaction starting e.g. from commercially accessible 2-amino-3-methylbenzoic acid and 2-chlorobenzoic acid in *N*-methyl-2-pyrrolidinone at 160 °C in a 98% yield [107]. In concentrated H_2SO_4, the key acridone intermediate was formed by ring closure reaction in 86% yield. This intermediate was transformed to asulacrine by common strategy using 9-chloroacridine route.

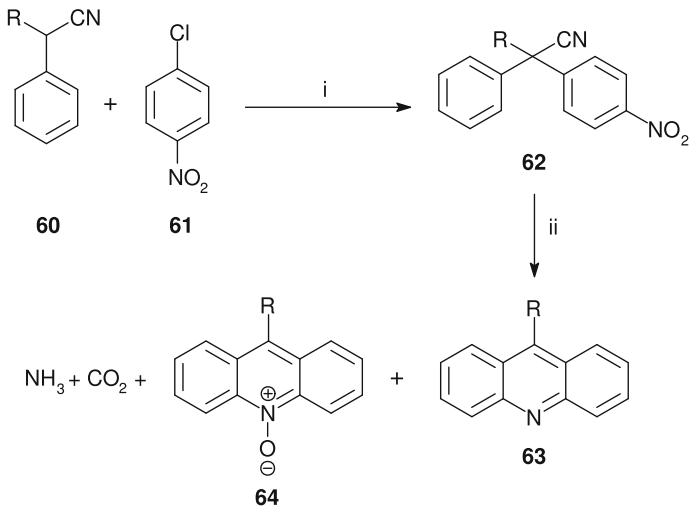

Fig. 3.11 Syntheses of acridines from nitriles [101, 102]. *i*. NaOH, $[BnNEt_3]^+ Cl^-$. *ii*. 90% H_2SO_4

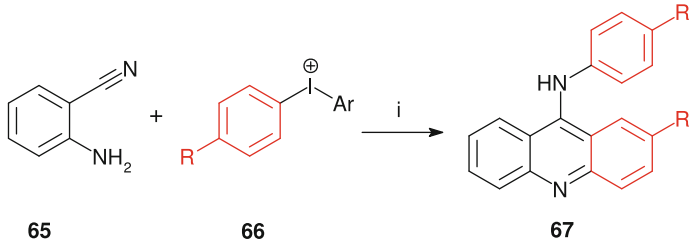

Fig. 3.12 Synthesis of acridine skeleton using diaryliodonium salts [112]. *i*. 10% $Cu(OTf)_2$/DCE 65 °C

Proflavine (**2**) is a good precursor for diazotization and synthesis of 3,6-diazoacridine [108–110] – a suitable building block for click chemistry and libraries of quadruplex binder [108]. Moreover, decorated DNA nanostructures can be prepared from proflavine analogues containing azides via click chemistry [111].

Two approaches to synthesize a range of acridines and quinazolin-4(*3H*)-imines by means of tandem cyclization from two kinds of easily accessible *o*-cyanoanilines and diaryliodonium salts were realized [112] (Fig. 3.12). When the ratio of *o*-cyanoanilines to diaryliodonium salts was 1:2, the reaction proceeded at 65 °C and provided acridines in 90% yield. These findings were further developed to a modular method [113] for synthesis of acridine derivatives by reaction of *o*-acylanilines and diaryliodonium salts. Moreover, the reaction to prepare acridones was successfully extended with *o*-aminobenzoates instead of *o*-aminophenones. The reactions proceeded readily under both Cu-catalyzed or metal-free reaction conditions as tandem arylation and Friedel–Crafts reactions. In the absence of metal catalysts, elevated temperature was required. The easy synthesis of the acridine skeleton and simple handling might be useful for design and generation of acridine libraries.

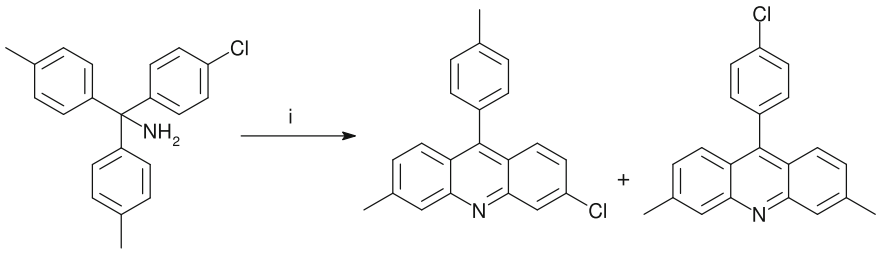

Fig. 3.13 Oxidative rearrangement of trityl amines to 9-phenylacridines [114]. *i*. $Cu(OAc)_2$/mesitylene, O_2 (1 atm), 170 °C, 6h. Yield 66% with **69**:**70** ratio 86:14

Tritylamines undergo an unusual copper-mediated cyclization including the splitting of two C-H bonds and one C-N bond leading to 9-arylacridine derivatives [114] (Fig. 3.13). Under an oxygen atmosphere, the yields are ca 3 times higher than those with air. This type of acridines is important due to their unique biological, optical, electrochemical and photochemical properties. Some of the synthesized acridine derivatives show intense fluorescence in the solid state.

Four 3-(acridin-9-yl)methyl-thiazolidin-4-one ligands were synthesized. The anticipated regioselective formation of two different 2-imino-1,3-thiazolidin-4-ones with methyl bromoacetate or bromoacetyl bromide, 3-(acridin-9-yl)methyl-substituted and 2-(acridin-9-yl)methyl-substituted, respectively, was not accomplished. Instead, only the first mentioned regioisomer had been obtained [115]. The mechanism of its formation including a spirocyclic intermediate was proposed.

The synthesis of 3,6-diamino-9-[(phenylalkyl)amino]acridines was developed by heating 3,6-di(butanoylamino)-9-[(phenylalkyl)amino]acridines in basic 2-propanol solutions [116] (Fig. 3.14). Formation of key intermediate – 3,6-diamino-9(*10H*)acridone (**74**) [117] – started from diphenylmethane (**71**) by nitration and subsequent oxidation to 2,2',4,4'-tetranitrobenzophenone (**73**). The acridone was formed by reductive cyclization. The side product 3,6,9-triaminoacridine was observed and its formation explained by cleavage of benzylic cation from the imine form of 9-aminoacridine.

When partial hydrogenation of acridine/acridone ring is required, saturated building blocks are needed. Thus, tacrine tricyclic ring is prepared from anthranilic acids and cyclohexanone (Fig. 3.15). Instead of *N*-phenylanthranilic acid, the spirocyclic ester of geminal amino alcohol is formed (**76**). This ester can be dehydrated to corresponding tetrahydroacridinone (**77**) [118, 119] or directly converted by $POCl_3$ to 9-chlorotetrahydroacridine (**78**) [16, 120, 121]. In our hands, the transformation of tetrahydroacridinone (**77**) with $POCl_3$ [122] provided purer chloroacridine than the direct reaction.

A potential anti-Alzheimer therapeutic – (±)-9-amino-1,2,3,4-tetrahydroacridin-1-ol (**13**) – with low toxicity was prepared by multi-step synthesis starting with condensation of anthranilonitrile (**79**) and cyclohexane-1,3-dion [123] (Fig. 3.16).

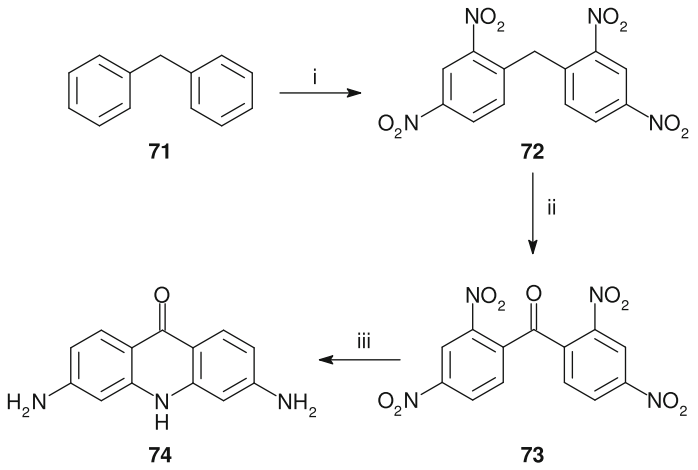

Fig. 3.14 Synthesis of key acridone for quadruplex binders [116, 117]. *i*. H_2SO_4, KNO_3, 70 °C, 40 min.; *ii*. CrO_3, CH_3CO_2H, reflux, 3 h; *iii*. $SnCl_2 \cdot 2H_2O$, HCl, EtOH, 90 °C, 18 h

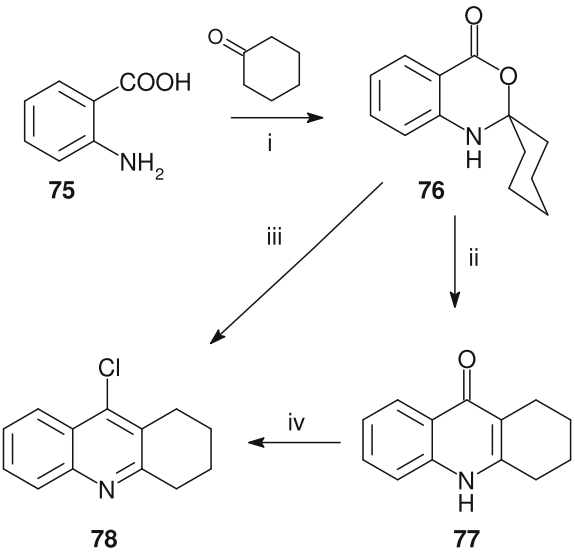

Fig. 3.15 Synthesis of tacrine precursor [16, 118–122]. *i*. heating of the reactants with/without solvent (toluene). *ii*. intensive heating to 230 °C without solvent. *iii*. and *iv*. $POCl_3$, 120 °C

From the enamine **80**, the 9-amino-1,2,3,4-tetrahydroacridin-1-one (**81**) ring was closed by CuCl mediated cyclization. The acridone was reduced to the desired alcohol **13** by $LiAlH_4$. The conversion of various anthranilonitriles to tacrine analogues can be also catalyzed by Lewis acids such as $ZnCl_2$ [124].

An effective one pot reductive cyclization mediated by iron/acetic acid was successfully elaborated [125] for the syntheses of acridin-1-one derivatives. It started with 2-nitrobenzaldehydes and dimedone. This very efficient method takes place under mild conditions, and affords various acridones in good to excellent yields.

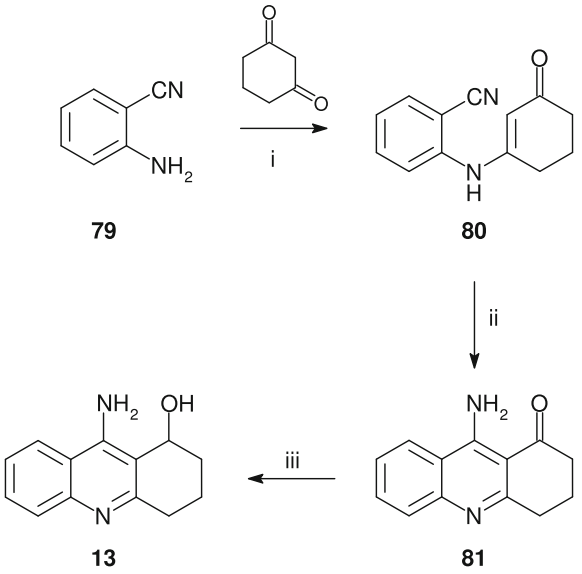

Fig. 3.16 Synthesis of anti-Alzheimer drug (±)-9-amino-1,2,3,4-tetrahydroacridin-1-ol [123]. *i*. pTSA. *ii*. CuCl, K_2CO_3. *iii*. $LiAlH_4$

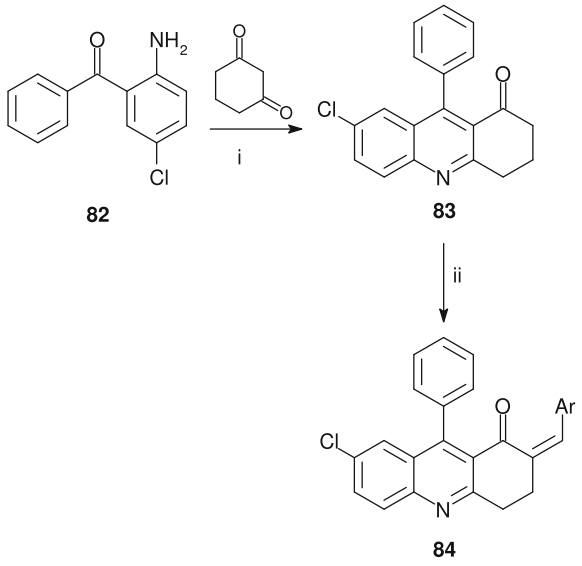

Fig. 3.17 Synthesis of 7-chloro-3,4-dihydro-9-phenyl-2-[(aryl)methyl-idene]acridin-1(*2H*)-one [127]. *i*. glacial AcOH, H_2SO_4, 150 °C, 6h. *ii*. Ar-CH=O, KOH, r.t., 5 h

Friedel–Crafts reaction of *N*-diphenylamine with acetone and subsequent alkylation provided a way towards antidepressant dimetacrin [126].

7-Chloro-3,4-dihydro-9-phenyl-2-[(pyridin-2-yl)methylidene]acridin-1(*2H*)-one (**84**) was synthesized from 7-chloro-3,4-dihydro-9-phenylacridin-1(*2H*)-one (**83**) and picolinaldehyde with involvement of KOH at room temperature [127] (Fig. 3.17). The acridine reactant and product were evaluated against the early fourth instar

larvae of Japanese encephalitis vector, *Culex gelidus* (Diptera: Culicidae) and lymphatic filariasis vector, *Culex quinquefasciatus*. The activities against mosquitoes were approximately 60 μM.

A category of substituted ethyl 10-chloro-4-(3,4-dimethoxyphenyl)-2-hydroxy-12-phenyl-1,4,5,6-tetrahydrobenzo[a]acridine-3-carboxylates, has been synthesized using NaOH mediated cyclocondensation of *(E)*-7-chloro-2-(3,4-dimethoxybenzylidene)-9-phenyl-3,4-dihydroacridin-1(*2H*)-ones with ethyl acetoacetate [128]. All the prepared derivatives were tested for their interactions with various metals. There were identified derivatives of acridine-3-carboxylate active against 4th instar larvae of *Anopheles stephensi* and *Hippobosca maculata*, which are non-toxic for water organisms. The same concept was applied also for *(E)*-2-benzylidene-7-chloro-9-phenyl-3,4-dihydroacridin-1(*2H*)-ones [129].

Acridinedione and its derivatives are both biologically and industrially attractive compounds [130, 131]. When dimedone is heated with 4-nitrobenzaldehyde in aq. MeOH, the 2,2'-(4-nitrobenzylidene)bis-dimedone precipitated out [132]. The tetraketone can react with primary amines under acid catalysis to 1,8-acridinediones [132, 133].

The synthesis of 1,8-acridinediones by an ecofriendly, highly efficient and improved methodology has been developed [130]. It includes a one-pot multicomponent condensation of 1,3-cyclohexanedione (**85**), aromatic aldehydes (**86**), and ammonium acetate under catalysis with poly(4-vinylpyridinium)hydrogen sulfate (**88**) in aqueous medium [131] (Fig. 3.18). Outstanding yields, simple work-up procedure, easy recovery, shorter reaction time, and repetitive use of the catalyst are tempting features of this green protocol. Another heterogeneous catalyst for the synthesis was based on SiO_2-I [134].

Previously, a similar synthesis was carried out with formic acid instead with polymeric catalysts [135]. The polymeric sulfonic acid can be substituted with chiral and soluble glucose sulfonic acid providing tetrahydrobenzo[α]xanthens and tetrahydrobenzo[α]acridines in water [136]. Analogous approach was applied to synthesis of the same skeleton by utilization of stoichiometric ratio of dimedone:aromatic aldehydes:amino alcohols 2:1:1 and microwave irradiation for a few min with superb

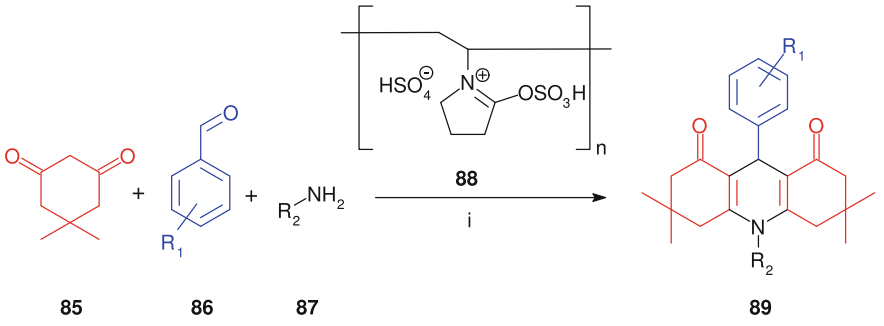

Fig. 3.18 Facile synthesis of acridinediones with polymeric catalyst [131]. *i*. EtOH/H_2O 3:1, reflux

yield (80–91%) [137]. The same strategy with dimedone was previously used also for syntheses of acridine alkaloids [138, 139]. 4-Phenyl analogue of diketone and derivatives of benzaldehyde provided branched acridinediones with three aromatic rings attached [140].

Reaction of dimedone, 4-amino-*N*-(diaminomethylene)benzenesulfonamide, and aromatic aldehydes proceeded with high yield under catalysis with sulfuric acid [141]. The acridine sulfonamide compounds were synthesized with high yields in water at ambient temperature.

Acridinedione derivatives were synthesized by thermal method using P_2O_5 ethanol catalyst. Identical compounds were prepared by a solvent free "green approach" using neat reactants (dimedone, aromatic aldehydes and amines) under microwave irradiation in a stoichiometric ratio 2:1:1 for a few minutes [142]. It provided stable acridinediones in an excellent yield (80–95%). The yield and purity of the greener method were better than those of the thermal one.

Another example for microwave assisted synthesis of 9-(substituted indolyl)-3,4,6,7-tetrahydro(*2H*,*5H*,*9H*,*10H*)acridine-1,8-dione was published [143].

When 5-trifluoromethyl-1,3-cyclohexanedione is used for this reaction, the acridinedione containing two trifluoromethyl groups at positions 3 and 6 is obtained [144].

When aldoses were used as sources of aldehyde group in the formation of acridinediones, polyhydroxyl functionalized inhibitors of α-glucosidase and α-amylase were prepared [145].

Synthesis of arylmethylene bis(3-hydroxy-2-cyclohexene-1-one) was efficiently promoted by catalytic amount of $ZnCl_2$ in water at room temperature in high yield [146]. The one-pot synthesis of 1,8-dioxo-octahydroxanthene and 1,8-dioxo-decahydroacridine derivatives was also catalyzed by $ZnCl_2$ at 120 °C providing good yield. In particular examples, uncommon dissociation of dimedone from the arylmethylene bis(3-hydroxy-2-cyclohexene-1-one) was observed in the reaction medium. It was suppressed by excess of dimedone in the reaction mixture in the one-pot synthesis of 1,8-dioxo-octahydroxanthene.

The acridinediones can be also prepared by catalysis of 1,3-disulfonic acid imidazolium carboxylate ionic liquids under solvent-free conditions [147].

Salicylic acid can serve also as catalyst for syntheses of acridinediones [148].

A highly effective and reusable Brønsted acidic ionic liquid 3-(carboxymethyl)-1-methyl-*1H*-imidazol-3-ium trifluoroacetate was applied with success for the synthesis of acridinediones in aqueous media [149]. Recyclability of the catalyst, high yields, use of environmentally friendly solvent, facile product isolation, high atom economy and avoiding column chromatography are the remarkable features of this method. The reaction was used for synthesis of an acridinedione library.

Mono-enamines prepared from 1,3-cyclohexanediones can be cyclized to acridinediones using CuBr in acetonitrile [150]. Thus, the acridinediones have the same substituents at *C9* and *N10* positions.

Another set of compounds containing hydrazide linkage was synthesized by a microwave assisted procedure using various benzyloxy benzaldehydes, dimedone, and appropriate phenyl hydrazines or aryl hydrazides in the ratio 1:2:1 in anhydrous

DMF and catalytic amount of HCl [151]. The utilization of microwave irradiation enhanced the yield and shortened the reaction time from 3–8 h to 5–8 min. These compounds serve as hSIRT inhibitors.

Usage of hydrazine instead of amines led to acridinediones conjugated to naphthalimide via N-N linkage [152]. The reaction was carried out in [bmim]HSO_4 ionic liquid as green medium.

Synthesis of acridinediones was carried out by the one-pot Hantzsch condensation of 5,5-dimethyl-1,3-cyclohexanedione with anilines in refluxing water [153]. This represents an environmentally friendly and efficient approach leading to acridinediones in good to excellent yields.

The most attractive biosynthesis of acridinediones has appeared recently [154]. It is based on biocatalysis with baker's yeast (*Saccharomyces cerevisiae*) under ultrasonication.

1,3-Dipolar cycloaddition reaction was used for a facile synthesis of acridinedione derived mono spiro-pyrrolidine and pyrrolizidine derivatives [155]. The dipolarophilic *O*-acryloylacridinediones reacted with azomethine ylide derived from di-/tri-ketones and *sec*-amino acids providing acridinedione derived mono spiropyrrolidine or pyrrolizidine derivatives in good yield. All reactions proceeded with high stereo- and regioselectivity.

A direct strategy for the synthesis of 9-(1-iodovinyl)acridin-1(*2H*)-one (**91**) was developed [156] (Fig. 3.19). This cascade transformation of 2-aminophenylpropynyloxyenone (**90**) utilized a iodocyclization and intramolecular condensation with subsequent 3,3-sigmatropic rearrangement. This method can be carried out under normal atmosphere at ambient temperature.

Two alternative syntheses of acridin-9(*10H*)-ones were designed [157]. One comprises a two-step conversion. The first one is the Diels–Alder reaction of *(E)*-1-methyl-2-styrylquinolin-4(*1H*)-ones with *N*-methylmaleimide in boiling toluene and the second one is oxidation of products. Next synthesis is a one-pot procedure utilizing 1,2,4-trichlorobenzene as solvent at 180 °C. The impact of microwave irradiation and Lewis acid catalysts in these cycloaddition reactions was also studied. In all transformations studied, the advantage of microwave irradiation was the shortening of reaction times.

A hydrogenation study of acridine derivatives has been performed using Rh/Al_2O_3 [158]. It was found, that the pyrrolidino-substituent had strong influence on the reduction route and reduction products. When the hydrogenation was carried out on the

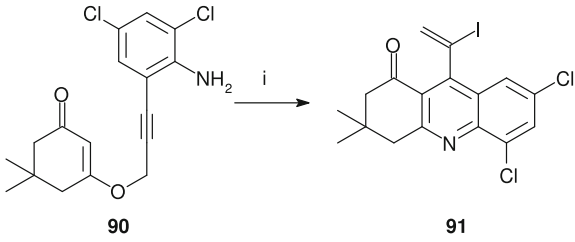

Fig. 3.19 Utilization of iodocyclization for acridone synthesis [156]. *i*. 1.2 eq I_2/DCM r.t

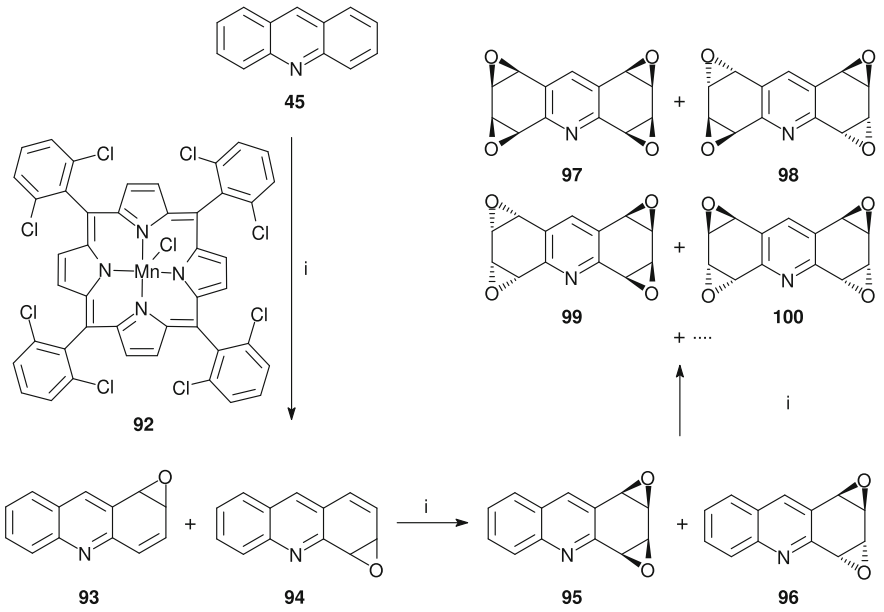

Fig. 3.20 Green oxidation of acridine ring to mixtures of various epoxides as suitable mimics of sugars [159]. *i*. cat. **92**, H_2O_2, $AcONH_4$, ACN, r.t

unsubstituted acridine, complete conversion was achieved in 8 h. Under identical conditions, when a pyrrolidine substituent was located at position 9 of acridine, a pure product was gained in which the two lateral carbocycles were reduced while the central heterocyclic ring was not. When the pyrrolidine substituent was located at the 1-position, pure compound with partially reduced central heterocyclic ring was obtained, but it was rapidly re-oxidized by air. In order to explain this substituent effect, theoretical calculations were carried out. With respect to the energies and thermodynamic data of each reaction component, as well as, the mutual interaction with the catalyst surface, the selectivity and variability in the reduced analogue production were partly explained and various reaction pathways were outlined according to the substitution pattern on the acridine scaffold.

The straightforward epoxidation of acridine on the edge sites was delineated [159] (Fig. 3.20). The epoxidation takes place under mild conditions employing a biomimetic catalytic system containing a Mn(III) porphyrin (**92**). The mono-, di-, and tetraepoxy derivatives (**93**-**100**) are prepared by oxidation with green oxidant – hydrogen peroxide – at ambient temperature.

In protic solvents acridine is photochemically reduced to derivatives of 9,10-dihydroacridine. At concentrations >1 mmol/L, in all cases a sparingly soluble solid product, 9,10,9',10'-tetrahydro-9,9'-biacridyl was formed [160]. Analogously, from 10-methylacridinium halogenide, 10,10'-dimethyl-9,10,9',10'-tetrahydro-9,9'-biacridyl was prepared. The photolysis of acridine affords also 9-substituted acridane

from which 9-cyclohexyl- and 9-dioxanylacridan were isolated and identified. The reaction proceeds via rearrangement of solvent radicals. The same biacridyls can be also prepared from acridine by $[H(thf)_2][V(CF_3CO_2)_3]$ catalyzed reaction [161].

3.2 Acridine-Peptide Conjugates

Syntheses of acridine-peptide conjugates have been described [1, 41, 106, 162, 163]. The acridine moiety is usually attached to peptides via amino groups to *C*-terminus or aspartate and glutamate side chains, via carboxyl or aldehyde to *N*-terminus or lysine and ornitine side chains. It can be introduced chemoselectively to cysteine side chains using *N*,*S*-displacement reaction or alkylation. Individual cases will be discussed below.

For small peptides, direct acridinylation with 9-chloroacridine in solution was described e.g. in diethanolamine Acr-Gly-Gly-Gly-OH was prepared [164]. Bis-acridinylated diamines with a peptide bond derived from Gly, β-Ala, and γ-Abu were obtained by similar reaction in PhOH [165] in the absence of base.

The tris-acridinylated peptide Acr-γ-Abu-Tyr-Lys-Lys-Gly-Dab(Acr)-Gly-Gly-Gly-Gly-NH$(CH_2)_3$NH-Acr [166] was prepared from appropriate segments combining Boc/Bzl, Nps/Z, and Z/Boc strategy by solution phase peptide synthesis. After hydrogenolysis, the entire peptide with three free amino groups and remaining Boc protection was treated with 9-methoxyacridine in MeOH.

A tetrapeptide with *C*-terminal lysine was derivatized at the side chain with 2-methyl-9-acridinecarboxaldehyde, whereas the *N*-terminus was protected with Fmoc group [167, 168]. The Fmoc-on/Fmoc-off procedure was applied for peptide-conjugate purification [169].

Another *C*-terminal lysine was derivatized with 3,6-bis(dimethylamino)-9-methylthioacridine (**103**) [35] in DCM/TEA/TFE at 40 °C (Fig. 3.21).

Amino acids were *N*-acridinylated with acridine-9-isothiocyanate [170, 171].

Direct acridinylation with 9-chloroacridine on solid phase was also reported [172–174]; however, the elevated temperature altered the resin properties such as mechanical stability, clarity, its particle size and separability.

Another approach based on use of suitable acridinylated building blocks such as protected amino acid, protected amine, or protected carboxylic acid is an alternative usable even on solid phase without affecting resin properties. Moreover, an introduction of acridine to required peptide position can be achieved.

Introduction of acridine moiety to amino group provided also an orthogonal protection for Boc and Fmoc approaches [1, 23, 175–177].

11-Aminoundecanoic acid after conjugation with 9-chloroacridine was used for synthesis of acridine-bZIP conjugate [178].

Nitroacridines or acridones equipped with diamine linkers containing 2–5 methylene groups were inserted to side chain of D-isoglutamine in muramyldipeptides by EEDQ coupling agent in solution [179, 180].

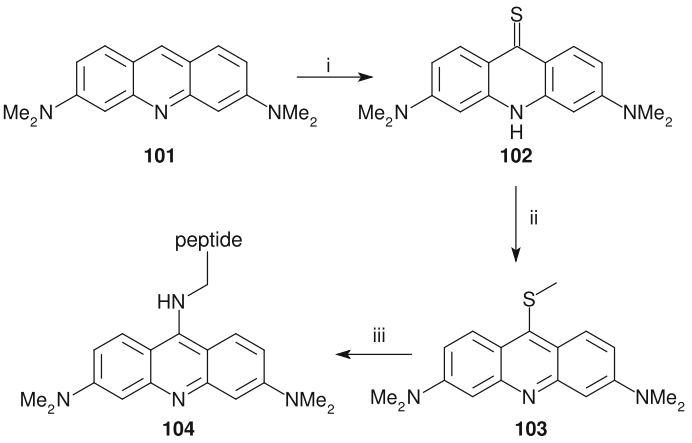

Fig. 3.21 Derivatization of ε-amino groups of lysine with acridines under mild condition [35] (iii). However, the activation of acridine precursor (AO, **101**) is quite harsh (i). *i*. S_8 220 – 230 °C. *ii*. EtONa, MeI, r.t. *iii*. peptide, DCM/TEA/TFE at 40 °C

Peptide conjugates with acridine-9-carboxylic acid at *N*-terminus were assembled on solid phase [181–183]. Acridine-9-carboxylic acid was also introduced to Lys side chain [184] by HATU coupling. The building block Fmoc-Lys(Acr-CO)-OH was used for peptide assembly. Combination of *N*-terminal acridinylation and use of the building block provided bis-acridinylated peptides.

Acridine-9-acetic acid (Ata) was used for synthesis of bradykinin conjugates with *N*-terminal modification [185].

N9-Anilinoacridines can be attached to solid support with building block acridin-9-yl-aminobenzenecarboxylic acids [186]. 3,6,9-Triaminoacridines were attached by this strategy to series of tetrapeptides [187].

1-Nitro-*N*-acridin-9-yl derivatized amino acids such as Gly, β-Ala and ε-Ahx were used for introduction of acridine moiety to *N*-terminus [188] of tuftsin analogues via TBTU/HOBt coupling. As well, the derivatives of acridone-4-carboxylic acid were coupled by the same agents.

The building of acridine systems suitable for *C*-terminal peptide modification was carried out by in-solution coupling of 9-activated acridines to corresponding amino group yielding mono acridinylated diamines [9, 11, 189–192], acridinylated aminoalcohols [193, 194] and acridinylated aminohalides [195, 196], respectively. Monoacridinylated diamine (**107**) was anchored to backbone acid labile linker (**106**) and used for syntheses of head-and-tail bis-acridinylated peptides on resin [175, 176] (Fig. 3.22). Monoacridinylated alcohols were converted to azides and used for click chemistry [194]. Click compatible building blocks were also prepared from 9-chloroacridines and propargyl amine or ethynylanilines [197].

Another approach is based on *C9* acridinylation of the side chain of basic amino acids such as Lys and Orn. Starting from free amino acid, the incorporation of acridine requires protection [198–200] of α-amino group by complexation with Cu^{2+}. Side chain acridinylation can be also carried out starting from Fmoc-Orn-OH and Fmoc-Lys-OH using 9-phenoxyacridines [201–206].

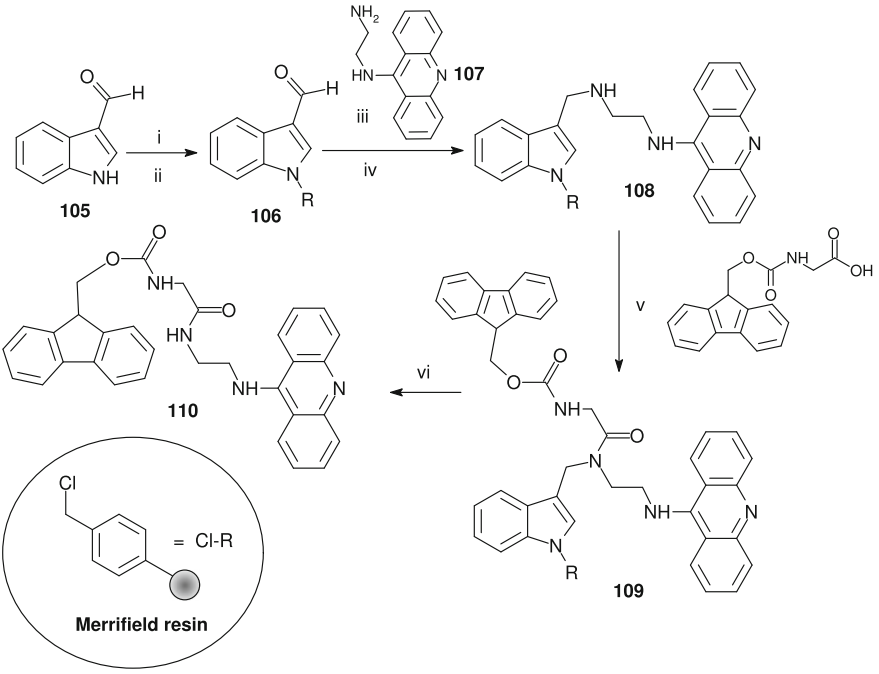

Fig. 3.22 Solid phase synthesis of *C*-acridinylated peptides [175, 176]. *i*. NaH, DMF, 0 °C. *ii*. R-Cl. *iii*. TEOF, THF. *iv*. $NaBH_4$, THF/EtOH 1:1. *v*. DIC, HOBt, DMF. *vi*.TFA/anisole/DCM 2:1:20

Very promising approach uses derivatives of 9-aminoacridine-4-carboxylic acid (**100**), which are advantageous for solid phase synthesis [18, 19, 207, 208] (Fig. 3.23). The strategy provided threading intercalator with aromatic moiety in the inner part of peptide sequence.

Noteworthy, the synthesis of chiral protected β-acridin-9-ylalanine (**118**) was carried out by Oppolzer's strategy [209] from 9-bromomethylacridine (**58**) and chiral glycinamide (**115**) (Fig. 3.24).

Another strategy for synthesis of *C*-acridonylated amino acids started from tyrosine (**119**) and led to β-(9,10*H*-acridon-2-yl)alanine (Acd, **122**) in five steps [210] (Fig. 3.25). Hydroxyl group of Boc-Tyr-OMe was converted to good leaving group OTf with Ph-N$(Tf)_2$. Subsequent Buchwald–Hartwig amination with $Pd(OAc)_2$ and racemic BINAP in the presence of base – Cs_2CO_3 – provided derivative of *N*-phenylanthranilic acid (**121**). This was cyclized with H_2SO_4 or PPA to corresponding acridone (**122**). The use of H_2SO_4 led to higher yields than that of PPA. This unnatural amino acid was incorporated into calmodulin via protein expressions carried out in DH10B cells transformed with pBad-sfGFP-150TAG and a synthetase-containing pDule vector producing UAA-sfGFP.

When acridine moiety is inserted as one ligand of transition metals and the peptide as another one, the peptide should be equipped with strongly complexing group e.g.

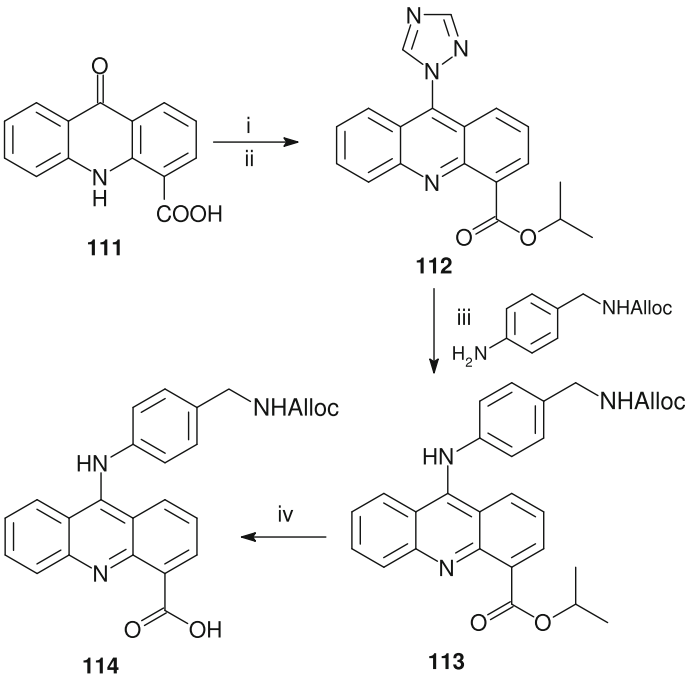

Fig. 3.23 Synthesis of an artificial acridine amino acid [18, 19, 207, 208]. *i*. CDI, THF, iPrOH. *ii*. 1,2,4-triazole, $POCl_3$, TEA, ACN. *iii*. TEA, ACN, reflux. *iv*. LiOH, H_2O, THF, r.t

with isonitrile for Tc or Re. By this approach, *N*10-alkylated proflavine was anchored via imidazole-4-carboxylic acid to Tc-bombesin complex [211].

Bi-nuclear amino acid constructs containing various drugs for conjugation to a peptide carrier were synthesized using solid phase organic synthesis (SPOS) [212]. Each branch of the platform contains different anticancer agent connected by the same or different functional group, affording discrete chemo- and bio-release profiles for each drug. This also enables "switch off/switch on" regulation of drug cytotoxicity by conjugation to the platform and to a cell targeting peptide. The generality of this approach leads to an efficient formation of drug-loaded platforms and design of favorable drug combinations/modes of linkage for consecutive conjugation to a carrier moiety for targeted cell therapy of cancer.

Acridine analogues of methyl cinnamate can add sulfhydryl group of cysteine with chiral induction leading to *(S)* enantiomer (Fig. 3.26) [213]. In future, analogues of this compound can be used as unnatural amino acid building blocks for SPPS. Furthermore, the double bond in precursor **123** opened a way for syntheses of heterocycles by means of 1,3-dipolar cycloadditions [214].

9-Aminoacridines can be connected to solid support by aromatic nucleophilic substitution. In the first approach, the resin bound amine acts as nucleophile and substitutes good leaving group of activated acridines [23]. In the second approach,

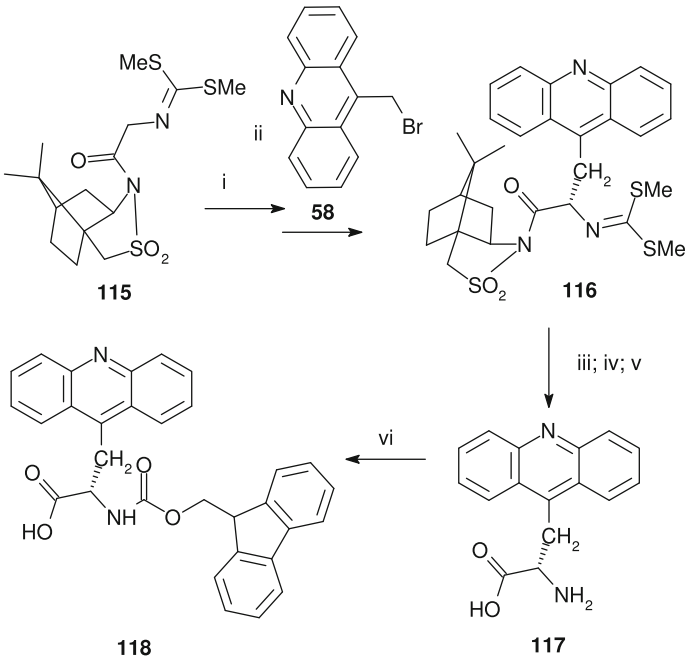

Fig. 3.24 Synthesis of acridinylated amino acids [209]. *i*. nBuLi, THF, −78 °C, Ar. *ii*. addition of acridine. *iii*. 1N HCl, THF. *iv*. NaOH. *v*. LiOH (aq), THF. *vi*. Fmoc-OSu, ACN, dixoane, H_2O

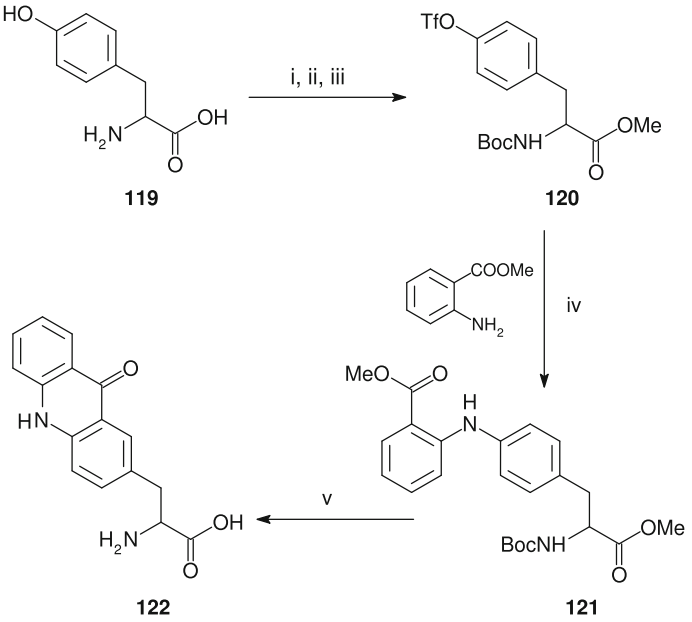

Fig. 3.25 Synthesis of amino acid with acridone in side chain [210]. *i*. $SOCl_2$/MeOH. *ii*. Boc_2O. *iii*. $PhN(Tf)_2$. *iv*. $Pd(OAc)_2$, *rac*-BINAP, Cs_2CO_3. *v*. H_2SO_4

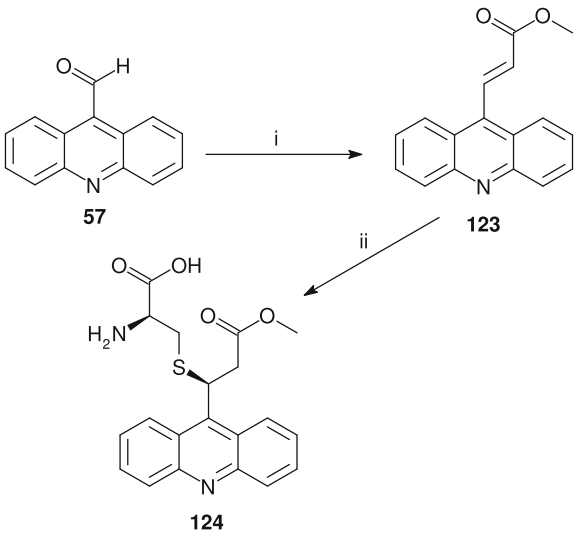

Fig. 3.26 Chiral induction during addition of cysteine to methyl cinnamate analogue [213]. *i*. $Ph_3P = CHCOOCH_3/CHCl_3$. *ii*. HCl.H-Cys-OH, Tris.HCl/MeOH

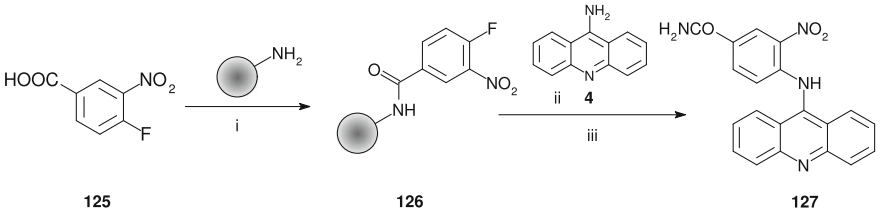

Fig. 3.27 Versatile solid phase synthesis of aminoacridines [216]. *i*. PyBOP, NMM, DMF, 40 min, r.t. *ii*. Cs_2CO_3/DMF 24 h, r.t. *iii*. TFA/H_2O/EDT

aminoacridine (**4**) serves as an amine and reacts with electron poor fluorinated benzene (**126**) (analogous to Sanger's reagents [215]) anchored to the resin [216]. In Sanger's reagent *p*-nitro moiety was substituted with carboxylate, which is also a good electron withdrawing group and can serve as anchoring point for solid phase synthesis (Fig. 3.27). The second approach is more versatile and provides broad spectrum of peptide conjugates.

3.3 Acridines as Catalysts

Acridines can serve as a platform for design of various catalytic systems using their colors for sequestration of energy from sun light and/or heterocyclic nitrogen as a part of metal coordination system.

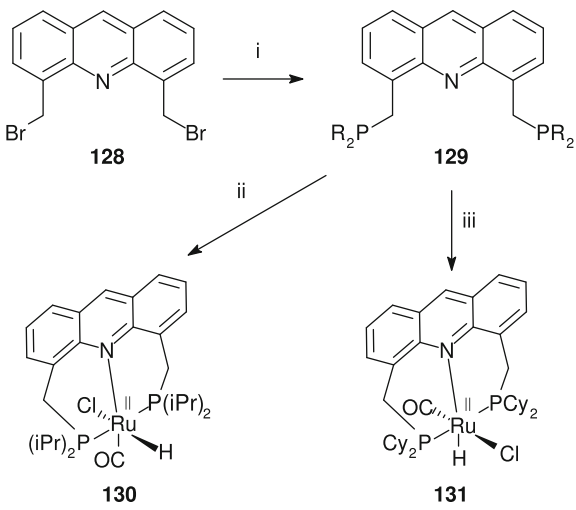

Fig. 3.28 Synthesis of acridine-based PNP-pincer Ru complexes suitable as catalysts of amination of alcohols with ammonia [217, 218]. *i*. R_2PH, MeOH, 50 °C, 48 h; *ii*. $[RuHCl(PPh_3)_3(CO)]$, THF, RT; *iii*. $[RuHCl(PPh_3)_3(CO)]$, toluene, 70 °C, 2 h

The mechanism of the amination of alcohols by a structurally altered Milstein's acridine-based PNP-pincer Ru complex [217] has been studied both experimentally and by DFT calculations [218] (Fig. 3.28). Substitution of isopropyl groups from Milstein's catalysts (**130**) with cyclohexyl groups led to improved conversions and selectivity of the catalysts (**131**). Some key Ru mediators have been prepared. The thorough analysis of many potential catalytic pathways such as with and without metal ligand cooperation, inner- and outer-sphere mechanisms was carried out. Interestingly, the most plausible pathway does not require metal ligand cooperation.

Reaction of $AgNO_3$ with a (S,N,S) pincer ligand – 4,5-bis-(phenylthiomethyl)-acridine (Phthac) – in acetonitrile for 12 h (at room temperature) and 24 h (at 90 °C) afforded complexes $[Ag(Phthac)(NO_3)(CH_3CN)]$ and $[Ag(Phthac)(NO_3)]$, respectively [219]. Phthac was prepared by the reaction of in situ generated PhS^- with 4,5-bis(bromomethyl)acridine (**128**) under inert atmosphere. These complexes were efficient catalysts for the three component coupling reaction of aldehyde, alkyne and amine at 2.0 mol% loading for $[Ag(Phthac)(NO_3)(CH_3CN)]$ and 0.5 mol% for $[Ag(Phthac)(NO_3)]$. The catalytic efficacy below 1 mol% is an exceptional activity for this reaction. Probably, $[Ag(Phthac)(NO_3)(CH_3CN)]$ belongs to the best activators of this reaction reported so far.

Acridine and acridinium-substituted complexes containing bis(terpyridine)-zinc(II) and ruthenium(II) were studied as photosensitizers for production of singlet oxygen [220]. The amount of singlet oxygen produced is better or comparable to that generated by common photocatalyst $[Ru(bpy)_3]^{2+}$.

Acridine yellow G served as photocatalyst for degradation of ferulic acid within 3 h [221].

Electrochemical properties of acridinium (Acr(+)) and flavinium (Et-Fl(+)) cations were studied [222], in order to examine their activity toward catalytic water oxidation. Cyclic voltammograms of both cations in acetonitrile are similar with

oxidation peaks at highly positive potentials. These oxidation peaks strongly depend on the type of the working electrode used. Nevertheless, both cations show different response in the presence of water. Whereas Et-Fl(+) enhanced electrocatalytic water oxidation through an electrode-assisted mechanism, the presence of Acr(+) does not speed up water oxidation. Acridines are inactive due to reaction of hydroxide ion at *C9* position, which is quite far from alkylated *N10* necessary for water oxidation.

9-Phenyl-10-methylacridinium iodide has shown catalytic wave at the reduction potential, which belongs to characteristic of the hydrogen evolution reaction [223].

The triplet excited state of AO goes through a proton-coupled electron transfer (PCET) reaction with tri-*tert*-butylphenol in acetonitrile [224]. Every reaction component has a spectroscopic fingerprint, providing a valuable chance to distinguish the individual proton transfer, electron transfer, and H-center dot-transfer feature by transient absorption spectroscopy. This allows understanding of reaction mechanism of this photocatalytic system. Finally, the photocatalytic process was used for reactivation of TEMPO catalysts. This extensive figure of excited state PCET activity describes the close coupling of light absorption with both electron and proton transfer. Possible application of this process will improve fuel production using solar energy. The role of acridine and AO in photocatalytic splitting of water was modeled theoretically [225, 226].

Interestingly, acridine red, which actually is not acridine, serves as a potent photocatalyst capable to activate C–H bond for thiolation [227]. Since it catalyzed thiolation of tetrahydrofuran and tetrahydropyran, it may influence chemistry of sugars.

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Chapter 4
Interactions of Acridines with Nucleic Acids

Abstract Acridines are moderate binders of nucleic acids due to hydrophobic interactions leading to auto-insertion between two adjacent base pairs of nucleic acids (intercalation). Use of multiple acridines in one molecule can cause polyintercalation and enhanced binding to the nucleic acids. Intercalation is widely employed for targeting of cargos to proximity of nucleic acids. Moreover, when the nucleic acid processing enzymes are inhibited by intercalated acridines, the cure of various cancer or viral diseases can be achieved. Usually, acridines preferred site rich on GC moieties at dsDNA and G4 at quadruplexes. Structural aspects of interaction with nucleic acids can help to the reader in design of specific binders, especially for targeting of quadruplexes involved in cancer.

Acridines are capable to bind and recognize nucleic acids by hydrophobic interactions namely intercalation, i.e. auto-insertion between two adjacent base pairs of nucleic acids [1–7]. This property is used not only for targeting of various species to proximity of nucleic acids but also for cancer treatment due to inhibition of various enzymes involved in processing of nucleic acids. Complexation of some acridines and nucleic acids could lead to precipitation of insoluble colloidal particles [8]. These particles can accumulate near the nucleolus. Acridines can also interact with nucleic acids in ribosomes and polysomes and cause structural dysfunction of them [8]. Polymersomes containing *N10*-alkylated AO were accumulated in the nuclei of pancreatic cancer cells more effectively than those without AO [9]. Higher concentration of reducing agents led to destabilization of polymeric vesicles.

4.1 DNA

Intercalation of positively charged acridines strongly depends on ionic strength [10, 11]. For monocationic acridine, 10-fold decrease of ionic strength led to one order of magnitude increase of binding constant. For dicationic intercalator, the same

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decrease of ionic strength provided only 3-times higher binding constant. When binding constants of differently charged compounds are compared, the influence of ionic strength has to be considered. For many acridine based intercalators, the ionic strength does not influence the unwinding angle [12]. However, there are some exceptions such as quinacrine. The 9-aminoacridine exhibits spectral properties corresponding to intercalative mode disregarding the salt concentration. At the low ionic strength, the 9-aminoacridine complexed DNA via both intercalative and the outside binding modes, where the positively charged acridine is associated randomly with the negatively charged phosphate groups of DNA [13].

For 9-aminoacridine, preferential intercalation between GC/GC base pairs was predicted by computational approach [14]. However, this was not proven experimentally for 9-aminoacridine. The closer experimental example was published for pentacyclic analogue of 9-aminoacridine with GC preference. If 9-aminoacridines are substituted, they can prefer either GC or AT base pairs [14]. The crystallographic study of complex between the conjugate Acr-Arg-Arg-Arg-Arg-OH and synthetic oligonucleotide showed intercalation into the center of duplex between the consecutive AT-base pairs. Thus, the preference of GC base pair was not corroborated [15]. On the other hand, we have shown that connection of aminoacridine with AT-binding motif led to decrease of dsDNA binding [16]. Our explanation was based on GC-acridine selectivity, which was not satisfied by AT-rich oligonucleotide. The system has been recently investigated by DFT [17, 18], which confirmed GC preference of acridine moiety. Connection of peptides with acridine unit may lead to distortion of base pair structure, which opens the phosphate group toward metal binding [19] (Fig. 4.1). 9-Aminoacridine is preferentially the minor-groove binder [20]. Some acridines can bind selectively abasic site of DNA, which can also contribute to their anticancer effect [21, 22].

A drug delivery vehicle – hydroxypropyl-β-cyclodextrin (HpβCD) – has differential binding affinity to the deprotonated and protonated forms of acridine [23]. This was used to study dye–drug transportation and pH-responsive transport to DNA. It was shown that at mildly alkaline solution (pH $\sim$ 8.5), the dye is mainly bound to the HpβCD. When the pH is reduced to a moderately acidic region (pH $\sim$ 4), the dye is efficiently released from the HpβCD cavity and nearly exclusively intercalated to

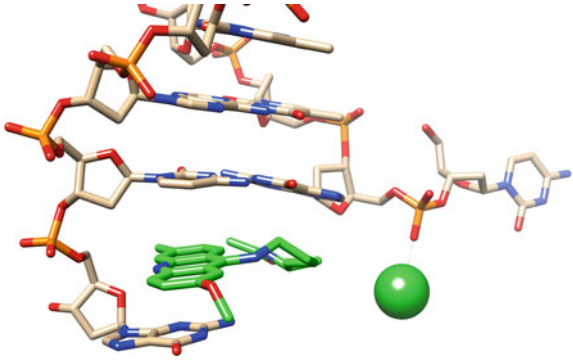


Fig. 4.1 Mono-intercalation of Ac-Lys(Qui)-Lys_4-OH opened the base pair for Ba^{2+} coordination [19], pdb id: 1XCS

DNA. It is possible to prepare a pH-sensitive supramolecular construct where pH serves as a simple impulse for controlled intake and release of the cargo.

A series of *N*-[2-(dialkylamino)alkyl]-acridine-4-carboxamides was reported to bind to DNA by intercalation [24]. Structure-activity relationships for antileukemic activity was described for the library of acridine-4-carboxamides with substituents at nearly all acridine positions. Selected compounds have also a remarkable activity against the Lewis lung solid tumor in vivo, with several analogues capable to cure nearly 100% of the advanced disease.

N-(4-aminobutyl)acridine-4-carboxamide (C_4-DACA) was synthesized by reduction of 9-oxoacridan-4-carboxylic acid to acridine-4-carboxylic acid which was transformed to C_4-DACA [25]. Data from molecular modeling studies indicated that a side chain length of four carbons in a DACA analogue was ideal to form formaldehyde-mediated drug–DNA complexes via the minor groove. An in vitro transcription assay has shown that formaldehyde-mediated C_4-DACA–DNA complexes are exclusively formed at CpG and CpA dinucleotide sequences. This fact is remarkably similar to that of formaldehyde-activated anthracenediones such as e.g. pixantrone.

For certain distance of two acridines in one molecule, called critical distance, the binder inserts both acridine units into DNA. These bis-intercalators have binding constant greater than $10^8 - 10^9\ M^{-1}$, and they can have also different selectivity in comparison with the parent acridine unit [26, 27]. The critical distance of two acridines is between 8.8 and 11.3 Å. The substituents of acridine ring at positions 2, 3 and 6 limit bis-intercalation to compounds capable of sandwiching of at least two base pairs in the complex. Unsubstituted bis-aminoacridines and acridines with 4-ethyl group could bis-intercalate with shorter linkers [28]. Enhanced binding constants of bis-intercalators is paid off by lower selectivity between AT and GC binding. Binding constant of [Lys(Acr)-Lys-$NHCH_2$-$CH_2CH_2CH_2$]$_2$ for DNA is similar to that of 9-aminoacridine itself [29], because the bis-acridine compound uses the mono-intercalative mode. In this case, the distance between acridine residues is 13 Å, i.e. longer than the critical distance for bis-intercalation. However, mono-intercalative mode was forced by a steric hindrance formed due to initial interaction of lysine cations with phosphate groups of DNA backbone. Due to rapid amide hydrolysis, many linkers of bis-intercalators with amide bonds led to lost of biological activity in L1210 cell line [30]. However, certain amides are resistant toward the hydrolysis e.g. the bis-intercalators with amide group in proximity to 1,4-piperazinediyl moiety belong to most potent inhibitors of L1210 cell proliferation [30]. Thus, nobody can exclude ad hoc the amide group from the linker of bis-intercalator design for in vivo assays [31]. Library based approach combined with structural data presented that tiny changes of bis-intercalating species can alter sequence specificity and that design of small ligands for targeting long DNA sequences in a selective manner is possible [32]. Theoretical analysis of bis-intercalative binding was published using distance geometry methods [33, 34]. Extensive study comparing several ways of enhancement of peptide–DNA binding by conjugation with one or two acridine units were published [35]. The computer assisted design in combination with combinatorial screening was used for tuning of intercalative binding of peptides [36]. Due to large

structural constrains for DNA binding of the tris-acridine, the trial of tris-intercalator design did not provide reasonable improvement of DNA binding constant in comparison with bis-acridine compound. The nature of the linking chain is crucial for a construction of high-affinity DNA polyintercalating agents [37, 38]. Empirically, the polyintercalators can be prepared and used for gene delivery [39].

The interaction of peptidic derivatives related to the antileukemic drug *m*-amsacrine and either the tetrapeptide SPKK (a nucleic acid-binding unit) or the octapeptide SPKKSPKK with DNA has been studied [40, 41]. Binding data are consistent with a model in which the acridine nucleus occupies an intercalation site and the tetrapeptidic or octapeptidic portion is located in the DNA minor groove. However, the tetrapeptide derivative fully intercalates into DNA and is therefore a 2.8 fold better inhibitor of DNA synthesis than the octapeptide one. DNA-binding sites of the conjugate are restricted to a few alternating AT-sequences proximal to GC-rich regions. Both drugs are weakly cytotoxic.

The same peptide structures – SPKK and SPKKSPKK - containing acridinyl chromophore were studied for their interaction with DNA minor groove by a combination of biochemical, hydrodynamic, and spectroscopic methods including diffusion-enhanced luminescence energy transfer measurements [42]. The acridinyl chromophore was much more accessible than that of a simple mono-intercalating drug such as AO or ethidium. The accessibility factor was increasing with the peptide length, reflecting the extent of perturbation imposed upon the intercalating chromophore by the binding to DNA in the hybrids. It was postulated that minimum of two SPKK motifs is required to mimic the AT-specific minor groove binding antibiotic netropsin and DNA-binding models were suggested, in which the acridinyl moiety of the tetrapeptide derivative protrudes slightly outside the double helix but remains more or less parallel to the plane of the base pairs. In the octapeptide, the chromophore lies only partially overlapped with the base pairs in the intercalation site and, in addition, the heterocyclic chromophore is significantly shifted with respect to the double helix axis.

A set of 3,6-bis(3-alkylguanidino)acridines was synthesized and its interactions with calf thymus DNA were studied using DNA melting techniques, UV-Vis, fluorescence and CD spectroscopy [43]. The binding constants K ranged from 1.25×10^5 to 5.26×10^5 M^{-1}, and the percentage of hypochromism was found to be 17–42%. UV-Vis, fluorescence and CD measurements showed that the compounds are effective DNA intercalating agents. Electrophoretic analyses showed that ligands influenced topoisomerase I at 60 μM concentration. From the compounds tested, only those with longer alkyl chains penetrated cellular membranes and suppressed cell proliferation efficiently. The biological profile of novel compounds was evaluated using various methods such as caspase-3 activation, cell cycle distribution, changes in mitochondrial membrane potential, and phosphatidylserine externalization. The assays revealed that cytostatic actions of the (acridine-3,6-diyldiimino)bis[(ethylamino)-methaniminium]dichloride tetrahydrate and (acridine-3,6-diyldiimino)bis[(*n*-pentylamino)methaniminium]dichloride tetrahydrate derivatives were mostly transient. The most cytotoxic was (acridine-3,6-diyldiimino)bis[(*n*-hexylamino)methaniminium]dichloride tetrahydrate. Different schemes of cell penetration were also noticed

for individual derivatives. Molecular dynamics was applied to study DNA-ligand interactions at molecular level. Generally, 3,6-bis(3-alkylguanidine)acridines were better DNA binders than structurally similar 3,6-bis(3-alkylurea)acridines. This is probably caused by presence of positively charged guanidinium which might increase electrostatic attractive forces between guanidine derivatives and DNA.

During reaction of quinacrine with glutathione in the body, an acridine-peptide conjugate was formed, which bound dsDNA 4.5 times more stronger than parent aminoacridine [44].

4.2 DNA Quadruplexes

Quadruplexes are secondary structures of nucleic acids organized into four-strands where guanine bases are stacked in planar tetrads stabilized by cation coordination and hydrogen bonds [45]. Many quadruplexes are part of telomeric repeats of DNA used for regulation of cell life. Available data regarding the therapeutic strategies suggested thus far and the current perspectives for the progress of innovative telomere-based therapeutic strategies with broad-spectrum anticancer activity and for their clinical applications were reviewed [46–49].

Acridines belong to one of pharmacophores used for targeting of telomeric DNA [50].

Anticancer drugs based on the trisubstituted acridine derivatives at the 2,6,9; 2,7,9; and 3,6,9 positions of acridine rings were evaluated for telomerase-inhibitory and quadruplex DNA binding properties [51]. The study revealed that the 3,6,9-trisubstituted derivatives are the most active of the three regioisomers examined. This behavior can be explained on the basis of variation in predicted binding to the intramolecular human quadruplex structure.

A series of trisubstituted acridines was prepared with modifications at positions 3 and 6 by thiourea, urea and guanidines, respectively [52]. The aim was to mimic the effects of BRACO19 (**9**) [53, 54]. The cellular activity of BRACO19 can be ascribed both to the uncapping of 3' telomere ends and to telomere shortening that may preferentially affect cells with short telomeres [54]. All of the tested derivatives stabilized human telomeric DNA quadruplexes. The data obtained show that all of the derivatives were capable to reorganize the single-stranded DNA sequences into antiparallel G-quadruplex structures. Thiourea derivatives provided the most active compounds. As revealed by cell cycle analysis, the cells were arrested in the S and G2/M-phases of the cell cycle during the first 72 h.

Trisubstituted acridine-peptide conjugates can serve for binding and recognition of various DNA quadruplexes including the human telomere, and the c-kit and N-ras proto-oncogenes [55]. According to surface plasmon resonance, all acridine conjugates bound to the human telomeric quadruplex with submicromolar affinity. The more selective towards the N-ras quadruplex were compounds capable of simultaneous binding of acridine and peptide units. The ligand **132** (Fig. 4.2) bound N-ras quadruplex with ca 4 nM dissociation constant, whereas for binding of the other quadruplexes the dissociation constants were in the range from 22–50 nM.

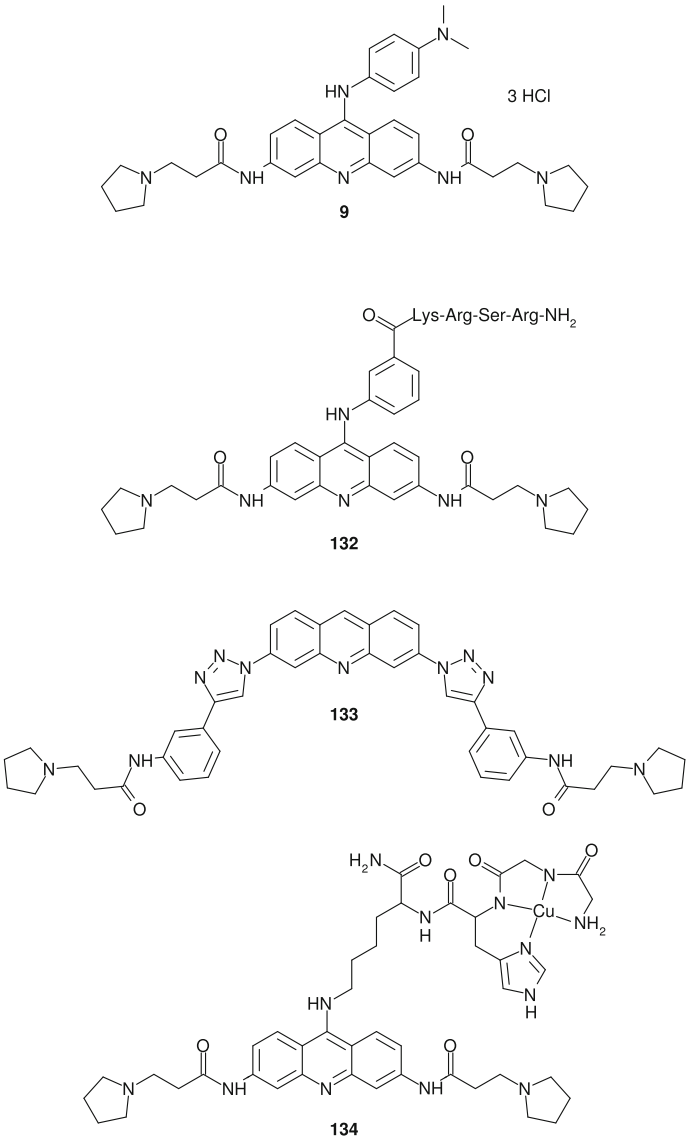


Fig. 4.2 Quadruplex binders. BRACO19 (**9**) widely-used inhibitor of telomerase with EC_{50} 60 nM [56]. The selective binder (**132**) of oncogenic N-ras quadruplex with K_D 4 nM [55]. The selective inhibitor (**133**) of human telomerase [57]. The artificial metallophosphatase (**134**) for cleavage of G quadruplex of telomeric DNA [58]

Improved inhibition of telomerase and hPot1 was achieved, when two units of BRACO19 analogues were linked together with diamine linker at position 9 and 9' [59].

When BRACO19 acridine scaffold (G-quadruplex binder) was conjugated with geldanamycin (Hsp90 binder) using click chemistry, potent inhibitors of telomerase were prepared [60] with activity similar to that of BRACO19. Interestingly, the conjugates can inhibit imatinib-resistant gastrointestinal cancer, which is resistant towards BRACO19. Thus, the mode of action of the conjugates has to be evaluated.

The preparation of an acridine-containing cyanine dye was described based on BRACO19 structure. The compound can serve as a pH-responsive colorimetric indicator and fluorescent probe [61]. This indicator binds firmly to DNA quadruplexes of oncogene promoter region like the parent structure.

Based on rational design a series of disubstituted triazole linked acridine compounds with selectivity for human telomeric quadruplex DNAs was prepared [57]. Following the design, a library of quadruplex binders was synthesized by click chemistry. Selected compound **133** (Fig. 4.2) had low affinity to the c-kit quadruplexes and dsDNA structures. This compound is not only powerful and selective binder of human telomeric quadruplex DNA but also selective inhibitor of the proliferation of cancer cell lines. Selectivity depends on the separation of the two alkylamino terminal groups and on the dimensions of the triazole-acridine compounds. Competition experiments confirmed very low binding to duplex DNA even at high duplex:telomeric quadruplex ratios. This anticancer effect was achieved by inhibition of telomerase enzyme, which is selectively expressed in cancer cells as a tool for telomere integrity and cellular immortalization.

A group of 9(*10H*)-acridone derivatives having terminal ammonium substituents at C2 (and C7) position(s) of the acridone ring was synthesized [62] and their relative affinities to G-quadruplex DNA have been tested. These compounds had a binding specificity for G-quadruplex over duplex sequences. In comparison with corresponding acridone derivatives with only one terminal ammonium substituent, the acridones having two terminal ammonium substituents were much more effective on the human telomeric G-quadruplex DNA. It seems, that more positive charges introduced to the side chains lead to improvement of the formation and stabilization of the G-quadruplex.

By utilization of a catalytic metallodrug approach, the copper–acridine–ATCUN complex (CuGGHK-Acr, **134**) has been proposed [58] that aims to G-quadruplex telomeric DNA (Fig. 4.2). Both gel sequencing and fluorescence solution assays show that the CuGGHK-Acr compound selectively interacts with the G-quadruplex telomere sequence. Moreover, the compound can catalytically cleave the recognized quadruplex. The CuGGHK-Acr system inhibited cancer cell proliferation and reduced the telomere length. Both apoptosis and senescence are evoked in the breast cancer cell line MCF7.

An electrochemical DNA biosensor was used to study redox mechanisms and in situ electrochemical interactions of two disubstituted triazole-linked acridine compounds [63] which were previously reported as quadruplex DNA-binding molecules [57]. It was shown that the binding of compounds to dsDNA is time-dependent and it caused condensation of dsDNA morphological structure. The voltammetry can be combined with AFM and the quadruplexes could be visualized as spherical or more complex aggregates [64].

4.3 RNA

Many biological pathways including the trafficking, editing, and maturation of cellular RNA, the interferon antiviral response, and RNA interference are key processes where recognition of double-stranded RNA is involved. The acridine-peptide conjugates play a role of artificial selectors capable to distinguish between dsRNA and dsDNA. Perhaps, they can lead to selective antiviral, anticancer, antimicrobial and others drugs. Tethered acridine peptide intercalators were reviewed by Beal's team [65]. Another review presents the role of 9-aminoacridine conjugates as small molecule ligands for RNA recognition [66]. Via interactions of acridines with mitochondrial RNA, they may also inhibit mitochondrial protein synthesis [67].

A series of tethered acridine peptide conjugates derivatized from the synthon **114** (Alloc-AAAcr-OH) (Fig. 3.23) revealed how a point of attachment onto solid support influenced RNA interactions. Most importantly, the resin or linker can completely suppress RNA-conjugate interaction. In order to prevent these false negative results, two similar libraries have to be prepared where peptides are attached on their both termini; and more than one strategy for screening of interactions between resin bound peptides and biomolecules is required [68]. The starting acridone **111** was also applied for syntheses of *N*-terminus substituted peptides, which can influence cancer processes [69].

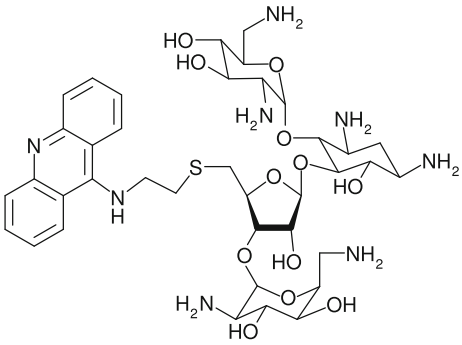
The ability of a library of structurally diverse acridine-peptide conjugates to target a complex formed between the dsRNA binding domain of RNA-dependent protein kinase and a viral RNA inhibitor was evaluated [70]. Selected compounds were able to inhibit the protein-RNA complex formation with an IC_{50} value of $10 \pm 5\,\mu M$.

The RNA specific for acridine conjugate H-Ser-Val-AAAcr-Arg-NH_2 was selected by SELEX assay. The binder selectively recognizes site 5'-CpG-3' in specific RNA loop surrounding without interference with DNA. Several viral RNA sequences that contain this site were identified [71].

Some of acridine peptide conjugates inhibit peptidyl transferase due to their interactions with RNA. However, the clear rules for rational design of a sequence, which distinguishes between RNA and DNA recognition, or even which inhibits peptidyl transferase, are not known [72].

Aminoacridine–glycoside conjugates were evaluated for potency against HIV-1 virus. The longer distance between acridine ring and aminoglycoside, the higher affinity for DNA was determined. This was explained as better availability of acridine moiety to DNA. As the most selective *au*-RNA binder, neo-*S*-acridine (**135**, Fig. 4.3) was found. The compounds with shorter linker are not capable of cooperative binding of aminoglycoside and acridine moiety. The size of linker can alter the selectivity for Rev-responsible-element of viral RNA and DNA [73].

Acridine scan on α-helical peptide LKKLLKLLKKLLKLKG was carried out with the aim to design selective and powerful binders of HIV-1 RRE and TAR [74]. The acridinylation on the second amino acid provided higher selectivity toward HIV-1 RRE than toward tRNA. On the other hand, acridinylation on the ninth position led to selective discrimination of TAR in contrast to tRNA. Bis-acridinylation on



135

Fig. 4.3 Neo-*S*-acridine — powerful inhibitor of Rev binding to Rev responsible element of HIV-1 RNA. This interaction causes arrest of HIV-1 replication

both positions provided subnanomolar dissociation constant, while offering 8-fold selectivity for HIV-1 RRE and TAR in comparison with tRNA. The most powerful binder was tris-acridinylated peptide at positions 2, 9, and 10; however, it lost the selectivity for HIV-1 RRE and TAR and bound the tRNA with almost the same efficacy.

High throughput screening of branched peptide library with 4096 members containing acridine and boronic acid units showed that dendrimers inhibited binding of HIV-1 RRE to RNA with low nanomolar dissociation constants [75]: for branched peptide boronic acids [H-Lys-Lys-Lys]$_2$-Lys-Lys(Acr)-Lys(Acr)-Lys-Tyr-OH, [H-Lys(Acr)-Lys-Lys]$_2$-Lys-Lys-Leu-Lys(Bba)-Tyr-OH, [H-Lys-Lys(Acr)-Lys]$_2$-Lys-Lys-Leu-Lys(Bba)-Tyr-OH electrophoretic mobility shift assay has shown dissociation constants 30, 60, and 70 nM, respectively. These peptides suppressed the generation of p24 (an HIV-1 capsid protein) with IC_{50} 10.2, 10.1, and 7.3 μM, respectively.

For treatment of viral infections, small molecule inhibitors of virus replication are used. Quinacrine influenced many steps of RNA virus infection such as binding of the p33 replication protein to the viral RNA template, recruitment of viral RNA for replication, reduction of minus-strand synthesis by the Tombusvirus replicase, and translation of the uncapped TBSV genomic RNA [76].

Combining Arg-rich peptides containing 11 and 22 amino acids and acridine intercalators provided enhanced RNA binder toward the bacteriophage λ N peptide-box B [77]. The peptide sequences were taken from RNA-binding domain of N protein. Introduction of acridine moiety increased the RNA binding affinity 80 times compared to the parent peptide. Moreover, 25-fold increase of specificity toward cognate RNA versus the noncognate one was observed. Thus, acridine-peptide conjugation led to successful design of specific and powerful RNA binding system.

NMR structure of proflavine intercalated into RNA duplex of GCCGCGGC sequence linked via 2', 5' atoms was described [78]. Comparison of two types of intercalated RNA duplexes, i.e. 2', 5' and 3', 5' linked, revealed distinct sugar pucker geometries, whereas the inter-phosphate distances were more or less constant. The phosphate backbone in both structures adopts trans-conformation whereas native

states prefer gauche-conformation. This can explain a probable contribution of the backbone to the origin of the nearest-neighbor exclusion principle.

Mode of binding of small molecules to DNAs and RNAs was studied by use of UV-Vis spectrometry. UV-Vis spectrometries are simple techniques that can give basic information on the manner of binding of small molecules (dyes, drugs) to DNA and RNA helices [79]. The spectral characteristics and interactions of three families of dyes with polynucleotides were reviewed. Each class represents one of the major binding modes: acridines for intercalation, Hoechst dyes for groove binding, and porphyrins for external dye stacking. Scope and limitation of the given techniques were discussed.

RNA molecules with acridine or quindoline residues at their 3'- and 5'-termini were synthesized [80]. These siRNA duplexes were tested for inhibition of the tumor necrosis factor. They are as powerful inhibitors as unaltered RNA duplexes in HeLa cells. The fluorescent features of acridines enable direct observation of the cytoplasmic distribution of modified siRNA within the cells. A clear cytoplasmic localization of siRNAs modified with acridine-containing moieties was proven in HeLa cells.

4.4 PNA

In contrast to common nucleic acids, peptide nucleic acids (PNA) are stable in living systems due to the lack of specific enzymes for their cleavage. This stability makes PNAs ideal tools for anti-sense therapy. Fluorescence of PNA-9-aminoacridine conjugate was influenced by hair-pin formation and/or unwinding [81]. 9-Aminoacridines increased selectivity of PNA–DNA binding [82]. The binding proceeded through invasive triplex formation. An excited singlet state of acridines can lead to oxidation of guanidines in PNA [83], which is similar to B-DNA. The recognition of DNA by PNAs was extensively reviewed [84, 85].

Conformationally preorganized γ-peptide nucleic acid (γ-PNA) with *(S)*-γ-methyl group containing one ε-acridin-9-yl-lysine at the *C*-terminus invaded B-DNA in a sequence-specific manner [86]. Recognition was carried out by Watson–Crick base pairing. Thus, the same principles that govern the recognition of single-stranded DNA and RNA are valid for recognition of double-stranded B-DNA. The process of invasion occurred both at physiological and elevated temperatures within 30 min. The role of the acridine unit is not fully understood; most probably, the acridine stabilized Y-junction of the melted region by intercalation.

5-Aminouracil (5-AU) is an easily available yet rarely starting material for the preparation of labeled nucleobases. The amino group of 5-AU was conjugated with 9-chloroacridine and potential of the conjugate was investigated as a base-discriminating fluorophore [87]. This conjugation proceeds smoothly at 120 °C. The fluorescent nucleobase served as a monomer usable for incorporation into PNA by Fmoc chemistry. Despite that the monomer was stable in organic solvents, its oligomers were thermally unstable and hydrolyzed partly to acridone in neutral aqueous solution during hybridization to cDNA.

4.5 Acridine Metal Complexes

Binding of 9-aminoacridines with nucleic acids was used for anchoring of Cu^{2+} metal complexes in proximity to chiral DNA [88]. These complexes were active catalysts for enantioselective Diels–Alder reaction. Application of this type of catalysis is partly covered by a review [89]. Intercalative role of metal complexes was also reviewed [90, 91].

Acridine conjugates mimicking cisplatin were presented as potential anti-neoplastic agents [49, 92–101]. Due to acridine systems, these conjugates can have different mode of action than cisplatin e.g. intercalation of tethered acridine derivatives with a simultaneous platination of nucleobase nitrogen [99]. Thus, they can serve as complementary therapeutics. Analogue $[Pt(en)(ACRAMTU)_2](NO_3)_4$ with ACRAMTU (1-[2-(acridine-9-yl-amino)ethyl]-1,3-dimethylthiourea) ligand can bis-intercalate with cooperative binding of Pt to minor groove. By binding to TATA-containing restriction site, the complex prevented endonucleolytic DNA cleavage [100, 102]. Pt-ACRAMTU derivatives are efficient inhibitors of leukemia (HL-60) and lung cancer (H460) cells growth [103].

Platinum–acridine hybrid complexes show low-nanomolar activity against chemoresistant non-small cell lung cancer (NSCLC); however, high systemic toxicity in vivo [104]. With the aim to decrease the promiscuous genotoxicity of these compounds and improve their pharmacological profile a "modular build–click–screen approach" was applied to test a library of twenty hybrid compounds comprising truncated and extended chromophores of variable basicities. Representative compounds were resynthesized and evaluated in five NSCLC cell lines. 7-Aminobenz[c]acridine was identified as a perspective scaffold with submicromolar efficacy in many of the DNA-repair capable and p53-mutant cancer models with enhanced tolerability in mice. It was 32-fold more active than the original platinum–acridine conjugate.

Platinum–acridine hybrid agents comprising carboxylic acid ester groups were described [105]. The most active analogs had up to 6-times higher activity in ovarian cancer (OVCAR-3) and breast cancer (MCF-7, MDA-MB-231) cell lines than cisplatin. Inhibition of cell proliferation at nanomolar concentrations was noticed in pancreatic (PANC-1) and non-small cell lung cancer cells (NSCLC, NCI-H460). Insertion of the ester groups did not changed the cytotoxic properties of the hybrids, which provide the same intercalative DNA adducts as the parent compounds. The ester groups underwent platinum-catalyzed hydrolysis in a chloride concentration-dependent manner to generate chelating carboxylate. The chloride-sensitive ester hydrolysis can be used as a self-immolative release mechanism for tumor-selective delivery of platinum acridines.

To boost the pharmacological character of the anticancer drug cisplatin, a series of new acridine-based tethered (ethane-1,2-diamine)platinum(II) complexes joined by a polymethylene chain was synthesized [106]. The compounds with hexamethylene chain with/without Pt were more potent than cisplatin and oxaliplatin in killing of cancer cells such as human colon carcinoma (HCT116), human colon adenocarci-

noma (SW480) and human colon carcinoma (HT29). Interestingly, the compound is ca 4 times more active without Pt metal than with it.

Acridine carboxamide Pt complexes and their potential as anticancer agents was reviewed [107].

Recently, 1-acridin-9-yl-3-methylthiourea complexes with Au(I) have been studied as DNA intercalators [108]. These complexes with structures $[Au(ACRTU)_2]Cl$ and $[Au(ACRTU)(PPh_3)]PF_6$ were highly toxic to human ovarian carcinoma cisplatin-sensitive A2780 cell line, possessing sub-micromolar IC_{50}. They were also active toward different phenotypes of breast cancer cell lines MDA-MB-231, SK-BR-3, and MCF-7. These complexes caused apoptosis via activation of caspase-3. Despite inhibition of thiol-containing enzymes is usually the main mechanism of cytotoxicity of gold complexes, these gold complexes shown a DNA-dependent mechanisms. They accumulated in cell nucleus, intercalated DNA, and inhibited topoisomerase I mediated DNA unwinding. Moreover, these complexes shown strong antiangiogenic effects, and prevented vasculogenic mimicry of highly invasive MDA-MB-231 cells.

Another field of the metal complexes is design of artificial nucleases. The acridine moiety serves as a targeting species, which brings the catalytically active transition metal complex to proximity of scised nucleic acids [109–111]. As catalytically active metals can be Cu(II) [110, 112, 113], Lu(III) [114, 115], Fe(II) [116–119], and Fe(III) [120]. The artificial nucleases can cut both DNA or RNA. Some of the nucleases are specific for certain region of DNA or RNA. There are also artificial nucleases without the metal [121–123] using the catalytic site of peptides [124–126] or photochemical cleavage caused by acridine itself [123].

Complexes of *N*,*N'*-bis(salicylidene)-9-(3,4-diaminophenyl)acridine with Ni(II)/Ni(III) and their interactions with DNA were studied by cyclic voltammetry and various spectroscopic and viscosimetric measurements [127]. The data revealed that complexes at both oxidation states bind to CT-DNA by intercalative mode. The intercalative mode of binding to the model duplex $d(CGCAATTGCG)_2$ possibly from the major groove with a slight preference for GC rich region was supported also by molecular-modeling studies. The apparent binding constants of both complexes are roughly $10^5\ M^{-1}$. Moreover, the complexes support the cleavage of plasmid pBR322 DNA during irradiation in the presence of oxone.

A sequence-specific DNA-cleaving molecule which conjugated a Cu(II)-chelating peptide, a netropsin residue, and an acridine chromophore was checked for binding to the minor groove of DNA, just like netropsin [112]. The DNA-binding configurations of these two moieties were found to be perfectly compatible due to synergistic effects of their components. In the presence of copper ions the conjugate GHK-NetGA induced site-specific cleavage of duplex DNA.

Radioactive ^{99m}Tc complexes with AO derivative were used for imaging of prostate cancer cells PC3 [128]. Bombesin like peptide was used for selective recognition of GRP receptor. The radionuclide was suitable for single-photon emission tomography, whereas the AO for fluorescence imaging. AO also targeted this probe to cell nucleus. When ^{188}Re is used, the probe can be also suitable for positron emission tomography.

Another conjugation of bombesin, AO, and Re or ^{99m}Tc was achieved [129] via imidazole as a linker between peptide and acridine moiety. This offered a way for selective delivery of radiochemicals to nucleus. Metal chelator can be also part of artificial amino acids connecting peptide with acridine label [130]. Acridinium isonitrile was also constructed for this purpose [131].

Bis(acridine-9-carboxylate)-nitro-europium(III) dihydrate complex – [Eu(Acr-COO)$_2$(NO$_3$)]·2H$_2$O – was prepared [132]. Its in vivo antiangiogenic effects against Ehrlich ascites carcinoma (EAC) were studied. The complex inhibited proliferation of EAC cells and ascites formation. The antitumor effect took place through antiangiogenic activity by the decrease of microvessel density in EAC solid tumors. The mediation of antiangiogenic effect proceeds through down-regulation of VEGF receptor type-2 (Flk-1). The complex also significantly increased the level of caspase-3 in laboratory animals in comparison with the acridine ligand and to the control group. The data are consistent with the DNA cleavage confirming induced apoptosis by the complex. The interaction of CT-DNA with this complex was studied by ethidium bromide competitive assay. Interaction mode with tyrosine kinases was described by molecular docking.

Complexes of Ru(II) bipyridyl analogues with linked acridine units through peptide linker allowed triplet–triplet energy transfer via biexponential luminescence decay [133].

4.6 Structural Aspects of Nucleic Acid Binding

According to analysis of PDB structures deposited in RCSB databank (August 2015), acridines bind mostly between GC-base pairs of dsDNA, or between G and C bases of ssDNA, or above planar potassium guanine quartet motif of DNA quadruplexes.

Binding to ssDNA is almost exclusively accompanied with recognition of G or C terminal nucleobase both from outside and from inside of oligonucleotide (e.g. pdb id: 1KCI [134]) with one exception where only intercalation was observed (pdb id: 2GJB) (Fig. 4.4).

In the case of dsDNA, the binding of acridines to terminal GC-base pair can be achieved with maintaining the double helix geometry (pdb id: 2GB9 [135]) or mostly the acridine binding lead to unwinding of double helix and one partner from the terminal base pair is repelled (pdb id: 1FD5 [136]). If the oligonucleotides without terminal GC-pairs are used, the intercalation can take place in the middle of the sequence, which can lead to unwinding of a half of the helix (pdb id: 2GWA [137]) (Fig. 4.5). This is used for recognition of DNA Holliday junctions by bis-acridines [137].

Proflavine can interact with d(CGCG) without insertion between nucleobases, it rather generated parallel structure alongside dsDNA helix (pdb id: 1VTF [138]). On the other hand, its tetramethylated analogue – AO – is intercalated to dsRNA d(GC) (ndb id: DRBB01 [139]) (Fig. 4.6).

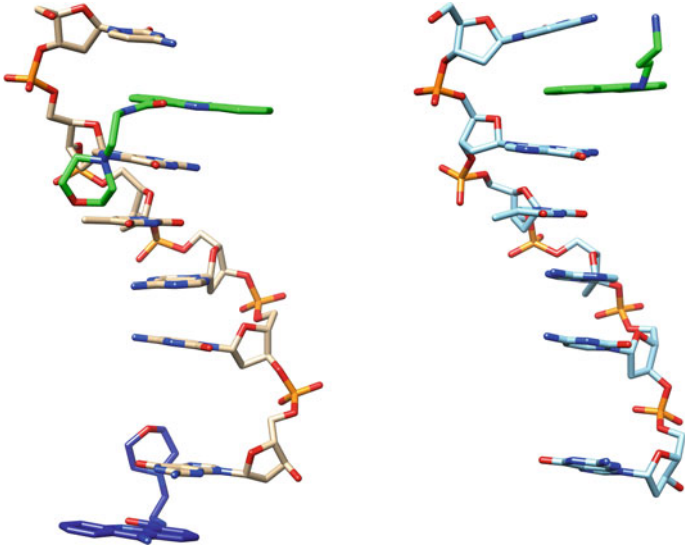


Fig. 4.4 Acridines can intercalate into ssDNA between G and C-bases (*green mode*). They can also stack to terminal G residue (*blue mode*). Image was created by program USCF chimera from pdb structures: 1KCI and 2GJB, respectively

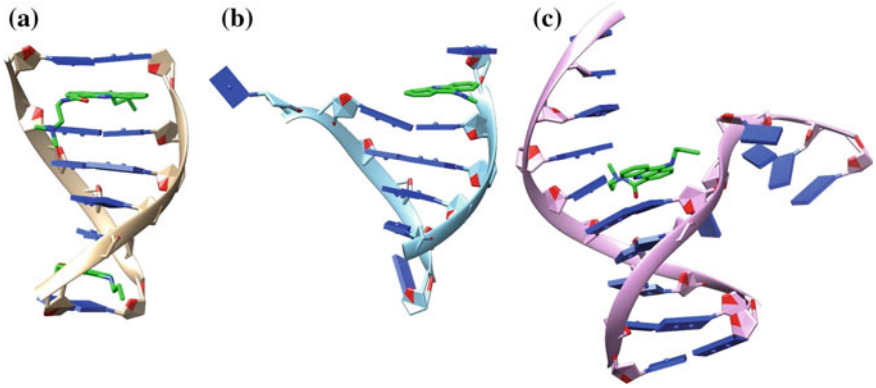


Fig. 4.5 Interaction of acridines with short dsDNAs can lead to **a** intercalation between two base pairs, **b** intercalation between base pair and base, and **c** stacking to GC-base pair and unwinding of remaining oligonucleotide. Image was created by program USCF chimera from pdb structures: 2GB9, 1FD5, and 2GWA, respectively

In two cases, complexes 1G3X [15] and 1XRW [98], the acridine derivative intercalates between the middle base pairs of the dsDNA oligonucleotide (Fig. 4.7a and b, respectively). In the first case, unexceptionally, the intercalation occurred between AT-base pairs. Intercalative binding to the middle of dsRNA between GC-base pairs was also observed for 2KD4 complex [78] (Fig. 4.7c).

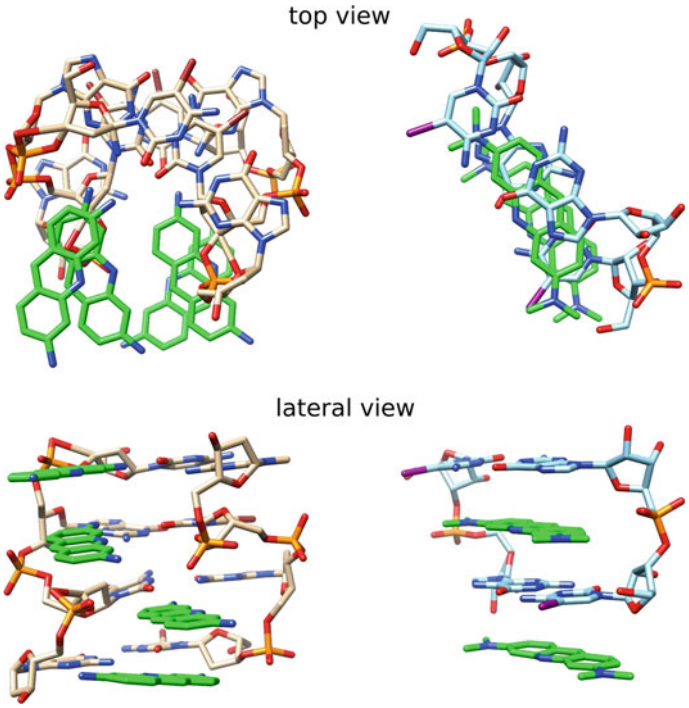


Fig. 4.6 Acridines can bind also outside of dsDNA forming self stack (*left*) as an alternative to common intercalation to dsRNA (*right*). Image was created by program USCF chimera from pdb structure 1VTF and ndb structure DRBB01, respectively

Derivatives of proflavine, lacking substituent at position 9, mostly bind DNA quadruplexes by stacking over planar quartets of guanine bases which are stabilized by cation coordination and hydrogen bonds (Fig. 4.8). Acylation of amino groups provided carbonyl residue which conservatively accepts hydrogen bond from H^3 of thymine. Another thymine serves as a lid covering acridine ring from upper side.

When a substituent is introduced at position 9 of acridine, the clashes between the acridine ligand and nucleotide backbone did not allow use of the same binding mode (Fig. 4.9). Thus, different secondary structure of quadruplex is selected by the ligand. Interestingly, again the acridine ring is above guanine quartet and has lid from thymine residue; however, the hydrogen bond is formed between carbonyl at position 4 of another thymine with amino group of proflavine scaffold.

Human telomeric DNA is transcribed into noncoding RNA sequences, called TERRAs. TERRAs can play roles in the regulation of telomere function and can form higher-order quadruplex structures. Thus, TERRAs can be the target of therapeutic intervention. The crystal structure of a TERRA quadruplex-acridine complex was reported and differed significantly from the structure of the analogous DNA quadruplex complexes [144]. The RNA complex possesses a parallel-stranded topol-

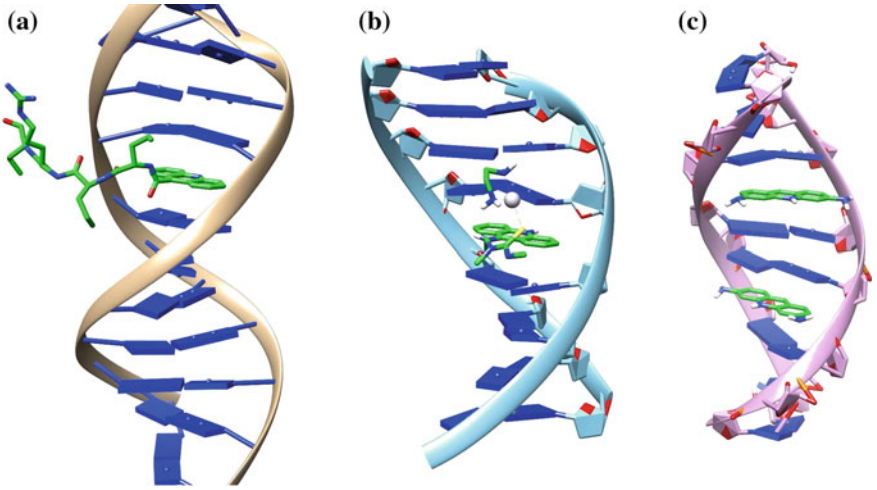


Fig. 4.7 Intercalative binding modes of various acridines into double stranded nucleic acids. **a** Intercalative binding of acridin-9-ylcarbonyl-tetraarginine between AT-base pairs of dsDNA; **b** Intercalative binding of acridine ligand from Pt-ACRAMTU between GC-base pairs of dsDNA combined with Pt binding to N7 of guanine; **c** Intercalative binding of proflavines between GC-base pairs of dsRNA. Image was created by program USCF chimera from pdb structures: 1G3X, 1XRW, and 2KD4, respectively

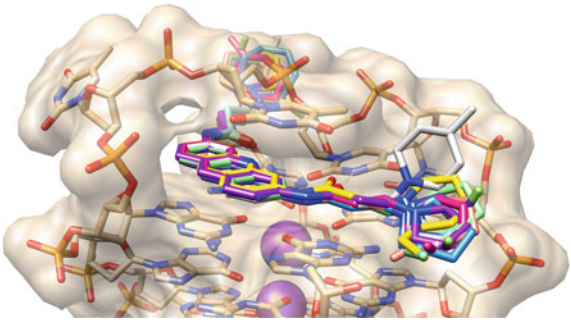


Fig. 4.8 Binding mode of proflavine derivatives with hydrogen at position 9 into DNA quadruplexes. Image was created by program USCF chimera from pdb structures: 1L1H, 3ERU, 3EUM, 3ES0, 3NYP, 3EM2, 3ET8, 3NZ7, 3EQW, and 3EUI [140–142]

ogy with propeller-like UUA loops. These loops play an active role in binding of the acridines to the RNA quadruplex. In contrast to similar DNA quadruplex complex with simpler 1:1 acridine binding, without loop involvement. Significant loop conformational changes were observed in the RNA quadruplex in comparison with the native TERRA quadruplex [145]. This has implications for the design of molecules targeting TERRA quadruplexes, and RNA quadruplexes more generally.

4-Aminoprolines can be used for construction of acridine containing dendrimers [146]. Quindoline oligomers based on 4-aminoproline were more selective G-quadruplex binders than corresponding acridine analogues [147].

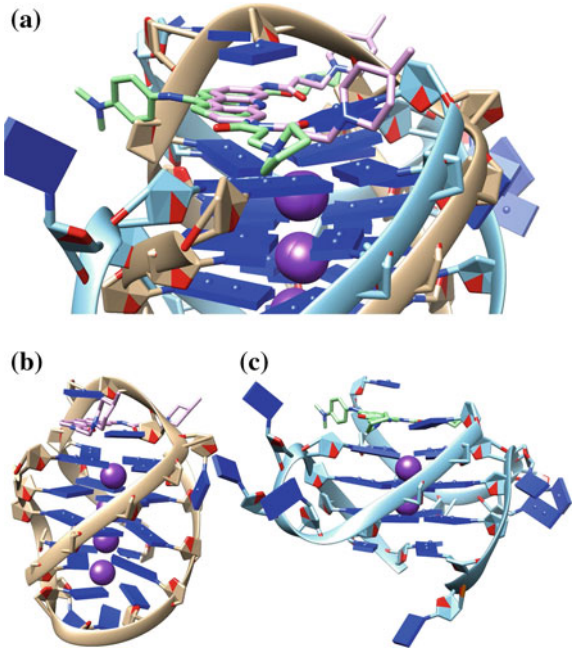


Fig. 4.9 Comparison of proflavine derivatives without and with substituent at position 9 binding to DNA quadruplexes. **a** Steric clashes of 9-substituted acridine (peas green) and nucleotide backbone of quadruplex implies selection of different quadruplex conformations. At **b** and **c**, there are shown conformations of quadruplexes biding acridine without and with 9 substituent, respectively. Image was created by program USCF chimera from pdb structures: 3ES0 [141] and 3CE5 [143], respectively

Increasing power of computational resources led to in silico investigation of various aspects of acridine nucleic acid interactions. By molecular dynamics, the interaction of 9-aminoacridine with oligonucleotide of DNA was studied [20]. The reversible process of complex dissociation was described for two binding sites containing AT or GC pairs in both minor or major grooves. The calculations have shown that the intercalation is most probably started by the minor groove binding, which is in accordance with published crystal structures of acridine DNA complexes. The computer simulations confirmed that acridine prefers GC-rich regions over AT sequences of DNA [20], which was previously shown experimentally [11].

Virtual ligand screening and structure-based design of drugs that recognize non-canonical nucleic acid structures such as G-quadruplex and triplex DNA have been reviewed [148].

Rational design of selective telomeric quadruplex binder was based on published crystal structures and CD and NMR study [57] (Fig. 4.2b). Similar approach taking G-quadruplex polymorphism into account was applied later [149]. Combination of NMR NOE constrains with molecular dynamics provided structures of quadruplex–binder complexes in solution [150].

The long-range electron transfer (ET) through dsDNA and Ac-Lys(Qui)-Lys_2-Lys(Qui)-NH_2 complex was reported [151]. Low distance dependence of ET process was explained by A-hopping mechanism, where intermediate AT base pairs served as temporary charge carriers. These dsDNA wires can probably serve for flexible design and construction of functional nanodevices.

Correlation of DNA-mode of binding and spectroscopic parameters, such as shift of absorbance maxima at UV-Vis, angle at linear dichroism, and induced circular dichroism spectra of achiral acridines were thoroughly reviewed [79].

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Chapter 5
Interactions with Proteins

Abstract Usually, the moderate binding of acridines to nucleic acid cannot compete with tight binding of transcription factors. Thus, other explanations for acridine biological activities are necessary. Biological activities of acridines can be significantly caused by direct interactions with proteins. Acridines interact with proteins both directly or as a component of nucleic acid-acridine complex. Acridines influence the living system by direct interaction with receptors, and also on genetic or epigenetic levels where more prolonged effects can be achieved. Influence of acridines on many kinases alters the signaling pathway and regulates apoptosis. Structural aspects of interactions with proteins are useful hints to design enzyme inhibitors involved in treatment of cancer, inflammation, and neurodegenerative diseases.

Many of acridine related biological activities are caused by direct interaction with proteins. Acridines interact with both type of proteins: binding of nucleic acids and non-binding of nucleic acids. Thus, they can influence the living system immediately by direct interaction with receptors, and also on genetic or epigenetic levels where more prolonged effect can be achieved. Influence of acridines on many kinases can alter signaling pathways and regulate apoptosis.

5.1 Interactions with Nucleic Acids Processing Proteins

Even the biological activities formerly associated with nucleic acid binding became under new discoveries attributed to co-interactions with proteins. It is obvious that mono-intercalating acridines dissociation constants are in range of μM, whereas many nucleic acid processing proteins have dissociation constants in sub-nM scale. Thus, acridines cannot mostly inhibit the relevant processes just by binding with nucleic acid in competition with nucleic acid binding proteins [1]. On the other hand, formation of stable complexes between acridines and proteins can lead to a blockage of active site. Sometimes, the nucleic acid can be involved in formation of a such stable complex.

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5.1.1 Topoisomerases

In the past, it was shown that dsDNA binding is not sufficient for cytostatic activity of 9-aminoacridines (amsacrines), but inhibition of topoisomerase II was required [2–8]. Nowadays, potent antitumor activity of acridines is attributed to inhibition of topoisomerases [9–11]. Eukaryotic topoisomerases and their roles in transcription, replication and genomic stability were reviewed in great detail [12]. Topoisomerases II in human cells are occurring as α- and β-isoenzymes. The methyl *N*-(4-(9-acridinylamino)-phenyl)carbamate is an inhibitor of topoisomerase II β. The enzyme mutation at residue Glu^{522} caused a resistance towards this anticancer drug. However, other anticancer agents can become more powerful by this mutation, which can be useful for multi-targeted cancer therapy [10]. Despite the importance of topoisomerases in anticancer effects of acridines, for long time, only X-ray data of complexes of acridines with nucleic acids were published and mode of binding evaluated [13–16]. Most recently, the crystal structure of amsacrine with DNA and topoisomerase II has been solved [17] (Fig. 5.1). The described intercalation of amsacrine into dsDNA, which led to inhibition of topoisomerase II (4G0U), took place between heterogeneous AT- and GC-base pair. Based on 3.5 Å distance from amsacrine, the binding involved also residues from the protein such as Arg^{503}, Lys^{505}, Glu^{522} (which occurred in two binding copies) and also Gln^{778} and Gly^{504} (one binding copy). The Glu^{522}, the mutation of which caused resistance toward some acridines, is involved in the binding [10]. This provides a tool for rational design of anticancer drugs.

Catalytic inhibitors of topoisomerase II could prevent G1→S transition during cell cycle [18] because proper function of topoisomerase II is needed for DNA unwinding during the startup of replication.

Evolution of topoisomerase II β resistant to amsacrine was observed after mutation G465D [19], which led to decreased ATP hydrolyzing activity.

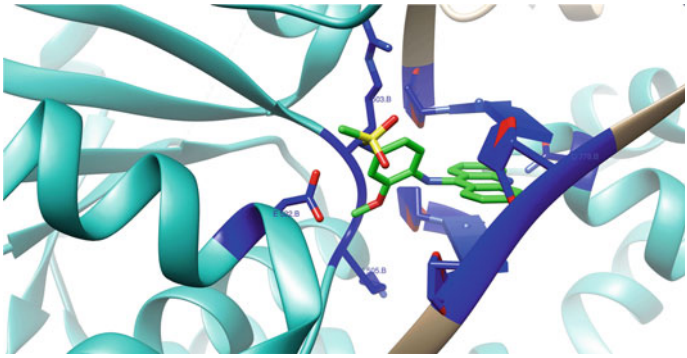

Fig. 5.1 *m*-Amsacrine (*green*) inhibits topoisomerase II (*cyan*) by stabilization of amsacrine–dsDNA–protein complex. Zone with distance 3.5 Å from amsacrine is highlighted by *blue color*. Image was created by program USCF chimera from pdb structure 4G0U

A series of 9-anilinoacridines, based on *m*-amsacrine, were also evaluated for their ability to inhibit both the growth of Jurkat leukemia cells and human DNA topoisomerase II in vitro [20]. Inhibition of topoisomerase II activity occurred via one of two mechanisms of drug action: (i) direct inhibition of the strand-passing activity or (ii) stabilization of cleavable complex formation. Some derivatives with different cytotoxicities stimulated DNA topoisomerase II-mediated DNA strand breaks in intact Jurkat cells by 2.2-11 fold, at a concentration of 10 μM. A good correlation between the ability of these derivatives to induce topoisomerase II-mediated DNA strand breaks *in vitro* or in whole cells, and their cytotoxicity to Jurkat cells was found.

A series of acridine derivatives was studied for acridine antiproliferative activity and the DNA-binding ability [21]. Among them, 2-methoxy-9-benzylaminoacridine was DNA-binder and topoisomerase I inhibitor. Moreover, it induced apoptosis by mitochondria-dependent intrinsic pathways in K562 cell lines and cytotoxicity also in HepG2 cells.

Hybrid derivatives of acridines and benzimidazoles were synthesized [22]. Acridines intercalate into DNA and benzimidazoles bind to the DNA minor groove. MTT assay showed that most of the tested compounds exhibited good antiproliferative activity. *N*-((*1H*-benzo[d]imidazol-2-yl)methyl)-2-butylacridin-9-amine demonstrated the highest activity against both K562 and HepG2 cells; it bound DNA and inhibited topoisomerase I activity. Besides, this compound induced apoptosis in K562 cell lines via mitochondrial pathway.

Poisons of topoisomerase II based on trifluoromethylated 9-amino-3,4-dihydroacridin-1(*2H*)-one scaffold were studied [23] with emphasis on their capability to improve DNA cleavage mediated by human topoisomerase IIα. Compounds containing a H, Cl, F, and Br at C7 improved enzyme catalyzed dsDNA cleavage cca 5.5- to 8.5-fold over baseline. However, they were less active than amsacrine. The presence of an 9-amino group was crucial for activity. The tested derivatives lost their topoisomerase IIα activity in the presence of a reducing agent. Thus, the compounds work as covalent, rather than interfacial, topoisomerase II poisons.

Pyrazoloacridine (PZA) was clinically tested as an anticancer agent. It was suggested that PZA might be a dual inhibitor of DNA topoisomerase I and DNA topoisomerase II that exert their effects by diminishing the formation of topoisomerase–DNA adducts [24, 25].

Derivatives of DACA (**5**) were thoroughly investigated for their toxicity related to topoisomerase poisoning [14, 15].

The DACA and its 7-chloro derivative (Cl-DACA) were highly active against solid tumors in mice. The topoisomerase specificity of DACA and Cl-DACA using camptothecin and amsacrine as control compounds was investigated and it was found that Cl-DACA, but not DACA, inhibited topoisomerase II action at 5 μM [26]. The activity of DACA and Cl-DACA may be seen by their action to form DNA–protein cross-links in H460 human lung carcinoma cells. It was concluded that both the drugs have dual topoisomerase I/II specificity, DACA preferentially inhibits topoisomerase II and Cl-DACA preferentially inhibits topoisomerase I. In addition, drug-induced inhibition of topoisomerase action at higher drug concentra-

tions might mask a poisoning in the cell-free assay as well as masking cytotoxicity in cultured cells. More hydrophilic 9-amino-5-sulphonylmethyl-DACA associates predominantly with DNA, whereas DACA binds predominantly to lipophilic sites such as proteins and cellular membranes [27].

6-Chloro-2-methoxy-*N*-(4-methoxybenzyl)acridin-9-amine strongly inhibited topoisomerase II and four tested tumor cell lines [28]. It caused severe DNA damage in tested cell lines.

5.1.2 Transcription Factors

Structure activity relationships of DNA-threading bis-acridines revealed that tuning of the lifetimes of the intercalated state and the degree of duplex unwinding are responsible for alteration of the cell killing mechanism. Due to quite weak acridine–DNA interaction, mono-intercalators do not usually prevent transcription factor–DNA binding [1]. Some bis-acridines act as strong cell poisons although they are not inhibitors of topoisomerases. The strong DNA binding causes competitive inhibition of transcription [29]. Unnatural polypeptides containing 4-amino-1-methylimidazole-3-carboxylic acids [30] were conjugated with 9-aminoacridine [31]. These intercalators selectively recognized binding site of transcription factors and suppressed their minor groove binding. Thus, related gene expression is switched off. The peptide sequence of conjugate has β-hair-pin conformation and interacts with both strands of dsDNA [31].

5.1.3 RecA Protein

RecA protein is responsible for DNA strand exchange reaction and for stimulation of synthesis and activity of proteins involved in DNA repair and mutagenesis. During cooperative RecA binding to DNA, the nucleotide cofactor with intercalative binding mode is required for complex formation. RecA to DNA binding is induced by 9-aminoacridine faster than that caused by the naturally occurring cofactor. The bis-intercalating acridine dimer causes RecA–DNA interaction much faster than parent 9-aminoacridine. Thus, destacking and/or unwinding should be related to initial rate determining step of RecA–dsDNA association. The negligible influence of tris-acridine on RecA–dsDNA complexation led to proposal that just a certain amount of DNA distortion associated with tris-intercalation is feasible for RecA binding [32].

5.1.4 Telomerase

Due to strong binding of acridine based telomerase inhibitors to DNA quadruplexes, they are described in the Sect. 4.2. See also reviews [8, 33].

5.2 Interactions with Other Proteins

Generally, acridines can bind to plasma proteins non-specifically, the binding negatively influences their bioavailability [34, 35]. E.g. acridines non-specifically bind into hydrophobic interior of globular proteins such as bovine serum albumin [36] or egg albumin [37], and they are unavailable for inhibition of desired enzymes. For discussion how non-specific binding to proteins or nucleic acids decreases bioavailability see also Chap. 8.

Lipophilicity or hydrophilicity belong to the factors deciding whether the acridine interacts with hydrophobic pocket of the proteins or with more hydrophilic nucleic acids [27]. However, modern structural aspects can provide more clues how the acridine is accepted by both proteins and/or nucleic acids (see Sects. 4.6 and 5.3).

In order to explain mechanisms of optical analysis of proteins, preliminary simulations of acridine yellow G and proflavine located at different binding positions within human serum albumin were done [38]. The two primary drug binding sites and a heme binding site were used, as well. The calculations were based on combination of molecular dynamics and density functional theory calculations of the excitation energies. Comparison of experimental and calculated shifts of maximum absorption energies revealed that the proflavine probably did not bind albumin at its drug binding site 1 nor at its heme binding site. Furthermore, computations clearly show that the change in optical response of the two acridines is caused by deprotonation, and not, as earlier proposed, by various binding modes. Thus, interpretation of spectroscopic data without combination with calculation can be misleading.

9-Aminoacridine and AO can bind hemoglobin with destabilization of the protein structure [39]. AO was found to be a stronger binder than 9-aminoacridine.

P-glycoprotein (P-gp) mediated multidrug resistance (MDR) belongs to the best known transporter-mediated barriers to successful cancer chemotherapy. In a search for MDR reversing agents, a set of acridine derivatives was generated and examined for their *in vitro* antiproliferative activities against K562 and K562/ADM cells [40]. Some of the tested compounds performed better than amsacrine, the reference compound. It was clearly shown that the *N*,*N*-diethylamine moiety plays a role to overcome the drug resistance of the 9-anilinoacridines. The study indicates that acridine structure is a potentially interesting candidate for developing new MDR reversing agents.

m-Amsacrine and its analogs inhibited tumor growth in the human leukemic cell line CCRF-CEM that expresses either P-gp-associated MDR (CEM/VLB100) or altered topoisomerase II associated MDR (at-MDR) (CEM/VM-1) genes [7]. Drug-topoisomerase II interactions and drug-stabilized DNA-topoisomerase II in an intact cell assay were measured. It was concluded that certain substitutions on the anilinoacridine ring have a nearly 30-fold effect on the biological potency of these agents, and make some of these compounds susceptible to P-gp mediated resistance.

N10-substituted acridones played a role of modulators of multiple drug resistance via interactions with P-gp [8, 41, 42] and potentially preventing efflux of drugs from the cells.

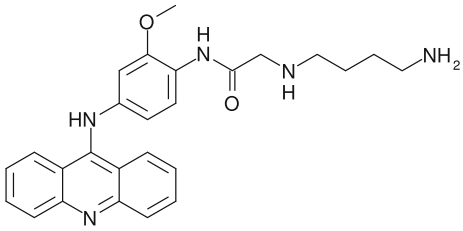

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Fig. 5.2 Inhibitor of P-gp [43]

Synthesis of a series of polyamine-based anilinoacridine derivatives was described [43]. The biological tests have shown that the 9-anilinoacridine-polyamines had reduced or negligible *in vitro* cytotoxicity to K562 cell line and to the drug-resistant cell line K562/ADM. Nevertheless, the examination of P-gp modulation revealed that one from these acridines is powerful P-gp inhibitor. The conjugate of putrescine with 9-anilinoacridine system (Fig. 5.2) had even greater effect on P-gp than the known P-gp modulator – verapamil. However, EC_{50} was not determined in the paper, where only preliminary results were published. Thus, 9-anilinoacridine-polyamines can be utilized as effective P-gp modulators.

Mycobacterium smegmatis uses an efflux pump LfrA for clearance of antibiotics. LfrA possesses two binding sites for proflavine with $K_{d,1}$ and $K_{d,2}$ values 79 nM and 4.3 μM, respectively [44] (see also Fig. 5.7).

Possible treatment of cancer could be done by derivatives of acridine yellow G, which were inhibitors of epidermal growth factor receptor and protein kinase C [45].

4-Amino-*N*-(4-sulfamoylphenyl)benzamide was prepared by reduction of 4-nitro-*N*-(4-sulfamoylphenyl)benzamide and utilized for synthesis of novel acridine sulfonamide derivatives, by a condensation reaction with cyclic-1,3-diketones and aromatic aldehydes [46]. The compounds prepared were tested as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), and more exactly the cytosolic isoforms hCA I, II and VII. hCA I was inhibited by the new compounds at K_Is of 0.16–9.64 μM while hCA II and VII showed higher affinity for these compounds, with K_Is above 15 nM for hCA II, and of 4 nM for hCA VII. Also bis-sulfonamides were used as inhibitors of various hCA forms [47].

4-Amino-*N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)benzamide was conjugated with acridine-1,8-diones [48]. These conjugates served as inhibitors of various hCA, where the best one was subnanomolar and selective inhibitor of hCA II. The good selectivity of the best inhibitor towards hCA II made it promising lead for therapy of glaucoma.

Hydratase and esterase activities of human carbonic anhydrase isoenzymes (hCA I and hCA II) are inhibited by synthetic acridinedione sulfonamides [49]. The IC_{50} values of these sulfonamides were in the ranges 47.2–230.1 μM for hCA I and 50.1–275.0 μM for hCA II.

Acridone alkaloids were identified as μM inhibitors of cathepsin V [50].

Dendrimeric tetra-acridines served as inhibitors of topoisomerase II and proteasome [51]. Due to inhibition of tumor cell resistant to topoisomerase II inhibitors, cytotoxic effect of these dendrimers was attributed to inhibition of proteasome rather than to inhibition of topoisomerase II.

The anticancer activity of amsacrine was attributed to its inhibitory effect on topoisomerase II in earlier studies. Nevertheless, 9-aminoacridine derivatives with the same structural motif as amsacrine induce apoptosis of cancer cells by influencing the expression of Bcl-2 family proteins. Thus, influence of Bcl-2 family proteins on the cytotoxic effects of amsacrine on human leukemia U937 cells was evaluated [52]. Apoptosis of U937 cells induced with amsacrine was characterized by activation of caspase-9 and caspase-3, by an increase of concentration of intracellular Ca^{2+}, by mitochondrial depolarization, and by Mcl-1 down-regulation. Amsacrine evoked Mcl-1 down-regulation by its destabilization. Additionally, amsacrine-treated U937 cells manifested Akt degradation and inactivation of Ca^{2+}-mediated ERK. Inhibition of ERK regulated phosphorylation of Mcl-1 affected a role of Pin1 in the stabilization of Mcl-1, and Akt degradation supported GSK3β-mediated degradation of Mcl-1. Renewal of ERK phosphorylation and Akt expression abolished amsacrine-induced Mcl-1 down-regulation. Mcl-1 overexpression reduced depolarization of mitochondrial membrane and increased the viability of the cells. This emphasizes an alternative pathway for antileukemic effect of amsacrine.

High cytochrome P450 3A4 (CYP3A4) expression makes tumor cells sensitive to some antitumor agents, whereas in other cases the expression lowers therapeutic efficacy. The impact of CYP3A4 overexpression to the cellular response caused by antitumor acridine derivatives 5-dimethylaminopropylamino-8-hydroxytriazoloacridinone (C-1305) and 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748) in two hepatocellular carcinoma (HepG2) cell lines (Hep3A4 producing CYP3A4 isoenzyme and HepC34 generating empty vector) was tested [53]. Both acridines caused necrosis and partially apoptosis, which were more obvious in Hep3A4 than in wild-type cells. In summary, CYP3A4-overexpressing cells generated higher levels of C-1748 metabolites, but they did not influence the cellular responses to the drug. On the other hand, cellular response was influenced after C-1305 treatment in CYP3A4-overexpressing cells, despite that metabolism of this drug was unaltered.

A cross-acridine scaffold [54] that displays functional groups with angular and spatial geometries that exactly mimic the i, i + 3, i + 5, and i + 7 side chains on a two-turn, two-face section of an α-helix of the Bim BH3 peptide were designed. Hotspot mutations could be imitated by different regions of the scaffold and combinations of side chains. The most effective compound (Fig. 5.3) can prevent protein–protein interaction and bind to Bcl-2 and Mcl-1 with K_i 56 and 79 nM, respectively.

Virtual screening of octahydroacridines has revealed that the compounds can inhibit hSIRT1 [55]. For verification, a library of acridines was prepared and evaluated for *in vitro* hSIRT1 activity. The most effective derivative 9-(2-(benzyloxy)phenyl)-10-(2,4-dinitrophenylamino)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetramethyl-(*9H*, *10H*)-acridine-1,8-dione displayed a significant inhibition of SIRT1 activity. Moreover, this compound caused a dose dependent increase of p53K382 acetylation and SIRT1 decrease with IC_{50} 0.25 μM in MDA-MB231 breast cancer cell lines.

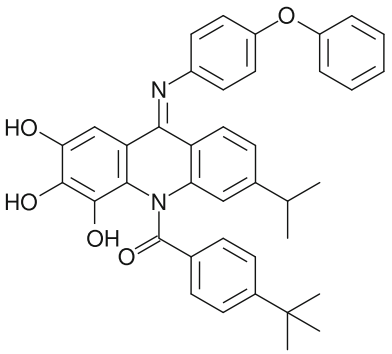

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Fig. 5.3 A mimic of the Bim BH3 peptide with K_i 56 and 79 nM for Bcl-2 and Mcl-1 binding, respectively [54]

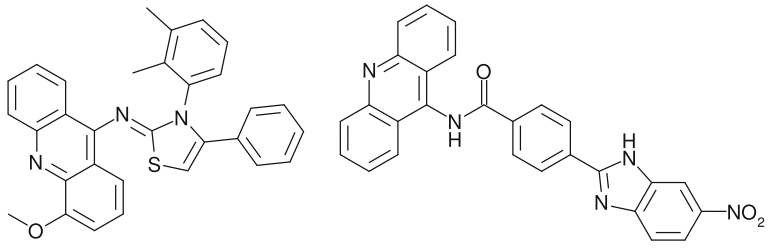

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Fig. 5.4 The inhibitors of cyclin dependent kinase 1 (**138**) with IC_{50} 8.5 μM [58] and cyclin dependent kinase 5 (**139**) with IC_{50} 4.6 μM [59]

3D-QSAR analysis by Schrödinger software showed that *ortho* substitution is indispensable for bioactivity.

The acridinyl-thiazole derivatives were synthesized and tested for their anti-inflammatory, analgesic, and some kinase inhibiting activities [56, 57]. Several of them are more effective analgesics than ibuprofen. One compound was identified as an inhibitor of cyclin dependent kinase 1, active at micromolar level [58] (Fig. 5.4). The same laboratory has identified a new derivative of the 9-aminoacridine, which acts as inhibitor of CDK-5 at 4.6 μM [59].

Acridines are inhibitors of wheat embryo calcium-dependent protein kinase with IC_{50} at 1.5–200 μM levels [60].

Inhibition of calmodulin-dependent myosin light chain kinase by acridines was selected as a useful criterion for the detection of potential antitumor compounds [61]. IC_{50} values were in the range 4–138 μM.

Acridines inhibit receptor tyrosine kinase (c-Met), haspin and dual-specificity tyrosine-regulated kinase [8].

1-(4-((3-Methoxyacridin-9-yl)amino)phenyl)-3-phenylurea can inhibit ca 60% of Src and 43% of MEK kinase activity at 10 μM concentration [62].

N-Acridine-9-yl-*N'*,*N'*-dimethylpropane-1,3-diamine prevented the unfolded protein response via inhibition of the inositol requiring kinase 1α (IRE1α)–X-box binding protein-1 (XBP1) pathway [63]. Its analogue N^9-(3-(dimethylamino)propyl)-N^3,N^3,N^6,N^6-tetramethylacridine-3,6,9-triamine inhibited not only IRE1α oligomerization but also *in vitro* endoribonuclease (RNase) activity. The inhibition of IRE1α led to cytotoxicity towards multiple myeloma cells.

The synthesis of 4-phenylethyl-acridinone and 4-phenylethyl-dioxophenothiazine derivatives together with their inhibition of tubulin polymerization and cytotoxicity were described [64]. Correlation between cytotoxic and antitubulin activity have shown that 4-methoxyphenylethyl derivatives were very toxic without significant antitubulin activity. On the other hand, derivatives after introduction of 3-hydroxy substituent possessed antitubulin activity without antiproliferative activity. This lack of correlation can be perhaps attributed to different mode of action of these acridones than the interaction with tubulin polymerization.

Synthetic 2-deoxy-2-fluoroglycosyl acridinone active site exploring compounds were tested and their kinetic parameters for inactivation of a β-galactosidase, a β-xylosidase and several cellulases were determined [65]. Active site titration with these reagents was able reliably detect active enzyme concentrations down to 3 nM by fluorescence spectroscopy. This sensitivity is sufficient for quantitation of glycosidases at typical protein expression levels.

Amsacrine and quinacrine served as micromollar inhibitors of aldehyde oxidase [66]. Inhibition of aldehyde oxidase [67] can lead to suppression of various metabolic pathways. DACA is oxidized with aldehyde oxidase, whereas 7-hydroxy-DACA is the powerful inhibitor of the aldehyde oxidase with IC_{50} 40 nM [66, 68].

The pharmacological results indicated that several 9-indolyl-1,8-acridinediones possess myorelaxant activity on smooth muscles precontracted by noradrenaline [69]. The relaxation effect of the tested compounds was not changed by glibenclamide and tetraethylammonium chloride. Thus, ATP sensitive and calcium activated potassium channels did not contribute to these myorelaxant effects. Further investigation is needed in order to understand the potassium channel opening by these acridines.

There has been substantial effort in exploring K_{ATP} channel openers in the therapy of various diseases e.g. cerebrovascular, cardiovascular, and urinary system disease and premature labor. A series of 3,3,6,6-tetramethyl-9-aryl-octahydro-1,8-acridinediones was synthesized [70]. Their influence on vascular potassium channels and mechanism of induced relaxations on phenylephrine-induced contractile responses in isolated rings of rat aortic smooth muscle was studied. However, the compounds were less potent than commercially available drug – minoxidil. The most active 3,3,6,6-tetramethyl-9-(3-nitrophenyl)-2,3,4,5,6,7,9,10-octahydro-1,8-acridinedione was ca one order of magnitude less potent than minoxidil. This effect was modulated by glibenclamide (the potassium channel blocker). As a consequence, the tested compounds work as ATP-sensitive potassium channel openers. Selectivity index of the tested compounds and minoxidil indicates that they are selective to ATP-sensitive potassium channels.

The history of quinacrine together with its pharmacokinetic characteristics and established safety record as an FDA-approved drug was reviewed [71]. The potential

applications of quinacrine as an anticancer agent, its actions on arachidonic acid pathway, nuclear proteins, and multidrug resistance, including its influence on signaling proteins in the cytoplasm were summarized. Data about quinacrine role on the NF-κB, p53, and Akt pathways were also discussed. The quinacrine is one of the best studied drugs ever introduced. Quinacrine is superior to quinine in antimalarial activity. However, later, it was replaced by chloroquine [71–73].

The quinacrine has been an efficient immunosuppressant with nearly seventy years of clinical employment. Patients with rheumatoid arthritis and lupus erythematosus can be treated with quinacrine even during pregnancy. Application of low doses easily prevents the side effects. The quinacrine action is multitargeted e.g. it inhibits DNA and RNA polymerases, forestalls prostaglandin synthesis, stabilizes neutrophils and lysosomes and acts as bradykinin and histamine antagonist [74]. This compound has strong trypanocidal activity against *Trypanosoma cruzi* and prevents the transfer of Chagas disease by blood transfusion. It is a competitive inhibitor of *T. cruzi* trypanothione reductase without effect on human glutathione reductase. Specific pair-wise interactions between functional groups of the drug and the trypanothione reductase concern those residues that are not conserved in human enzyme [75].

The quinacrine inhibits botulinum toxin metalloprotease, the active species of one of the most lethal biological poison. Besides, it can reduce botulinum protease delivery to target cell cytoplasm. The simultaneous action of these two properties led to deceleration of muscle paralysis [76]. Quinacrine blocks the channels created by the transmembrane pores of *Clostridium botulinum* C2 and anthrax toxins in lipid bilayers [77]. It prevents the intoxication of HeLa cells with C2 toxin and anthrax lethal toxin by enclosing the C2IIa translocation pores and the translocation pores of anthrax toxin in cell membranes, respectively.

The α_1-adrenergic receptors serve as targets for many central nervous and cardiovascular events. Unfortunately the current drugs for these receptors are not specific. An alternative mechanism of action is offered by allosteric modulators in comparison to traditional α_1-adrenergic ligands. Regrettably there is insufficient information characterizing this class of drugs at the α_1-adrenergic receptors. A series of 9-aminoacridine compounds [78] that demonstrate allosteric modulation of the α_{1A} and α_{1B}-adrenergic receptors were identified [79]. E.g. C4 and C5 bis(9-aminoacridine)'s bound α_{1A} receptor with K_i 21 nM and 33 nM, respectively. The inhibition constants for α_{1B} receptor were opposite i.e. 210 and 94 nM, respectively. The 9-aminoacridines improve the speed of [^{3}H]prazosin dissociation from the α_{1A}- and α_{1B}-adrenergic receptors and noncompetitively prevent receptor activation by the endogenous agonist norepinephrine. Tacrine, an allosteric modulator of the muscarinic receptors, also modulates the α_1-adrenergic receptors. This indicates a lack of selectivity for allosteric binding sites within aminergic G protein-coupled receptor. Conjugation of two 9-aminoacridines with variable methylene linkers augmented the allosteric effects of these ligands. A bivalent strategy may offer a way for fine tuning of allosteric compound efficacy in drug design.

Acridines can be used as modifiers of stationary phase for chromatographic separation of proteins involved in neurodegenerative diseases such as various forms of

acetylcholinesterase (AChE) [80, 81] or different forms of prion proteins [82]; in tumor diseases such as epidermal growth factor receptor [83].

Further examples of acridine–protein interactions are described in sections dealing with structural aspects of protein binding and applications for treatment of protein misfolding diseases i.e. Sects. 5.3 and 6, respectively.

5.3 Structural Aspects of Acridine Interactions with Proteins

Depending on substituents, acridine ring is hydrophobic and is mostly protonated under physiological pH. Thus, expected interactions are with aromatic and hydrophobic amino acids. Positive charge can be recognized by carboxylate of Asp and Glu residues.

When acridine was extended by extremely reactive azo-analogue of yperite, it reacted rapidly with available protein side-chains such as sulfhydryl group of Cys and carboxylate of Asp and Glu leading to formation of thioether or ester linkages, respectively [84]. Rapid reaction proceeded without appropriate selectivity, thus, one hydrophobic acridine residue is anchored to hydrophilic pocket which is in contrast to the concept "like-dissolve-like" (Fig. 5.5, orange acridine). The second acridine unit (green) is stabilized by intercalation between indole ring and first acridine unit. The order first and second was denoted according to reactivity of yperite with nucleophiles, where Cys residue reacts much faster than the carboxylates. Thus, one can hypothesize that the first unit reacted rapidly with Cys residue, then, the second unit was stabilized by intercalation and slowly reacted with available carboxylates. On the other hand, previously solved structure of quinacrine with the enzyme described interaction of hydrophobic acridine ring with the hydrophobic wall built from Trp^{21} and Met^{113} [85]. Unfortunately, the PDB file is not available via the database.

In order to understand structural basis of simultaneous binding of two different drugs to a multidrug binding protein, the crystal structures of the *Staphylococcus aureus* multidrug binding transcription repressor (QacR) with ethidium and

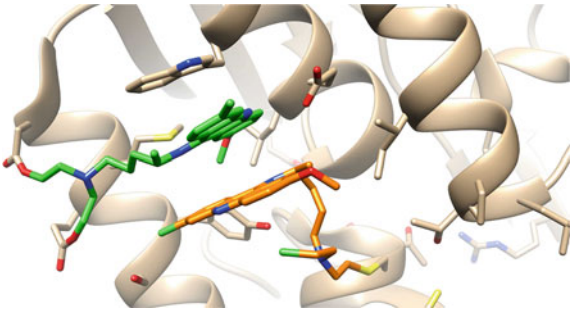

Fig. 5.5 Binding of quinacrine analogue to trypanothione reductase. The *orange* unit is covalently linked to Cys residue, whereas the *green* unit is covalently linked to Asp and Glu residues [84], pdb id: 1GXF

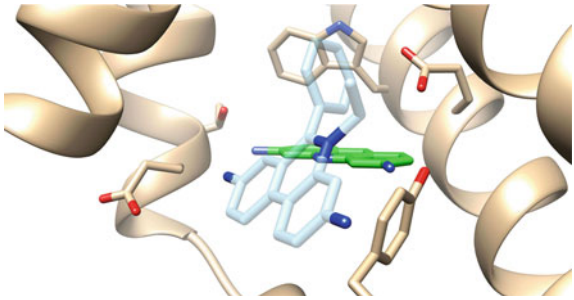

Fig. 5.6 Simultaneous binding of ethidium (*cyan*, transparent) and proflavine (*green*) to multidrug-binding transcription repressor [86], pdb id: 1QVT and 1QVU

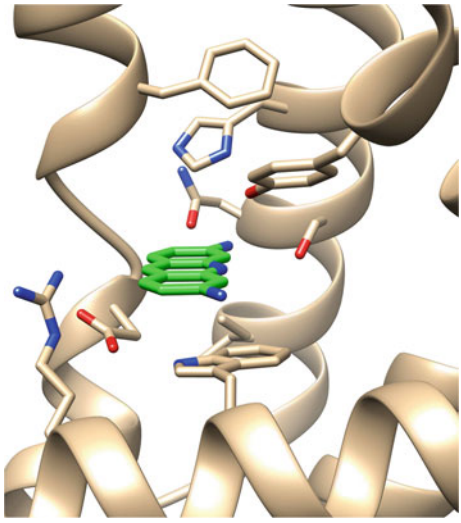

Fig. 5.7 Binding of proflavine to *M. smegmatis* efflux pump LfrA [44], pdb id: 2V57

proflavine were studied [86] (Fig. 5.6). Simultaneous binding led to plasticity of binding pocket caused by reorganization of aromatic amino acid side chains, thus, the affinity for ethidium decreased when proflavine had been bound.

Another multidrug binding protein, which serves as *M. smegmatis* efflux pump LfrA for clearance of antibiotics, was also crystallized with proflavine [44], (Fig. 5.7). In the structure, polar residues such as Tyr^{106}, Ser^{70}, Asn^{71} recognized polar side of proflavine by formation of hydrogen bonds. Moreover, proflavine is stacked over indole ring of Trp^{152}.

Binding of proflavine with EbrA repressor of *Streptomyces lividans* was also studied [87]. Proflavine is stacked between indole rings of Trp^{95} and Trp^{126}. Despite the proximity of Phe^{67} and acridine ring, the orientation of phenyl group is perpendicular to the acridine plane.

Acridines can serve as inhibitors of ribosomal protein S6 kinase 1 involved in diseases such as obesity, diabetes and cancer [88]. Derivative of tetrahydroacridine carboxylate interacts with hydrophobic residues of ribosomal protein S6 kinase

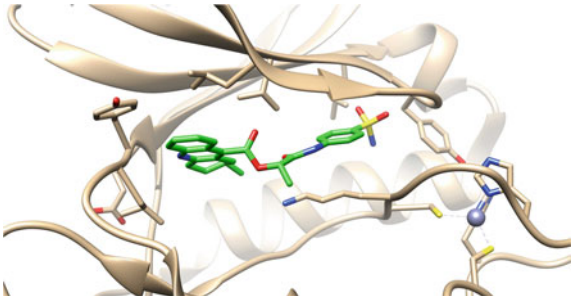

Fig. 5.8 Binding of acridine to hydrophobic pocket of ribosomal protein S6 kinase β-1 [88]. pdb id: 3WF9

β-1 such as Leu^{97}, Tyr^{102}, Val^{105}, Leu^{125}, Leu^{172}, Tyr^{174} and Leu^{175}. However, no intercalation like mode is involved (Fig. 5.8).

Human carboxylesterase 1 (hCE1) plays a role of general bioscavenger involved in narcotic metabolism, clinical prodrug activation, and the processing of fatty acid and cholesterol derivatives. Tacrine mostly binds to liver carboxylase I by hydrophobic interactions with Val^{1254}, Leu^{1255}, Leu^{1304}, Leu^{1363}, Phe^{1101}, Leu^{1097}, Met^{1364}, and polar interactions with Ser^{1221}, His^{1468}, and Glu^{1220} (pdb id: 1MX1). There are multiple orientations of tacrine in the catalytic gorge of hCE1. This enzyme flexibility or possibility to interact with the ligand in various orientations paw the way for broad applicability of the enzyme in xenobiotic metabolism [89].

Interaction of tacrine with AChE was one of the most important for AD treatment with acridine family [90, 91]. In the contrast to the studies of chemical warfare agents, which modified the Ser^{200} in active site directly without major reorganization of an active site of the enzyme [92, 93], tacrine binding led to non-covalent allosteric adaptation of the enzyme cavity [94–97] (Fig. 5.9). Binding of tacrine to Trp^{84} by $\pi - \pi$ stacking influenced mostly aromatic residues in the cavity. First, Phe^{330} has to move its benzene ring from clashes to coplanarity with tacrine allowing another $\pi - \pi$ stacking interaction. Furthermore, Phe^{442} was slightly moved, as well, which pushes away Trp^{432}. The reorganization led also to shift of distance between catalytic pair of His^{440} and Ser^{200} from 2.96 to 3.26 Å. The binding of tacrine prevents interaction of acetylcholine quaternary ammonium group with Glu^{199}.

Introduction of mutation S203A to mouse AChE led to stable crystal structure of AChE with its native substrate acetylcholine and also with competing substrate butyrylcholine [97].

Structures of *Drosophila melanogaster* AChE in native state and with inhibitors such as 1,2,3,4-tetrahydro-*N*-(phenylmethyl)-9-acridinamine and 1,2,3,4-tetrahydro-*N*-(3-iodophenylmethyl)-9-acridinamine were solved at 2.7 Å resolution [98]. Interactions of the enzyme with acridine inhibitors led to reorganization of aromatic residues at active site: Tyr^{71}, Tyr^{324}, Tyr^{370}, Tyr^{374}, Phe^{330}, Phe^{371}, His^{480}, Trp^{83}, and Trp^{472}. Importantly, variability of specific enzyme activity between the insect and vertebrate proteins can lead to rational design of selective insecticides.

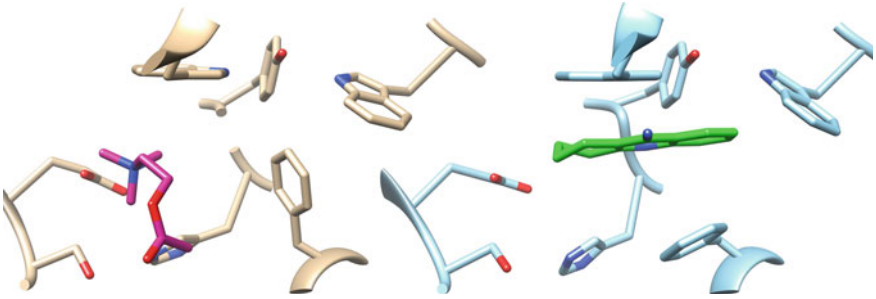

Fig. 5.9 Active site of AChE in native state and influenced by tacrine binding depicted with *light brown* and *cyan colors*, respectively [94, 96, 97]. Tacrine is shown with *green color* and native enzyme substrate acetylcholine is *purple*. pdb id: 1EA5, 1ACJ and 2HA4

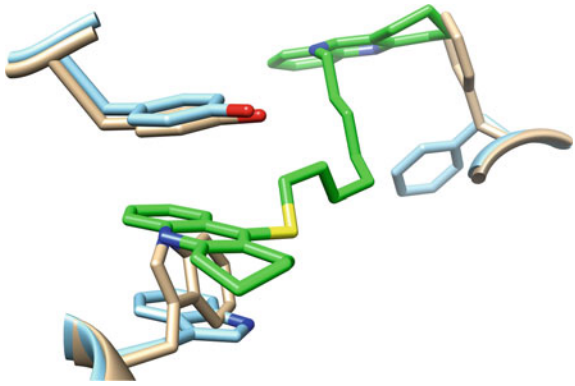

Fig. 5.10 Distortion of aromatic residues in peripheral site of AChE caused by bis-acridinylated compound [96, 101]. pdb id: 1EA5 and 2CEK

Quinone-tacrine based conjugates were designed [99], which bind AChE similarly to monomeric tacrine.

5,6,7,8-Tetrahydroquinolin-2-one analogue of (–)-huperzine A (an alkaloid obtained from *Huperzia serrata*) can occupy the active site of AChE [100]. If two of these quinolinones are connected by appropriate linker, they can occupy both AChE active site and peripheral site. The binding is then more powerful than that of (–)-huperzine A. Interestingly, when tacrine is linked with 5,6,7,8-tetrahydroquinolin-2-one with the same linker, acridine ring binds to the AChE active site, whereas the weaker quinolinone binders remain at the peripheral site.

Tacrine alone can induce conformational changes in AChE active site [94, 97]. Moreover, when two tacrine analogues are linked with oligomethylenes, major reorganization of peripheral site is achieved, as well [101]. By distortion of indole (Trp^{279}) and phenyl (Phe^{330}) from the native state, the acridine ring can be stacked between Trp^{279} and Tyr^{70} (Fig. 5.10).

In order to better understand interactions of bis-tacrines with AChE, a series of bis-tacrines containing linker with 2 to 10 methylene units were investigated [102]. The optimal zone lays between 5 and 9 units having IC_{50} approximately less than

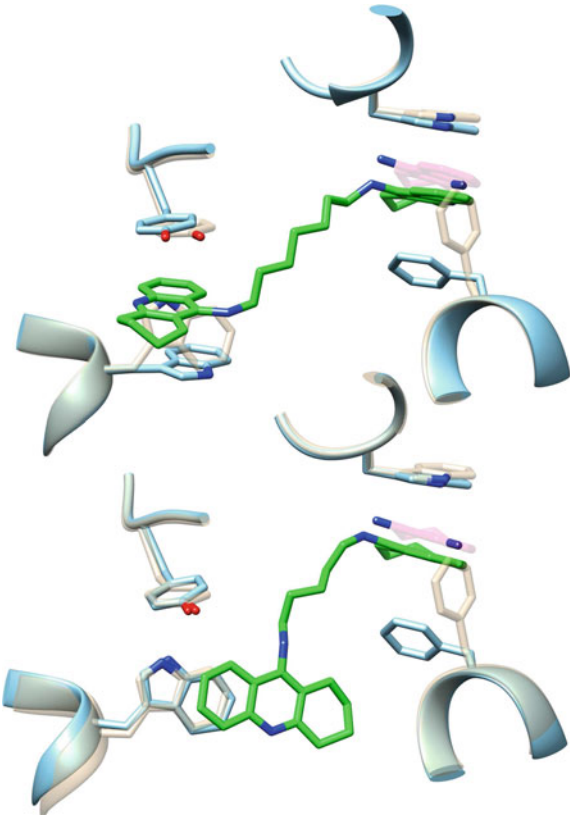

Fig. 5.11 Significant remodeling of AChE upon binding of bis-tacrine with 7 methylene units (*green*, *top*). Only tacrine binding site is remodeled when the linker comprises of 5 units (*green*, *bottom*). Selected residues of AChE in native and bound states are depicted with *brown* and *cyan colors*, respectively. The *purple* structure shows monomeric tacrine binding site [96, 102]. pdb id: 1EA5, 2CKM, and 2CMF

one order of magnitude below the monomeric tacrine. Optimal AChE binding was achieved for 7 units with IC_{50} 1.5 nM and 99 times higher selectivity for AChE than for BChE. The inhibitor with 7 units reorganizes both active site (Trp^{84} and Phe^{330}) and peripheral binding site (Trp^{279} and Tyr^{70}) in order to achieve almost perfect $\pi - \pi$ stacking interaction at both sites (Fig. 5.11). The inhibitor with 5 units was also crystallized; however, it reorganizes only catalytic site but cannot fully modify peripheral binding site. The capability to cause large-scale structural alterations in the active-site gorge of AChE is a challenge for structure-based drug design because this conformational flexibility in the target enzyme is hardly predictable in models.

Phenomenon of enzyme catalyzed click reaction [103, 104] was partly explained by crystallographic study of tacrine analogue linked with ethidium analogue by triazole moiety [105]. Both possible products of 1,3-dipolar cycloaddition were firstly crystallized with AChE (Fig. 5.12) and their binding affinity evaluated. Interestingly, the tighter binder – *syn* product – is the only one obtained by AChE catalyzed reaction of tacrine azide and "ethidium" alkyne. It is explained by reorganization of peripheral site during *anti* product binding with ethidium moiety, whereas the *syn* product fitted

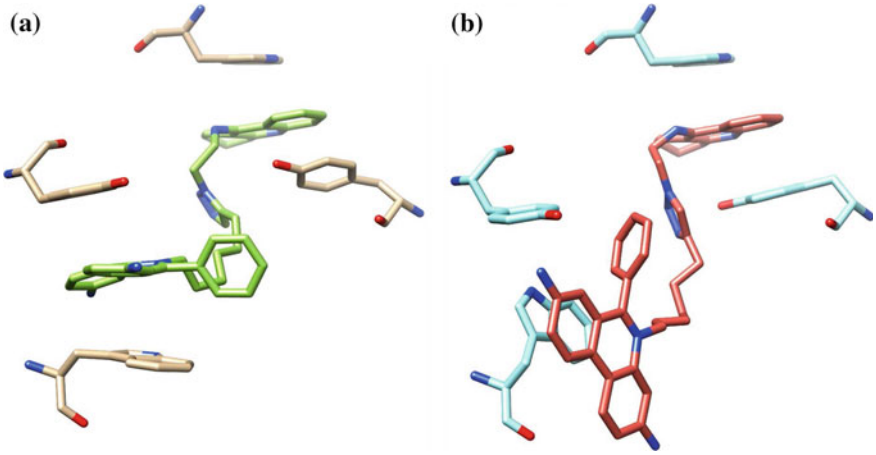

Fig. 5.12 Two possible products of click chemistry crystallized with AChE: **a** *syn* and **b** *anti* product [105]. pdb id:1Q83 and 1Q84

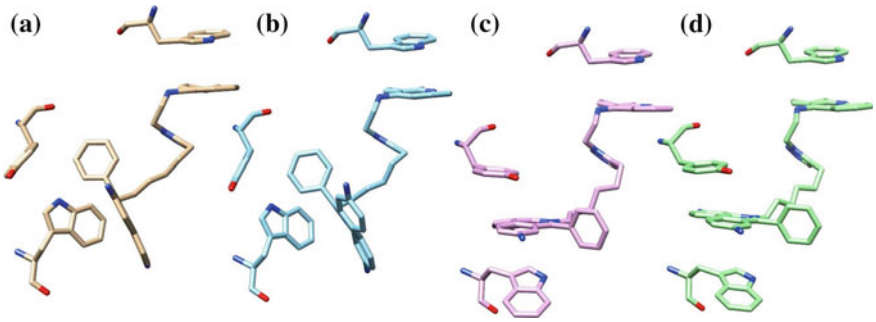

Fig. 5.13 Maturation of enzyme inhibitor complex for *syn* isomer: **a** soaking **b** 1 week **c** 1 month, and **d** 10 months [106]. The c case corresponds to dissociation half-time. pdb id: 2XUP, 2XUI, 2XUJ, and 2XUK

correctly and required only reorganization of active site by tacrine residue, which is also required by *anti* product.

Another achievement of click chemistry based prospecting of mutant mouse AChE (mAChE) binding sites was revelation of time dependent crystal structure formation [106]. The high affinities of ligands to mAChE, with more longer dissociation times than times of nucleation and crystal growth, provided a unique way to evaluate conformational states driven by ligand association, a kinetically controlled process, and those obtained from thermodynamic equilibrium, reached by slow isomerization of the complex. The maturation of the complex is mostly achieved after dissociation half-time related to the substrate–enzyme kinetics (Fig. 5.13).

Tacrine binding to butyrylcholinesterase (BChE) is driven by $\pi - \pi$ stacking with Trp^{82}, hydrophobic interactions with Trp^{430}, Tyr^{332}, His^{438}, and polar interactions

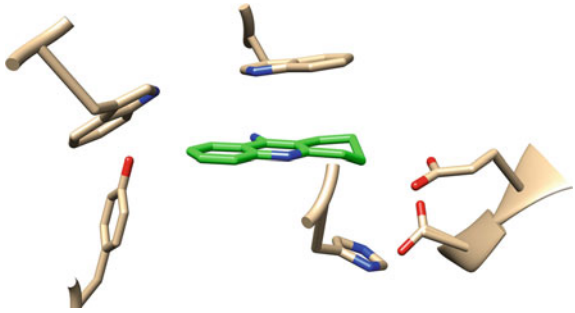

Fig. 5.14 Structure of tacrine with BChE [107]

with Glu[197], and Ser[198] (Fig. 5.14) [107]. There is also a hydrogen bond with the carbonyl of the catalytic histidine residue.

Recently, computational trial to explain AChE/BChE selectivity has been published [108].

For further reading, the Sect. 6 is also recommended.

Imidazoacridin-6-ones inhibit quinone oxidoreductase 2 at nanomolar levels [109]. However, the structure contained "polycyclic" acridines and thus it is omitted in this review. These acridines $\pi - \pi$ stacked to FAD cofactor without significant alteration of residues in the binding site. The same stacking mode was also observed for 9-aminoacridine [110].

One of important enzymes used in clearance of histamine is histamine *N*-methyltransferase. Histamine not only influences smooth muscle contraction, increases vascular permeability, and stimulates secretion of gastric acid, but is also crucial for neurotransmission, immunomodulation, and regulation of cell proliferation [111]. Acridines can serve as inhibitors of histamine *N*-methyltransferase and lead to increased levels of histamine. The enzyme was crystallized with two drugs from acridine family: quinacrine [111] and tacrine [112]. Tacrine binds to the same site as native substrate histamine, whereas acridine moiety of quinacrine occupies entry site of the enzyme causing dramatic reorganization of the α-helix Hnmt(12–25) (Fig. 5.15). The helix reorganization is required by $\pi - \pi$ stacking interaction between acridine ring of quinacrine and Tyr[15]. Another partner of the stacking is Tyr[147], but its position is mostly not altered.

Proflavine served as inhibitor of α-thrombin protease by binding to S1 pocket of the active site [113]. In contrast to other acridines, proflavine bound as dication to hydrophilic site without any $\pi - \pi$ stacking interaction (Fig. 5.16). Also, the active site is almost unaltered with an exception of Glu[192], which is expulsed from the active site [113, 114].

ER-resident protein STING plays an important role in a cell-intrinsic type I IFN response to viral infections. High polarity of 10-carboxymethyl-9-acridone (CMA) led to preferential binding of two CMA units into hydrophilic cavity in STING protein, which normally suits to binding of c-diGMP [116]. Like in the proflavine case, $\pi - \pi$ stacking interactions are not involved in complex stabilization.

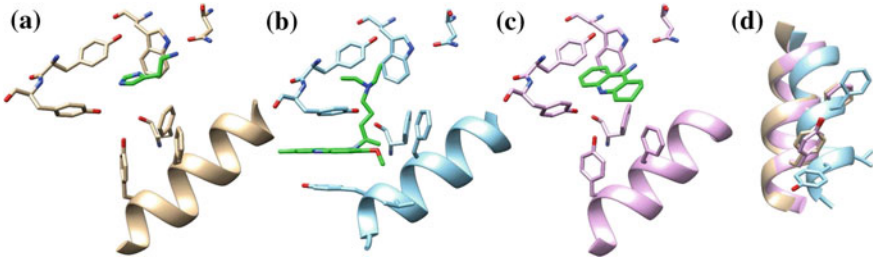

Fig. 5.15 Histamine *N*-methyltransferase complexed with **a** native substrate, **b** quinacrine, and **c** tacrine. **d** The distortion of helix of Hnmt(12-25) caused by complexation with quinacrine (*cyan*) and in native state (*brown*). pdb id: 1JQD, 1JQE, and, 2AOW

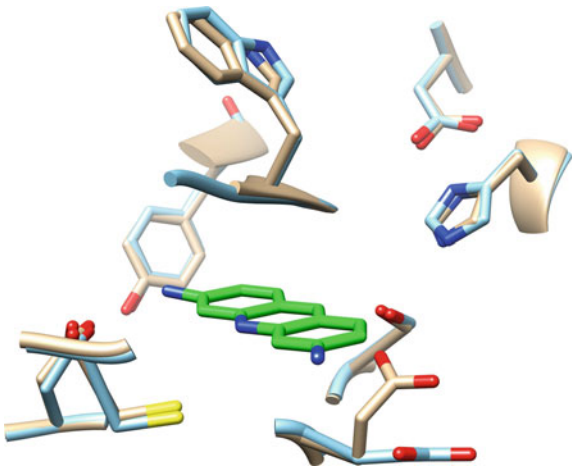

Fig. 5.16 Binding of proflavine to S1 pocket of α-thrombin. Native α-thrombin is *brown*, whereas the bound one is *cyan* [113–115]. pdb id:1SG8 and 1BCU

Structural aspects could also be investigated by other means than the X-ray crystallography. E.g. quenching of Trp fluorescence in combination with CD spectroscopy, provided indicated structural changes of human serum albumin interacting with *N*-((*N*-(2-dimethylamino)ethyl) acridine-4-carboxamide)-α-alanine [117]. However, without deep computational analysis structural details cannot be obtained [38].

Statistical approaches for evaluation of AChE drug bindings were discussed [118]. In silico screening and design of dual site inhibitors of AChE were described [119].

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Chapter 6
Applications for Treatment of Neurodegenerative Diseases

Abstract Hallmark of neurodegenerative disorders such as Alzheimer's, Parkinson's, and prion diseases is aggregation of various proteins, which accumulate in the brain. The early stages of aggregation are responsible for toxicity of the protein to neurons. Later, the protein precipitates out from the system and can be unavailable for its original purpose. The diseases are accompanied with higher oxidative stress and accumulation of various metals. Since acridines can interact with proteins, they can influence the aggregation process or lead to dissociation of the aggregates. Furthermore, acridines can inhibit various enzymes involved in neurodegenerative diseases. In order to tackle multiple factors involved in neurodegenerative diseases, acridine conjugates with anti-oxidants, metal chelators, or anion binders are constructed. Moreover, the reader can see how subtle alterations of acridine structure can serve for detection of prion strains i.e. most probably various conformations of pathogenic proteins.

Neurodegenerative diseases are related to deposition of abnormal protein aggregates in the brain, which are mostly composed of amyloid-like fibrils. These diseases are often called "diseases of protein misfolding" [1]. If we consider deposition of the abnormal protein in other target organs than brain such as pancreas, the profile of the diseases can increase from neurodegenerative to e.g. type II diabetes. Another remarkable attribute of prion diseases is that they can be also spread as an infection [2]. In accord with Prusiner the infectious pathogen responsible for these diseases is not a virus but a misfolded protein. Prusiner coined the term "prion" to describe such a highly unusual proteinaceous infectious agent [3]. In connection with this "protein-only" model, the conformational conversion of a ubiquitous cellular prion protein, termed PrP^{C}, into a misfolded form, PrP^{Sc} is a central molecular event in the pathogenesis of transmissible spongiform encephalopathies (TSE) [2]. The ability of peptides and proteins to form amyloid aggregates was thoroughly studied [4].

The presence of proteinaceous deposits with a fibrillar structure is characteristic for many human diseases such as Alzheimer's disease, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Lafora disease, Friedreich's ataxia (FRDA), Huntington's disease (HD), impairments of working memory or mild cognitive impairment (MCI), familial fatal insomnia (FFI), Gerstmann-Sträussler-Scheinker

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syndrome (GSS), dementia with Lewy bodies (DLB), Down syndrome, Creutzfeldt–Jakob disease (CJD) in humans, TSE, astrogliosis, spinal and bulbar muscular atrophy (SBMA) also known as Kennedy's disease, frontotemporal dementia, supranuclear palsy, multiple system atrophy (MSA), spinocerebellar ataxias (SCA), neuronal intranuclear inclusion disease (NIID), fronto-temporal lobar degeneration (FTLD), and type II diabetes [3, 5–27].

Animal prion diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle also known as "mad cow disease", and chronic wasting disease (CWD) in deer, elk, moose, goat, mule deer, cat, and mink [21], transmissible mink encephalopathy, transmissible spongiform encephalopathies, exotic ungulate encephalopathy. In recent years, CWD has been recognized as a significant epidemic among wildlife populations across certain regions of North America [28–30]. It was found that domestic cats can be infected with prions, known as feline spongiform encephalopathy [3, 5–9, 11, 12, 15, 18, 20–22, 26, 31].

The number of proteins involved in amyloid diseases is much lower than the number of proteins that can misfold and aggregate into amyloid assemblies in vitro [4, 32]. Besides, the majority of proteins are able to form fibrils under specific physicochemical conditions. It is suggested that fibrillization is a common attribute of polypeptide chains [32]. In general in vitro protein fibrillization displays a sigmoid growth curve which is characteristic for autocatalytic processes [4]. The primary risk factor for many neurodegenerative disorders is age. These diseases have a growing social impact [33].

New discoveries in the treatment strategies or pathology for one of neurodegenerative diseases may have importance for other neurodegenerative diseases. Because change of several different pathways can improve phenotypes related to disease, it is probable that therapeutic interventions in the future will be based on connections of medications which improve many features of cellular and organismal dysfunction [33].

In 2003, both the United States and Canada published their first confirmed cases of BSE in cattle within their borders [34]. This spread of variant CJD (vCJD) in North America is the human version of "mad cow disease" that affected Europe, particularly the United Kingdom, since the late 1980s. It is widely believed that this devastating disease results from the consumption of beef contaminated with a pathogenic form of a cellular protein called the prion protein. The way of transmission is still not clear and the incubation period of the disease could be many decades [35]. We are witness of expansion of many other diseases that were unknown even a few decades ago. The most serious both from the social and economical point of view is AD and type II diabetes [24, 27, 36]. These two diseases differ in the symptoms, but their basic principles are in fact similar to vCJD [37]. The common feature of all these diseases is, that proteins, which are normally soluble switch into insoluble aggregates that can form toxic deposits in muscular and skeletal tissue and in organs such as the liver, heart and brain. However, it is believed that soluble aggregates from the early stage of the process are responsible for the toxicity [1, 38]. The most dangerous form of the prion protein aggregates in mammals has been identified as an oligomer containing about 14–28 molecules. These small aggregates belong to

the most effective agents of TSE [1, 39]. The small aggregates play also crucial roles in AD and PD [1]. In spite of facts that a lot of things must be learned to explain mechanisms of these diseases, it looks like that they are finally caused by a failure of the complex process that normally guarantees that proteins remain stable in their correctly folded functional states [32]. These mechanisms, which contain the actions of folding catalysts, molecular chaperones, and degrading enzymes, are normally able to detect misfolded or damaged proteins and either repair or destroy them before they can cause any harm. If these mechanisms fail, these aberrant proteins self-assemble into the highly tough structures known as amyloid fibrils. It is obvious that fibrillar aggregates are not limited to a small number of proteins, but instead represent a general form of polypeptide structure that results from the prevalence of interactions involving the main chain common to all such molecules [32]. On the contrary, the structures of the normally soluble forms of proteins are controlled by the specific folding of the side chains that recognize one protein sequence from another. We can expect that the amyloid diseases are a result of the "reversion" of the highly evolved biologically functional forms of peptides and proteins into alternate and unwanted structures that exist as a consequence of the inherent physicochemical character of polypeptide chains. The normal regulatory processes that prevent such events e.g. by maintaining the conditions necessary for proteins to stay correctly folded–can fail owing to many different factors including genetic mutations, ingestion of pathogenic forms of prion proteins, or simply old age [40]. The growing importance of amyloid diseases to human health has started great research efforts devoted to clarify the molecular mechanisms leading to pathological symptoms and to study strategies to prevent or reverse the amyloid diseases [40]. The principle of the protein aggregation mechanism is that monomeric species formed the oligomers, which further aggregate to fibrillar structures. There are some moments at which intervention can prevent or reverse the aggregation process. Moreover, there has been progress in the screening methods and examination of amyloid deposits [41]. Advanced imaging can enable better monitoring of the biochemical and medical effects of drugs targeted against these diseases. Partial or complete unfolding of proteins having functional state in a form of a tightly packed globular fold are necessary for the first step of fibril formation. On the other hand, the same unfolding can serve for protection from aggregation. Thus, the folding to the native state is quite fragile process with high risk to be misleading to toxic isoform.

Therefore, many of the inherited forms of amyloid diseases are related with genetic mutations that reduce protein stability and promote unfolding. For these instances, one access to therapy is to stabilize the native states of disease-associated representations of amyloidogenic proteins. Quinacrine, a drug originally developed to fight malaria, reduces the replication of the pathogenic prions in cell culture in vCJD [42]. However, quinacrine failed in clinical trials [43, 44] and also in animal models [45, 46].

Other types of amyloid disease are caused by the aggregation of peptide fragments. These peptide fragments are generated by incomplete degradation or the natural processing of the full-length protein. They are unable to fold when the rest of the polypeptide chain is lacking. One of therapeutic strategies is to diminish the

amount of the amyloidogenic peptide fragments by blocking the enzymes that produced them. AD can serve as an example of this approach where the 40- or 42-residue amyloid β (Aβ) peptides are produced from the amyloid precursor protein (APP). Many studies have been focused to the secretase enzymes processing APP. Many secretase inhibitors are in clinical trials for treatment of AD [47]. Risk of possible side effects given by the huge variety of secretase substrates must not be underestimated [48]; however, the recent results from the clinical trials for β-secretase inhibitors are promising [47]. Another strategy for the future could be to use techniques based on introduction of mutations into an amyloidogenic peptide or protein by gene therapy. These mutations can suppress tendency of peptides for aggregation. The knowledge necessary to modify aggregation behavior in a desired manner now exists as a consequence of detailed analysis of the influence of a wide range of mutations on aggregation rates [49].

Another possibility for reduction of aggregating proteins in an organism is to boost their clearance. The action of antibodies produced against specific amyloid-forming polypeptides stands for one of the most exciting strategies [50]. Very important is the finding that extensive clearance of amyloid deposits in transgenic mice can be achieved by active immunization with Aβ peptides. Even though the clinical trial with the Aβ vaccine in Alzheimer's patients was discontinued due to an inflammatory response in some individuals, the vaccination strategy evidently has potential [51]. One useful modification of the original technique is to use passive immunotherapy. The clearance can be stimulated also without the direct action of antibodies. It remains to be determined how such molecules will succeed in clinical trials. Definitely the general idea of using small molecules to target specific proteins for degradation is a remarkable progress.

Of particular importance may be enhancement of the clearance of amyloidogenic species in other diseases where peptides and proteins are accumulated untouched, but appear not to assemble into globular structures even under physiological conditions. As a model can serve α-synuclein, whose aggregation is connected with PD, and amylin, which is participating in type II diabetes [27, 37]. Another approach is to interfere with the aggregation process directly by application of small peptides or peptide derivatives designed to bind tightly to fibrils during their formation, preventing their further growth. Many such species are under exploration, especially in the context of AD [52]. A possible problem with this strategy is that suppression of amyloid fibril growth can lead to the increase of small aggregates, which are more toxic than the corresponding fibrils [37]. When such problems can be kept off, this general strategy would be widely effective.

Taking into account possible intervention at many stages of the aggregation process, there is reason for optimism that new and powerful forms of treatment will appear soon. Furthermore, increasing evidence for the characteristic nature of amyloid disorders induced considerations that it might be possible to block various amyloid diseases with a single drug. Antibodies developed against small aggregates of Aβ served not only for recognition, but also for suppression of the toxicity of similar aggregates formed by other proteins [53]. This discovery has high impact for all amyloid disorders that endanger aging populations and provides an additional

time for natural defenses to combat aggregation, which postpones the onset of these diseases. Despite that Dobson coined this strategy as "a first step toward the dream of the medieval alchemists to produce an elixir of life" [54], the fight will be never done mainly due to resistance of various prion strains [55] and the problem with blood–brain barrier crossing [56, 57]. The second problem can be solved by multifunctional nanocarriers [58].

Topoisomerases are unrivaled enzymes that control torsional stress in DNA to allow indispensable genome functions encompassing DNA replication and transcription [59]. In spite of the fact, that all cells in an organism need topoisomerases to guarantee normal function, the nervous system especially asked for a vital requirement of these enzymes. Of course, a variety of inherited human neurologic syndromes, inclusive schizophrenia, neurodegeneration, and intellectual impairment, are related to abnormal topoisomerase function. Many problems remain unknown concerning the tissue-specific function of neural topoisomerases or the relationships between these enzymes and disease etiology. The precise knowledge how topoisomerases regulate genome dynamics within the framework of the nervous system represents therefore a decisive research question. Significantly, substantially chemical manipulations of topoisomerases provide great possibility for therapeutic strategy to help in the medication of cognitive and neurodegenerative diseases. Thus, acridines as powerful inhibitors and modulators of topoisomerases can influence significantly neurodegenerative disorders (see Sect. 5.1.1).

A crucial factor of neurodegenerative diseases is an oxidative stress. Oxidative stress and mitochondrial dysfunction can cause a variety of pathophysiological conditions including neurodegenerative disorders such as e.g. AD, PD, ALS, and FRDA [60–63]. There are many reactive oxygen species (ROS) and reactive nitrogen species (RNS) involved in the oxidative stress [64]. The main culprits of oxidative stress ROS and RNS can be formed by cell lysis, oxidative burst, and/or an excess of free transition metals [61]. Thus, a plethora of natural products with different activities [65–69] can be applied for therapy of diseases caused by oxidative stress. Natural products act in a common way and are able to suppress oxidative stress related to neurodegenerative diseases. Applications of anti-oxidants against neurodegenerative diseases were described for epigallocatechin gallate from green tea [70], eriodictyol from the Chinese herb *Dracocephalum rupestre* [71], (–)-clausenamide from *Clausena lansium* (lour) skeel [72], derivatives of caffeic and rosmarinic acids [73], etc.

No feasible cure is currently available for amyloid-related diseases. Computational and experimental methods were employed to examine the ability of glycoacridines to prevent lysozyme amyloid fibrillization in vitro [25]. Combination of atomic force microscopy and fluorescence spectroscopy revealed inhibition of amyloid aggregation of lysozyme by glyco-acridines. Submicromolar levels of IC_{50} were observed for these inhibitors. These experiments were confirmed by semi-empirical quantum simulation using PM6-DH+ . The data obtained provide a groundwork for the evolution of new low molecular weight inhibitors which are efficient in therapy of amyloid-related diseases. Surprisingly, hydrogen bonds are not the crucial factor governing the binding affinity. The magnitude of the role of van der Waals and electrostatic interactions is a matter of ligands [25]. The biological activities

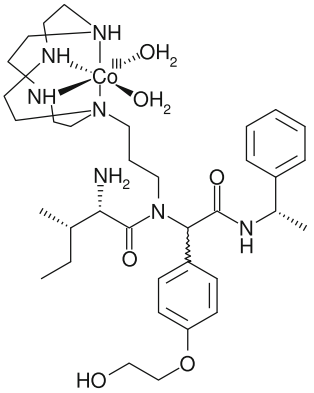

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Fig. 6.1 The artificial metalloprotease capable of amyloid peptide cleavage [76, 77]

qualify glyco-acridines to be viewed as potential therapeutic agents against diseases connected with the lysozyme amyloid aggregation and amyloid-related diseases in general.

In series of planar, spiro, and tetrahydroacridines, the planar ones are most potent in suppression of lysozyme amyloidogenesis [74].

Acridines are able to depolymerize amyloidogenic aggregates of lysozyme [75]. The depolymerization decreases in series from most potent tetrahydroacridines, via spiro-acridines to planar acridines.

The target-selective artificial proteases could provide a therapeutic option for amyloid diseases such as AD, prion diseases, and diabetes [76]. The therapeutic potential lays in cleavage of causative agents of the neurodegenerative diseases which are soluble oligomers of amyloid peptides. Many peptide-cleavage agents have been described which are capable to decrease the level of the pathogenic species of neurodegenerative diseases. The most promising compound (Fig. 6.1) inhibits apoptosis caused by amyloid peptides in the presence of the polymeric aggregates and is not captured by the polymeric aggregates of amyloid peptides [76, 77]. Therapeutically safe peptide cleavage agents could be obtained by selecting the organic pendants properly. The activity may be modulated by the metal-chelating agents. Since currently no cure is available for amyloid diseases, it is necessary to extensively explore the area of peptide-cleavage agents. The possibility to design peptide-cleavage drugs capable to block a number of different amyloid diseases as proposed previously is a challenge [54, 76, 78].

6.1 Alzheimer's Disease

AD is a neurodegenerative process that affects 36 million people worldwide and for which there are currently no causative and effective treatments [79]. In United States alone, the cost of care is estimated to be >US$220 billion per year. AD is a leading

cause of dementia in the elderly. It was estimated that the number of people suffering from AD will double till 2050 [80]. AD is an irreversible, progressive neurodegenerative disorder clinically characterized by memory loss and cognitive decline [80–84]. The patients invariably die usually within 7–10 years after diagnosis. Currently, there is no cure for AD nor proven way to slow the rate of neurodegeneration.

It is supposed that AD is associated with the deposition of insoluble plaques in the brain mainly by two 4 kDa Aβ peptides (Aβ40 and Aβ42). The more dangerous is Aβ42 which easily aggregates. Both these peptides are produced by the action of β- and γ-secretases on the APP, which is an integral membrane protein. AD is going to become one of the most extensive and significant medical threat in the near future and there is a continuing need for development of more effective medication and diagnostics [47, 83, 85, 86].

Computational assay for quantitative prediction of Aβ aggregation inhibitors was designed [87].

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) and confocal fluorescence microscopy were used to study the spatial distributions of lipids, Aβ deposits, markers of neurons and glial cells at submicrometer lateral resolution [88]. The brain structure imaging was carried out using AD mouse model. Presence of cholesterol granules of micrometer-size was observed in hippocampal regions with Aβ deposition. These granules or specific cholesterol patterns were viewed as hallmarks for regions undergoing amyloidogenesis. TOF-SIMS appeared to be a useful technique for the investigation of molecular species in healthy and diseased mouse brain tissue.

The structure of Aβ – a model of AD – can be studied by NMR. However, the expense of the isotopically enriched peptide used for most NMR studies is problematic. Biomolecular NMR studies are mostly based on biosynthetic approaches, which provided isotopically enriched biomolecules in reasonable prices. Recently, the biosynthetic strategy has been also published for synthesis of uniformly enriched ^{15}N and ^{13}C, ^{15}N Aβ42 with yield of ca 10 mg per L of cell culture [89]. This strategy reduced the common problem — the tendency of Aβ to aggregate during purification. So prepared and purified product was stable for long periods. These achievements should enhance investigations in the field of AD.

A surface-based approach to inhibit the binding of proteins to Alzheimer's-related amyloid β (Aβ) fibrils with small molecules is reported [90]. It uses an intracellular, disease-related fibril as a material the surface of which can be coated with small molecules. Coating with 3,6-diaminoacridine demonstrated 76 $\pm$ 10% inhibition of the binding of an anti-Aβ IgG to Aβ fibrils. These protein–amyloid interactions are best inhibited in the low to mid-micromolar range. Thus, molecular surface coatings can be used to attenuate the interactions of proteins with these fibrils. This approach represents a potentially new method for therapeutics of neurodegenerative amyloid diseases.

N,*N*-Bis-(2,4-dimethoxy-9-acridinyl)-1,4-bis-(3-aminopropyl)piperazine was the best inhibitor of Aβ42 and *N*,*N*-bis-(5-methoxy-3-nitro-9-acridinyl)-1,4-bis-(3-aminopropyl)piperazine was the best inhibitor of prion propagation from the tested library [91].

Tetravalent quinacrine-like peptide conjugate based on RAFT cyclic peptide concept [92] was successfully applied as inhibitor of Aβ40 fibrillization [93]. The acridine moiety contained the same 9-amino component as quinacrine, but the acridine skeleton had only carboxylate at position 4. The dendrimer inhibited Aβ40 fibril formation at 20 μM concentration, whereas the monomeric acridine is inactive.

The accumulation of Aβ peptides in AD brain is caused by excessive production or low clearance and failings in the proteolytic degradation of Aβ peptides. Therefore, Aβ peptide degradation is a hopeful therapeutic strategy in AD therapy. Aminopeptidase from *Streptomyces griseus* KK565 (SGAK) degrades Aβ peptides but the interactive residues are needed to be understand in more details [86, 94]. The three-dimensional model of aminopeptidase (SGAK) was built using SWISS-MODEL, Geno3D and MODELLER. The interactions of Aβ peptides with the aminopeptidase were investigated by molecular docking. Further MD simulations have shown that the residues Glu^{140}, Asp^{168} and His^{255} are involved in the degradation of Aβ peptides. Therefore, the predicted three-dimensional structure of aminopeptidase (SGAK) can serve to identify the mechanism of Aβ peptide cleavage as well as to propose new lead structures to eliminate AD.

Currently many amyloid degrading enzymes (ADEs) are known such as a lot of zinc metallopeptidases, insulin degrading enzyme, neprilysin (NEP), many cysteine and serine proteinases [86]. Some of them individually in mouse models of AD have demonstrated effectively amyloid and plaque clearance. The cognitive enhancement was improved, too. Insulin-degrading enzyme, NEP, and its homologs remain as principal ADEs and recently revealed mechanisms of epigenetic regulation of NEP expression pave new avenues in handling of AD-related genes, comprising ADEs.

Experimental evidence has shown that GCPII does not degrade Aβ42 [95]. It was proven that there are elemental structural reasons for the lack of endoproteolytic activity of this enzyme. As a result, there is no direct causative link between the GCPII inhibition and the amyloid formation in vivo, as it was previously suggested [96].

On the other hand, AChE has an higher intrinsic capacity to facilitate the aggregation of Aβ40 [97]. However, when the enzyme is pure enough, it lacks protease activity responsible for cleavage of the APP to Aβ40. It seems that purification of AChE with acridinium-based affinity chromatography provided an enzyme contaminated with APP processing protease.

The neuronal enzyme AChE is considered as another target for treatment of AD. AChE also served as a nice model for X-ray studies of acridine–protein interaction (see also Sect. 5.3). AChE is inhibited by tacrine (**8**), a submicromolar non-competitive inhibitor owing to binding to hydrophobic area external of the catalytic sites. The action between tacrine and the enzyme shields the enzyme active site towards high-affinity phosphorylating agent isoflurophate [98]. Reversible inhibition of AChE can be used as protection against intoxication with organophosphates – war agent [99].

Tacrine (**8**) was licensed for the treatment of AD [100, 101]. Tacrine hydrochloride forms needle-shaped crystals soluble in water and is yellow in color. It has a bitter taste. The mechanisms of the tacrine actions are still not fully understood. However, it

is believed that the enhancement of cholinergic system is responsible for its activity. By inhibition of both AChE and BChE, tacrine increases the level of acetylcholine in CNS. Moreover, tacrine serves as a blocker of potassium channels leading to increased release of acetylcholine from cholinergic neurons [102, 103].

The anti-Alzheimer drug tacrine was incorporated into chitosan nanoparticles as a new delivery system [104]. Chitosan nanoparticles were examined in a preclinical study as a fine-tuned transport system for tacrine and the formulation exhibited optimal pharmacokinetic characteristics in a rat model of AD. Interactions of tacrine with chitosan were also studied by MD simulation [105].

Tacrine interferes with erythrocyte membranes [106]. Positive charge at position 9 of acridines was responsible for increased protein–protein interactions in both erythrocyte and synaptosomal membranes caused by tacrine and velnacrine [107]. Interaction of tacrines with membranes led also to their accumulation in the brain, i.e. their concentrations in brain were 10-fold higher than that in plasma.

The effect of tacrine on AChE and BChE was first described by Heilbronn in 1961 [108]. It was established that the compound works as a reversible inhibitor, which is partly competitive with the substrate acetylcholine and a more powerful inhibitor of BChE than AChE [109]. Besides being an enzyme inhibitor, it was found that tacrine has other mechanisms which can explain its cholinergic activity. In electrophysiological experiments with *Lymnaea stagnalis* neurons, tacrine reduced the slow outward K^+ current and therefore prolonged the action potentials. Additionally, tacrine decreased the absorption of dopamine, noradrenaline, and serotonin. Since this effect occurred at monoaminergic storage granules [110], tacrine could have monoaminergic effect [111]. Another activities of tacrine were found later: blocking of monoamine oxidase activity [112], interaction with muscarinic acetylcholine receptors [113], blockade of certain potassium ion channels [114], inhibition of neuronal uptake of 5-hydroxytryptamine (5-HT), and dopamine receptors [115].

Targeting of an anti-Alzheimer's drug tacrine in the brain utilizing polymeric nanoparticles was investigated [116]. Tacrine was applied conjugated to poly(*N*-butylcyanoacrylate) nanoparticles with or without 1% polysorbate 80 (Tween 80) coating, respectively. With the nanoparticles, higher concentration of tacrine was observed in liver, spleen and lungs in comparison with the free drug. The coating with polysorbate led to decrease of tacrine concentration in the liver and spleen, whereas a significant increase in tacrine concentration in the brain was achieved in comparison with the uncoated nanoparticles and the free drug. High tacrine concentrations reached in the brain can be used for treatment of AD. The developed formulations may also decrease the total dose needed for the therapy and reduce the dose related toxicity.

According to mouse model, tacrine can reduce self-administration of psychostimulant drugs such as cocaine and serve as a help for addicted individuals [117]. The effect was observed for doses, which are well tolerated by liver and kidney.

Tacrine is broadly used as an anti-Alzheimer's standard in many studies [118–120]. However, we can not completely cover it in details.

Recently, the tacrine–AChE inhibition assay has been improved by use of nitrogen-doped graphene quantum dots; the fluorescence of which was inhibited by products of AChE catalyzed cleavage reaction [121].

Non-parasitic platyhelminth – *Dugesia tigrina* – is a simple and low cost experimental model with the nervous system for screening of AChE inhibitor in vivo [122].

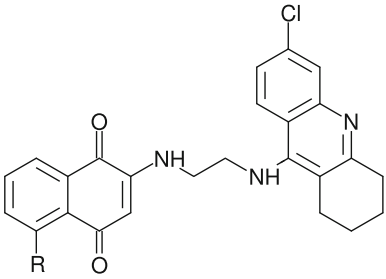
6.1.1 Multitargeted Strategy in AD

In order to combat the multifactorial nature and heterogeneity of AD the multitargeted approach has been suggested as exceptionally suitable [123–128]. The leading idea is founded on the assumption that "drugs hitting a single target may be inadequate for the treatment of diseases like neurodegenerative syndromes, which involve multiple pathogenic factors" [124, 125, 128, 129]. AD, characterized by amyloid plaques, neurofibrillary tangles, inflammatory intermediates and reactive oxygen species (ROS), causes neuronal death via a complicated array of network processes [130]. Therefore, to succeed, the therapeutic tools should be similarly complex, in order to be able to target multiple parts of the diseased network [127–129, 131–136]. Construction on this strong basis and a steadily increasing number of papers about multitargeted drug discovery have appeared in the AD field [127, 128, 137]. Memoquin was reported as one of the first small molecules rationally designed using a multitargeted strategy [138]. Its outstanding biological activities supported validity of such a strategy for obtaining valuable drug candidates against AD [139]. In vitro memoquin effectively inhibited Aβ aggregation and AChE and showed powerful free-radical scavenger activity [138]. Cognitive enhancement in many AD mouse models was observed by application of memoquin in vivo [140, 141].

Neuroactive multifunctional tacrine analogues with cholinesterase, anti-amyloid aggregation and neuroprotective properties were reviewed [84, 125–128, 142].

Tacrine–melatonin conjugate with IC_{50} 8 pmol has been identified [143].

Nepovimova et al. [144] described the design and identification of multitargeted anti-Alzheimer compounds based on combination of a naphthoquinone function and a tacrine fragment. In vitro, ca 17 compounds displayed excellent AChE inhibitory potencies and interesting capabilities to block Aβ aggregation. The X-ray analysis of AChE–**142** complex permitted logical explanation of superb activities (IC_{50} 0.72 nM) and **141** (IC_{50} 1.93 nM) (Fig. 6.2). From the compounds tested, the **141** and **142** ones belonged to the most selective in respect to discrimination between AChE and BChE. Insignificant toxicity in immortalized mouse cortical neurons Neuro2A and primary rat cerebellar granule neurons was found for both **141** and **142**. Nevertheless, only **141** was less hepatotoxic than tacrine in HepG2 cells. In T67 cells, **141** and **142** demonstrated antioxidant activity, following NQO1 induction. Moreover, in Neuro2A, they were able to totally revert the decrease in viability induced by Aβ42. A greatly significant fact is, that they crossed the blood–brain barrier, as proven in



R = H (**141**), OH (**142**)

Fig. 6.2 Multitargeted compounds suitable for AD treatment **141** and **142** [144]

ex vivo experiments with rats. Combination of ex vivo results with in vitro studies has proven that **141** and **142** are perspective multitarget lead candidates deserving of further quest.

A new group of tacrine/acridine and tacrine/tacrine dimers substituted with aliphatic or alkylene-thiourea linkers was prepared and their inhibition of human acetylcholinesterase (hAChE) and human butyrylcholinesterase (hBChE) with activity in the nanomolar range was evaluated [145]. The best AChE inhibitor with an IC_{50} value of 2 nM was 1-(1,2,3,4-tetrahydroacridin-9-yl)-3-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl] thiourea. This activity is 250-times higher than that of tacrine and 7500-times higher than 9-amino-7-methoxy-1,2,3,4-tetrahydroacridine, the compounds which were utilized as standards. Data from binding assays correlate with a dual binding site model. However, bis-tacrines possessed enhanced inhibition of topoisomerase I and II [146].

Bis-tacrine linked with 3 methylenes interacts with multiple targets [147]. It can serve as a therapeutic drug for AD and various neurodegenerative diseases. Memory and spatial learning were greatly reduced in the AD model mice generated by hippocampal injection of Aβ. Chronic application of the bis-tacrine for 21 days suppressed the memory impairments in a dose-dependent way. Moreover, the bis-tacrine analogue halted the decrease of the filopodia and synapse amounts caused by Aβ. This neuroprotective activity can be partially suppressed by a specific phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 (50 μM). Thus, these activities of bis-tacrine analogue are partially regulated via the PI3-K pathway.

Hybrids of tacrines with indanones or phthalimides were also investigated as AChE inhibitors [148]. IC_{50}s of the best inhibitors were between 2 and 3 nM i.e. ca 8-times more effective than the approved drug donepezil and ca 70-times more potent than parent tacrine.

The pro-cognitive impact of four inhibitors based on 7-methoxytacrine and mimicking donepezil was tested [149]. Tacrine and donepezil were used as common standards in T-maze assay using toxic effect of 3-quinuclidinyl benzilate. The tested compounds suppressed the toxicity of 3-quinuclidinyl benzilate at 1, 24 and 48 h test intervals. Their effect on brain cholinesterase inhibition was in the range from 5.4 to 11.3%. The most effective was *N*9-hexyl-7-methoxytacrine i.e. mono-targeted one

not resembling donepezil. (*N*-(2-{4-[(2-Bromophenyl)methyl]piperazin-1-yl}ethyl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine trihydrochloride performed in T-maze assay ca 2-times worse than tacrine [150]; however, it was ca 2-times less toxic than tacrine.

The work of Bolognesi and co-workers [144] clearly confirms the multitargeted strategy approach and indicates that structurally new and characteristic tacrine hybrids with improved AD-character can be proposed. It is now necessary to further design these hybrid compounds and scrutinize their full capacity for investigation and cure of AD. Especially, fundamental questions such as activity balancing and toxicity should be carefully inspected in the subsequent hit score to lead optimization studies.

Inhibitors of AChE based on hybrids between tacrine and coumarin were prepared with IC_{50} 15.4 and 26.3 nM, respectively [151]. Recently, others have reached IC_{50} 38 nM with simultaneous inhibition of Aβ40 aggregation [152]. These hybrids were capable to reduce metals involved in neurodegenerative diseases e.g. Cu(II) to Cu(I). Another team prepared tacrine–coumarin hybrids with inhibition of AChE with IC_{50} 33.63 nM and selective inhibition of hMAO-B with IC_{50} 0.24 μM [153]. Tacrine–chromenone inhibitors were evaluated with IC_{50} 16.17 and 7.99 μM for AChE and BACE1, respectively [154].

6-Chlorotacrine–trolox conjugate with octamethylenediamine linker provided inhibitor of AChE, which can inhibit also neurotoxic cascade via AChE induced Aβ aggregation [155]. Non-chlorinated tacrine analogues are nM inhibitors of various AChEs [156].

Subnanomolar tacrine–benzofuran inhibitors of AChE were designed [157]. *N1*-(Benzofuran-2-ylmethyl)-*N7*-(1,2,3,4-tetrahydroacridin-9-yl)-heptane-1,7-diamine inhibited hAChE (0.86 nM), hBACE-1 (1.35 μM), and Aβ aggregation both spontaneous and AChE induced ones.

A series of 9-amino-1,2,3,4-tetrahydroacridines with 4-dimethylaminobenzoic acid was prepared and evaluated as dual inhibitors of cholinesterases and Aβ aggregation [158]. Despite 4-dimethylamino-*N*-[3-(1,2,3,4-tetrahydroacridin-9-ylamino) propyl]benzamide hydrochloride was the most powerful inhibitor of AChE (19 nM), the compound 4-dimethylamino-*N*-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl] benzamide hydrochloride was advantageous candidate for development of new multifunctional drugs in the therapy of AD.

Conjugation of lipoic acid with 6-chlorotacrine provided lipocrine [159], which can exist in *(R)*- and *(S)*-isomers. *(R)*-Isomer is twice more potent inhibitor of AChE than the *(S)*-one [160]. Lipocrine is also potent inhibitor of ROS formation. When tacrine or 6-chlorotacrine were extended with 1,3-diaminopropan-2-ol, they provided scaffold for conjugations with caffeic, ferulic, and lipoic acids, respectively [161]. The conjugate of 6-chlorotacrine with ferulic acid was 20 nM inhibitor of AChE and it inhibited also Aβ42 self-aggregation. Whereas the caffeic acid analogue is weaker inhibitor of AChE (IC_{50} 150 nM), it is 7 times more potent inhibitor of BACE1 than ferulic compound.

Rational design afforded a series of tacrine–resveratrol hybrids effective as potent multitargeted ligands against AD [127]. Some compounds were submicromolar inhibitors of AChE. *(E)*-5-(4-((6-Chloro-1,2,3,4-tetrahydroacridin-9-yl)amino)-styryl)benzene-1,3-diol efficiently replaced propidium from peripheral anionic site. It was also more powerful anti-aggregating agent than the parent resveratrol. Concerning the anti-aggregation, 5-(4-(6-chloro-1,2,3,4-tetrahydroacridin-9-ylamino) phenethyl)benzene-1,3-diol was even more active. 6-Chloro-*N*-(4-(3,5-dimethoxyphenethyl)phenyl)-1,2,3,4-tetrahydroacridin-9-amine served as a promising antioxidant. However, all tested compounds were more hepatotoxic than tacrine.

Adduct of Ugi reaction (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-(8-((7-methoxy-1, 2, 3,4-tetrahydroacridin-9-yl)amino)octyl)-*N*-(2-(naphthalen-2-ylamino)-2-oxoethyl)acrylamide served as multipotent target capable to inhibit AChE (IC_{50} 51.9 nM), to penetrate into the CNS, to prevent oxidative stress, to inhibit Aβ42 self-aggregation (65.6%), and to be less hepatotoxic than tacrine profile (59.4%) [162]. Moreover, it provided promising neuroprotection against Aβ40 and Aβ42 in SH-SY5Y cells.

A set of nature-based conjugates was synthesized by connection of tacrine with *S*-allylcysteine (garlic constituent) or *S*-propargylcysteine moiety with the aim to improve the cholinergic system and neuroprotective capacity [163]. The docking studies enabled the choice of linkers to fine-tune the bimodal drug interaction with AChE. The compounds were tested for some characteristic biological properties e.g. AChE activity and Aβ aggregation inhibition. Their neuroprotective activity to Aβ and ROS-induced cellular toxicity was also examined. The best compounds for the AChE inhibition and for the prevention of superoxide production and Aβ-induced cellular toxicity contained allyl and propargyl substituted cysteine, respectively. The conjugates could interact with Cu(II) [164].

Tacrine conjugate with thioester moiety improved cognitive functions and glucose metabolism in rats suffering from vascular dementia [165].

Tacrine–(hydroxybenzoyl-pyridone) hybrids were submicromolar inhibitors of AChE and sequestrators of metals involved in aggregation of amyloids [166].

N-(1,2,3,4-Tetrahydroacridin-9-yl)-2-(3,4,5-trimethoxyphenyl)methylcarbonyl-oxyacetamide inhibited AChE and Aβ self-aggregation with IC_{50} 5.63 and 51.8 nM, respectively [167].

Trihybrids combining inhibitor of AChE, anti-oxidant agent, and inhibitor of Aβ were prepared and tested using molecular motives of tacrine, cysteine, and benzothiazole, respectively [168]. It was a successful extension of previous dihybrids between tacrine and benzothiazole [169].

1-[6-(Acridine-9-carbonyloxy)hexyl]pyridinium chloride was designed as dual inhibitor of AChE [170]. It combines esters of alkylpyridinium with acridine recognition unit. Although its activity is better than those of both individual units, it is worse inhibitor than tacrine and 9-aminoacridine.

Tacrine derivatives having piperazino-ethyl spacer connected with appropriate secondary amines and tacrine homodimer were prepared [171] and examined as cholinesterase inhibitors on hAChE and plasmic hBChE. The majority of synthesized derivatives shows a high AChE and BChE inhibitory activity with IC_{50} in the low-nanomolar range. They are obviously better than the reference standard tacrine and

7-MEOTA (7-methoxytacrine). Among them, 3-{[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]amino}-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]piperazino}-1-propanone and 3-[cyclohexyl(ethyl)amino]-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]piperazino}-1-propanone were powerful inhibitors of hAChE (IC_{50} 4.49 and 4.97 nM, respectively) and had a high selectivity to hAChE. The compound 3-(methylanilino)-1-{4-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]-piperazino}-1-propanone was the most potent inhibitor of hBChE (IC_{50} 33.7 nM). It manifested a good selectivity in relation to hBChE. The binding modes between individual derivatives and hAChE/hBChE were studied by molecular modeling.

In order to control AChE, tacrine hybrids with photoswitchable azobenzene were prepared [172]. These AChE inhibitors can be deactivated/reactivated by light. Thus, the AChE inhibition assay can have better controlled kinetic measurements.

6.2 Parkinson's Disease

PD was first described in 1817 by James Parkinson [173]. PD is the second most prevalent neurodegenerative disease, which in industrialized countries affects 1 and 4% of the population over 60 and 80 years of age, respectively [19, 174, 175]. PD is a motion disease causing tremor, stiffness bradykinesia and postural instability. Nevertheless independent studies have recently associated non-motion symptoms with PD, i.e. cognitive and behavioral changes including sleep impairments, constipation, impotence, and loss of olfaction, and neuropsychiatric disorders, suggesting the damage of multiple brain areas [176]. A substantial loss of dopaminergic neurons in the substantia nigra and accumulation of intracytoplasmic Lewy bodies that contain α-synuclein and ubiquitin are characteristic markers of PD [19, 176]. The first genetically identified PD-associated protein was α-synuclein [177]. Recent progress in the knowledge of the cooperation between the α-synuclein and intracellular trafficking in the context of PD were reviewed [175]. α-Synuclein represents the main component of Lewy bodies—intracellular protein reservoirs which are an important attribute of PD pathology—and has also been associated to early onset of familial cases of PD. α-Synuclein probably participates to disturbances in intracellular trafficking. This leads to neuronal dysfunction and, finally to death of these cells [33].

PD ranks to the group of protein misfolding diseases. Two biochemical mechanisms have been suggested for the pathogenesis of PD: (1) the intracellular accumulation and aggregation of misfolded proteins, such as α-synuclein and parkin, which are involved in the core of Lewy bodies; (2) oxidative stress produced by reactive metabolites of dopamine and changes in the concentrations of glutathione and iron in the substantia nigra leading to mitochondrial dysfunction [178–181]. Two mitochondrial proteins related to PD i.e. PINK1 and parkin have been recently identified as inhibitors of an immune-response-eliciting pathway during inflammation [182]. These proteins can serve as regulators of immune system, which can explain e.g. imbalance of CD8+ and regulatory T cells causing autoimmune mechanisms during PD [183].

In PD, besides muscle movement disturbance the affective disorders such as anxiety, depression, and phobia are observed. Depression is found in 20–40% of patients [184, 185]. The depression in PD patients seems to accelerate cognitive disability and motor slowness [186]. Therefore, use of antidepressants could reduce the problems in patients. PD is incurable, but improvement of some symptoms can be achieved by medications and surgery [175, 187, 188].

The influence of antidepressants seems to be connected with the inhibition of the formation of ROS, RNS, and the depletion of glutathione [184].

One of the main environmental factors associated with human neurodegenerative disorders such as PD are herbicides [189]. In addition, it was described [190] that the exposure to glyphosate is most probably linked to parkinsonism. Glyphosate (*N*-(phosphonomethyl)glycine) is a broad-spectrum systemic herbicide used to kill weeds, which compete with crops grown widely. Glyphosate acts by interference with the synthesis of the amino acids phenylalanine, tyrosine and tryptophan. Glyphosate causes not only apoptosis, but also autophagy in PC12 cells. This gives a new link between use of herbicide and PD.

Results from human and animal research have suggested that oxidative damage fundamentally influences neuronal loss in PD [191]. Curcumin, a strong antioxidant, obtained from the curry spice turmeric, suppresses cell death of PC12 cell induced by mutant A53T α-synuclein. Moreover, curcumin reduces intracellular reactive oxygen species (ROS) levels, cytochrome c release, mitochondrial depolarization, and caspase-3 and caspase-9 activation. Thus, curcumin is a perspective neuroprotective agent for Parkinsonism linked to A53T α-synuclein [191].

One of the products of oxidative stress – 3-nitrotyrosine – changes biophysical properties of α-synuclein derived peptides [192]. The role of site-specific nitration of α-synuclein in the pathogenesis of PD was studied using protein semi-synthesis and mutagenesis [193]. Influence of other post-translational modifications of α-synuclein on the progress of PD was reviewed [194].

Acridines are mostly used for induction of tremulous jaw movements as a model of PD [195, 196]. There are only few examples of acridine uses against PD. Despite our review omits most applications of polycyclic acridines, this was presented as an exception owing to treatment of PD.

A set of multifunctional targeted derivatives of 3-arylcoumarin-tetracyclic tacrine was prepared for the sake of PD treatment [197]. A number of tested derivatives showed significant decrease of aggregation of "human" α-synuclein protein, expressing on transgenic *Caenorhabditis elegans* model NL5901. Furthermore, many compounds were also good antioxidants and greatly increased the dopamine content in the model system. Surprisingly the protective effect of these conjugates could be arranged via activation of longevity promoting transcription factor DAF-16. Outstanding interaction of some active compounds with α-synuclein was studied in silico.

6.3 Prion Diseases

The originally heretical idea that a protein can be infectious is now generally accepted. It is promoted by many biochemical experiments including latest progress in generating TSE infectivity in vitro, and by fascinating findings related to protein conformation-based inheritance in yeast and other fungi [1–3, 50, 198, 199].

Human PrP is encoded as a polypeptide containing 253 amino acids. Cleavage of 22-amino acids from *N*-terminal and 23-amino acids from *C*-terminal signal sequences affords a functional protein with 208 amino acids which is linked to cell membrane via a glycophosphatidylinositol (GPI) anchor [2].

The paramount of the replication of mammalian prions within the concept of the protein-only theory, is the self-propagating conformational transformation of PrP^{C} to the misfolded PrP^{Sc} isomer. For this protein-based replication process two principally different mechanisms have been proposed. In agreement with the heterodimer refolding hypothesis (also termed as the template assistance hypothesis), PrP^{Sc} exists as a monomer which is thermodynamically more favorable than PrP^{C}; however, this thermodynamically preferred conformer is kinetically unavailable. Therefore, a critical step in the transformation is the complexation of PrP^{Sc} and PrP^{C} followed by PrP^{Sc} catalyzed refolding of PrP^{C} to a thermodynamically more stable PrP^{Sc}. However, there is no experimental proof for the existence of a stable PrP^{Sc} monomer. On the contrary, accessible data show that prion protein conversion is closely connected with the aggregation process, and the infectious species is oligomeric in nature. This second model is called nucleated polymerization mechanism. It supposes that the conversion between PrP^{C} and PrP^{Sc} is reversible, and the PrP^{Sc} monomer is less stable than PrP^{C}. Stabilization of PrP^{Sc} can be observed only upon formation of a stable oligomeric core. Despite its formation is thermodynamically not favorable, once the core is formed other PrP^{C} join it easily. In this mechanism, the rate-limiting step is the nucleation step [2].

The preparation of synthetic prions sensitive to proteolysis causing transmissible diseases, represents an important step for understanding the role of protease-sensitive forms of PrP^{Sc} in the pathogenesis of prion diseases. The pathogenicity of $sPrP^{Sc}$ (soluble PrP^{Sc}) requires to redefine some concepts for description of different isoforms of PrP, such as PrP^{res} and PrP^{sen} [200, 201]. PrP^{res} is often called as PrP^{Sc}, and PrP^{sen} as PrP^{C}. However, it was shown that PrP^{Sc} was presented in both forms as protease-resistant and protease-sensitive prion. Thus, protease resistance cannot serve itself to label prion protein as prion. In order to label it as prion, infectivity assays are necessary requisite to avoid confusion and separate the terms $sPrP^{Sc}$, $rPrP^{Sc}$, and PrP^{C} [200, 201].

In the absence of yet unknown factor X, the transmission of prion disease has not been possible [9, 202, 203]. The mechanism of transmission with factor X is depicted in Fig. 6.3.

Recently, several connections among neurodegenerative diseases have been described such as induction of AD-like neuropathologic changes caused by prions [204], and impairment of synaptic plasticity caused by prion protein and Aβ

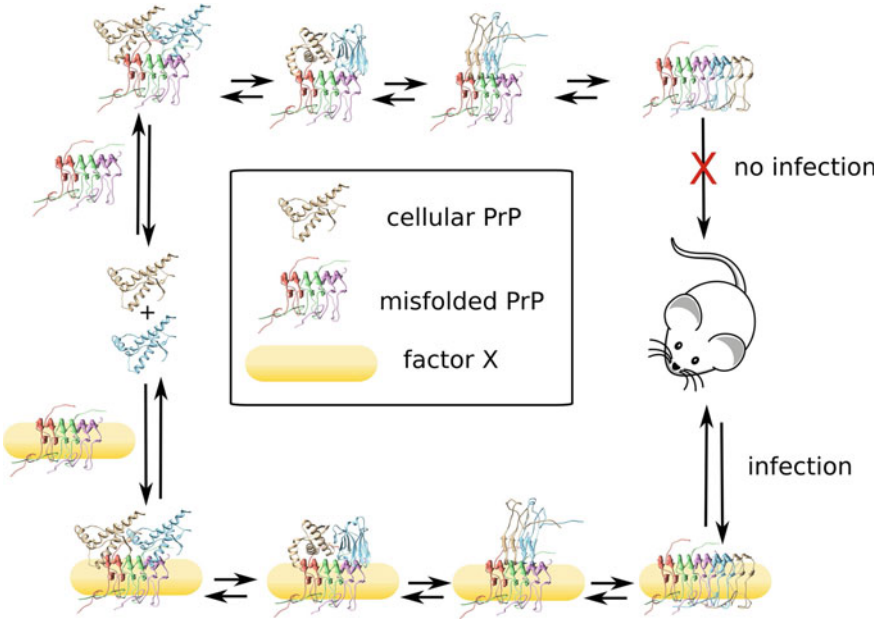


Fig. 6.3 Plausible function of factor X during conversion of PrP^{C} into PrP^{Sc}. The generation of an infectious PrP depends on permanent binding of factor X to PrP^{Sc}, causing stabilization of this structure. The resulting complex propagates and produces disease upon in vivo transmission, whereas in the absence of factor X, PrP^{Sc} only aggregates into biologically inactive rubbish [9, 202, 203]

oligomers interactions [205]. However, long term memory impairment was independent of cellular prion protein [206].

Detection methods of prion proteins and prions were developed to be as sensitive as ca 10^4 to 10^5 molecules i.e. 0.02–0.2 amol [15].

At present, prion inhibitors have mainly been tested using PrP^{Sc}-infected cells. Real time-quaking-induced conversion (RT-QuIC) is a complementary screening assay [199]. The spreading of inhibition effects of known antiprion compounds was evaluated using RT-QuIC and was compared with the results from a PrP^{Sc}-infected cell test. After 22 h treatment, amplified signal dropped down in wells treated with either acridine or tannic acid. Observed activities in RT-QuIC were confirmed by western blot in a dye-independent conversion assay. Therefore, RT-QuIC allowed more specific classification of inhibitors based on their action: suppression of prion propagation versus decline of amplified aggregates. RT-QuIC shows the restrictions of cell-based screening assays and applies further aids for comprehension of the mode of action of prion inhibitors.

Very interesting, although poorly understood attribute of TSE is existence of multiple prion strains. This typically leads to distinct disease phenotypes which can be distinguished by clinical symptoms, incubation times, and neuropathological

patterns. Because multiple strains are able to coexist within the same species, the phenomenon of prion strains cannot be explained by different amino acid sequences. Prion strains can also undergo to mutations and/or adaptation to external selection pressure [2].

It was proven that quinacrine is able to reduce occurrence of prion toxicity in cell culture chronically intoxicated with prion [207]. Interestingly, one dose of quinacrine can heal the cell culture overexpressing prion protein from prion infection [208]. Quinacrine binds to *C*-terminal helix of prion protein most probably due to interaction with abundant aromatic amino acids such as Tyr^{225} and Tyr^{226} [209]. However, the drug failed in clinical trials for patients with CJD or new variant of CJD [43, 44] and also in animal models [45, 46].

A complex with 4.6 mm dissociation constant between quinacrine and isotopically labeled human prion protein globular domain 121–230 is formed. The constant is nearly four orders of magnitude weaker than the effective antiprion concentration. Thus, the drug can be accumulated in cells where it displays its activity [209]. The range of binding was 1–5 mm at various laboratories [209–213]. However, with better models of prion protein HuPrP90-231 [214] and His-mPrP23-231 [208], μM and sub-μM dissociation constants were observed. The affinity of quinacrine to prion protein was utilized for design of acridine radio tracer with potential to visualise prion plaques in brain [215]. In the most potent radio tracing binder, the ^{125}I is used instead of 6-chloro substituent in quinacrine and the amino substituent at position 9 is exchanged with dimethylamine. The dissociation constant was ca 43 nM.

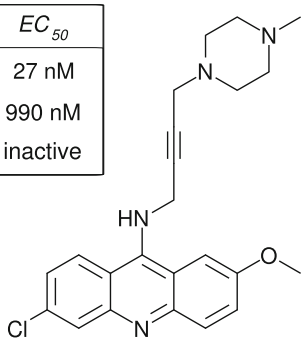
We have shown that quinacrine and other 9-aminoacridine derivatives could serve as a source of acridine moiety for aromatic nucleophilic substitution on C9 of acridine ring. The peptides and proteins can covalently modify the ε-amino group of lysine and sulfhydryl group of cysteine residues [208, 216–218]. Such reaction could explain an accumulation of quinacrine-like drugs in living systems. Moreover, the deactivation of 9-aminoacridines in prion treatment by glutathione was described by the same mechanism [208, 218]. Furthermore, the acridine–glutathione conjugate spontaneously decomposed to practically insoluble acridone [208]. Interestingly, cysteine protease inhibitors suppressed also scrapie-associated prion protein accumulation [219]. Another pathways of quinacrine metabolism lay in *N*-desethylation and *O*-demethylation [220]. Human cytochrome P450 isoforms CYP3A4/5 were responsible for the mono desethylation. Quinacrine is effectively cleared from brain by P-gp transportation.

The prion protein model – peptide HuPrP106-126 – is capable to form cation channels into phospholipid bilayers. Quinacrine plays a role of inhibitor of these channels [221].

Screening of a number of compounds on a variety of cell culture models that accumulate and chronically propagate PrP^{Sc} [222] lead to discovery of a number of substances with antiprion properties. An antiprotozoal drug quinacrine belongs among the most effective substances. Several groups have reported an IC_{50} in the range from ~0.25 to 0.5 μM in neuroblastoma cells [207, 218, 219, 223–225].

Quinacrine derivatives more potent than quinacrine in protection of neuronal cells against prion caused cell death were discovered [214, 225]. Some identified

Prion Strain	EC_{50}
ScN2a	27 nM
N167	990 nM
F3	inactive



143

Fig. 6.4 Comparison of activity of quinacrine and compound **143** can show information about specific prion strain [225]

analogues possessed prion strain specific response [225] (Fig. 6.4), i.e. **143** is ca 10-times more potent than quinacrine at ScN2a strain, ca 2-times less potent than quinacrine for N167 strain, and practically inactive for F3 strain, whereas quinacrine retained ca one tenth of its activity against ScN2a strain also for F3 strain [225].

Multitargeted drug strategy (see Sect. 6.1.1) is also applied for treatment of prion diseases. Quinpramine - a hybrid compound between quinacrine and tricyclic antidepressant - is potent inhibitor of prion disease via destabilization of detergent-resistant membrane compartments [226]. Further a library of related compounds was evaluated [26].

Several bis-acridinylated derivatives were tested for their antiprion activity [42]. The results have proven that the minimal distance between two 9-aminoacridine amino groups of highly active compounds is 10 Å. Due to Le Pecq limit of critical region [227, 228], this value gives evidence in favor of requirement that nearly all active antiprion bis-acridinylated agents could form bis-intercalators.

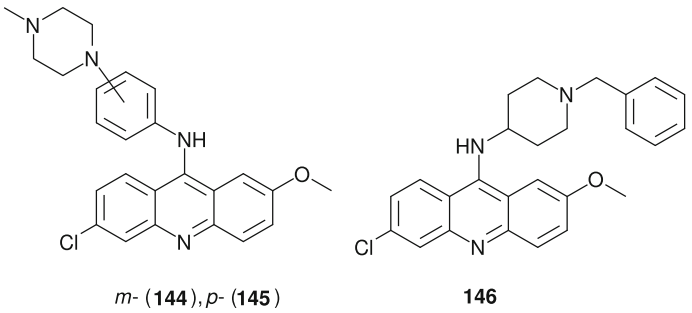
Because quinacrine is a non-competitive inhibitor of the nicotinic acetylcholine receptor, it can also interfere with neuronal signal transmission [229].

Structure-activity study of quinacrine derivatives was performed [230]. It has shown that replacement of diethylamino group by di-*N*-propylamino group, methoxy group by trifluoromethyl group and chloro group by methoxy group afforded derivatives with activity similar to quinacrine, nevertheless, with reduced toxicity.

Tetravalent acridine conjugate with tetraphenylporphyrin core had 16 fold enhancement of antiprion activity than the quinacrine itself [231]. Generally, these dendrimers are multivalent inhibitors of prion propagation. This opens new ways towards structure-based understanding of prion biology. Quinoline or acridine containing dendrimers were only sparingly soluble in water. This is an obstacle for their delivery via the bloodstream to central nervous system.

Conjugates of anti-oxidant lipoic acid and 9-aminoacridine moieties resembling quinacrine or 6-chlorotacrine were active against prions [232]. Quinacrine resembling derivatives were ca 2–3 times more active than the quinacrine itself.

2,5-Diamino-1,4-benzoquinones linked with 3 methylene units to 6-chlorotacrine provided antiprion activity with IC_{50} 0.17 μM [233].



m- (**144**), *p*- (**145**) **146**

Fig. 6.5 Lead compounds with good antiprion activity from quinacrine-like library [26]

A library of synthetic 6-chloro-2-methoxyacridin-9-amines structurally related to quinacrine was synthesized and screened for antiprion activity on four cell models permanently infected with scrapie prion strains (ScN2a, Ch2, N167) or a human disease prion strain (F3) [26] (Fig. 6.5). The side chains such as dialkylaminoalkyl and phenyl were attached to 9-amino group of the acridine ring. The most favorable derivative was 6-chloro-2-methoxy-*N*-(4-(4-methylpiperazin-1-yl)phenyl)acridin-9-amine (**145**) with EC_{50} values in the submicromolar range (0.1–0.7 μM) on all cell models studied. Besides, it was able to clear PrP^{Sc} at non-toxic concentrations of 1.2–2.5 μM, and its activity was better than that of quinacrine in terms of EC_{50} values. Other lead compounds were **144** (a regioisomer of **145**) and **146** which had a 1-benzylpiperidin-4-yl substituent attached to the 9-amino function. These side chains have not been previously assigned to antiprion activity and can be perspective leads for further optimization of antiprion activity.

Libraries of acridines, 2-methylquinolines, and 2-phenylquinazolines with same amine modifiers were tested for prion proteins binding by surface plasmon resonance [234]. The acridines outperformed other series.

6.4 Other Neurodegenerative Diseases

ALS belongs to fatal neurodegenerative diseases characterized by aggregation of TAR DNA-binding protein-43 (TDP-43) in neuronal cells [235], which leads to symptoms such as motor neuron dysfunction and muscle atrophy. The carboxyl-terminal prion-like domain of TDP-43 has propensity to form toxic β-sheet containing amyloid-like structures. Up to now, treatment possibilities for ALS are very restricted to riluzole, which influences glutamate receptors and is the unique but mostly ineffective drug. From this reason, great concern exists in development of ALS therapy. Certain acridine derivatives including the same substituents at position 4 and 5, were examined as inhibitors of TDP-43 aggregation. [4,5-Bis(*N*-carboxymethylimidazolium)methylacridine] dibromide substantially suppresses

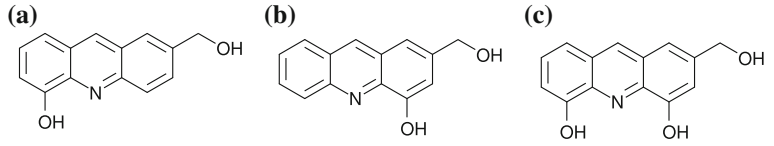


Fig. 6.6 Acridine alkaloids as potential neural regenerative medicine [239]. Inubosin A (**147**), B (**148**), and C (**149**)

TDP-43-YFP aggregation in yeast model and also eliminates in vitro amyloid-like aggregation of carboxyl terminal domain of TDP-43. Therefore, it can serve as a lead structure for further therapeutic research of ALS.

Multiple sclerosis belongs to the most common inflammatory demyelinating and neurodegenerative diseases of CNS in humans [236]. Acridine–iminodibenzyl hybrids were previously used as a class of cholesterol-redistributing substances with antiprion effects [26, 226], where the quinpramine was identified as a lead compound. Quinpramine was applied for treatment of an animal model of multiple sclerosis with preventive and therapeutic functions [237]. Quinpramine suppressed inflammatory CNS lesions, antigen-specific T-cell proliferation, and concentration of proinflammatory cytokines IFNγ and IL-17. It has potential to become a pharmaceutical candidate for multiple sclerosis treatment.

Formation of pancreatic islet amyloid correlates with occurrence of advanced type II diabetes mellitus. AO can inhibit formation of amyloids from human amylin in dose dependent manner [238].

Bioassay-guided fractionation afforded three new acridine alkaloids, inubosins A, B, and C [239] (Fig. 6.6). These alkaloids induced neurogenin 2 (Ngn2) activity. Ngn2 is an activator-type transcription factor responsible for neural stem cell differentiation. The structures of inubosins were proven using 1D and 2D-NMR experiments. Inubosin B showed the highest Ngn2 promoter activity from all three compounds tested. Furthermore inubosin B increased mRNA expression of genes related to neural stem cell differentiation. These alkaloids can serve as new therapies promoting neurogenesis and can improve nervous system diseases by supporting growth of new neurons. Thus, neural regenerative medicine for clinical treatment can be achieved soon.

Glutamate-induced neuronal damage plays a role in several neurological disorders such as stroke, epilepsy and neurodegenerative diseases such as AD, PD and HD [240]. Ionotropic glutamate receptors can cause apoptosis or necrosis, when they are over-stimulated [241, 242].

A useful identification of neuroprotective compounds against oxidative glutamate toxicity in vitro is the neuronal cell death model based on the HT22 mouse hippocampal cells. Substituted acridin-9-yl-phenylamines protected HT22 cells from glutamate toxicity at concentrations less than 1 μM [243]. The Aryl-NH-Aryl moiety that is incorporated in these compounds served as the minimal pharmacophore for desired activity. The mechanism of protection against the endogenous oxidative

stress caused by glutamate did not include up-regulation of glutathione concentration but weakening of mitochondrial ROS production and reduction of intracellular calcium concentrations. In this regard the NH group in the pharmacophore plays a decisive role as seen from the disappearance of neuroprotection in its absence. That the same NH was essential for radical scavenging in cell-free and cell-based systems pointed to an antioxidant basis for the neuroprotective activities of these compounds. The essential role of the same NH for radical scavenging in both cell-free and cell-based systems indicates an antioxidant basis for the neuroprotective activities of these compounds. However, the acridine derivatives were less effective than nanomolar inhibitor *N*,*N*-diphenyl-*p*-phenylendiamine.

Conjugate of 6-chlorotacrine with caffeic acid and propylene diamine linker suppressed glutamate-induced cell death in HT22 cells via stimulation of Nrf2/ARE/HO-1 pathway, an endogenous prosurvival signaling pathway [244].

3-Amino-9-thioacridone via inhibition of cyclin-dependent kinase 4 (CDK4) influenced CDK/pRb/E2F pathway of neuronal cell death [245]. In cerebellar granule neurons, 3-amino-9-thioacridone suppressed the kainic acid-induced apoptosis [246].

Bis-tacrine linked with 7 methylenes prevented glutamate-induced neuronal apoptosis through NMDA receptors. In cultured rat cortical neurons, the bis-tacrine suppressed glutamate-induced excitotoxicity with IC_{50} 20 nM i.e. it was ca 35-fold more active than memantine [247]. Moreover, the bis-tacrine was more potent than memantine in buffering the intracellular Ca^{2+} triggered by glutamate.

Bis-tacrine linked with 3 methylenes is a non-competitive NMDA receptor antagonist with fast off-rate characteristic. In cultures of rat cerebellar granule neurons, 2 h pre-incubation with this bis-tacrine (IC_{50} 0.45 μM) suppressed glutamate-induced excitotoxicity 10 times stronger than memantine (IC_{50} 4.58 μM) [248]. Moreover, this pre-incubation inhibited the increase of intracellular nitric oxide and the activation of phosphorylated ERK, and converted the suppression of phosphorylated Akt and GSK3β generated by glutamate. Additionally, this neuroprotection was eliminated by phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002. The bis-tacrine generated neuroprotection against glutamate-induced neurotoxicity via pathway independent on inhibition of NO and MAPK/ERK.

The overexpression of the G protein-gated inwardly rectifying K^+ channel subunit ($K_{ir}3.2$) occurs in various phenotypes of Down syndrome. Blockers of $K_{ir}3.2$ could cure the symptoms of Down syndrome. Therefore, screening assay for evalution of $K_{ir}3.2$ blockers activity was developed [249] using a K^+ transporter-deficient yeast strain. The strain produced a constitutively active $K_{ir}3.2$ mutant. The mode of action of potent blockers was electrophysiologically examined using K_{ir} channels expressed in *Xenopus* oocytes. Proflavine was studied as an inhibitor of the growth of $K_{ir}3.2$-transformant cells and $K_{ir}3.2$ function. The current blockade was powerful when membrane potentials were higher than equilibrium potential of K^+. In the opposite case, the blockage was influenced by the difference between membrane potential and $[K^+]$. Various acridines served as prototypes of $K_{ir}3.2$ blockers ordered with decreasing potency: aminacrine > proflavine > acridine yellow > acriflavine ≫ acridine.

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Chapter 7
Some Application of Selective Toxicities of Acridines

Abstract Inhibitions of pathogen specific enzymes with acridines play roles in cure of various diseases. Thus, acridines can serve for treatment of various tropical diseases caused by protozoal parasites e.g. African sleeping sickness, antibacterial agents for combating e.g. tuberculosis, antiviral agent for combating HIV. Moreover, interactions with immune system can overcome failure of human immune system suppressing either cancer or massive autoimmune response. In this chapter, we briefly describe the role of acridines in fight with tropical diseases, cancer, and bacterial infections.

Acridines can inhibit various enzymes, when inhibition of specific enzyme for pathogenic system is achieved, the inhibitors can play important roles as potential drugs for eradication of unwanted pathogens or pathogenic conditions. Thus, acridines can be used as a cure for various tropical diseases caused by protozoal parasites e.g. African sleeping sickness, antibacterial agents for combating e.g. tuberculosis, antiviral agent for combating HIV, but also for dealing with failure of human immune system suppressing either cancer or massive autoimmune response. In this chapter, we briefly describe the role of acridines in fight with tropical diseases, cancer, and bacterial infections.

7.1 Antiparasitic Drug

Hundreds of millions of people worldwide suffer from parasitic diseases which constitute major health problems, especially in underdeveloped countries. Medication is extremely difficult owing to the existence of drug resistance, the absence of efficacious vaccines, and the dissemination of insecticide-resistant vectors. Therefore, identification of affordable and readily accessible drugs against resistant parasites is of worldwide demand [1–5].

Parasitic protozoa of the Trypanosomatidae family cause many tropical diseases such as African sleeping sickness (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*), Chagas disease (*Trypanosoma cruzi*), Nagana cattle disease

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(*Trypanosoma congolense* and *Trypanosoma brucei brucei*), leishmaniasis, Kala-azar (*Leishmania donovani*) and oriental sore (*Leishmania tropica*) [1–4]. *Trypanosoma*, *Leishmania* and *Crithidia* are protozoan parasites which belong to the order kinetoplastidae [6]. They originate from the most ancient eukaryotic lineages. The mortality of these diseases mainly occurred due to lack of both accessibility and availability of treatment and growing resistance of the protozoa to the limited variety of approved drugs. Sleeping sickness (human African trypanosomiasis) is spread in sub-Saharan Africa. The tsetse flies serve as main vector for transmission of the infective protozoan parasite *T. brucei* [7, 8]. Nearly all cases are caused by *T. b. gambiense*, which is native to west and central Africa. With less than 12,000 cases of this severe disease annually described, trypanosomiasis is one of the most neglected tropical diseases. An excellent overview of clinical and epidemiological features of Chagas disease, with an emphasis on information concerning *T. cruzi* and Chagas disease in the USA, was published [9]. Cardiomyopathy in the Americas is mainly consequence of Chagas heart disease [10]. Another threat of society is caused by malaria and related diseases caused by parasites from *Plasmodium* [11]. *Plasmodium vivax* can be also transmitted by transfusion [12]. The suppression and eradication of malaria is especially important in Amazonia located countries like Brazil. The clinical manifestation is complex, and exact diagnosis and effective treatment difficult. Nowadays used drugs are old, their administration is complicated, and they are connected with severe adverse reactions [13, 14]. Therefore, new diagnostic techniques and effective and safe drugs are crucially needed [7, 8, 15].

A hopeful access for development of antiparasitic drugs is to focus to enzymes with unique metabolic pathways that are present solely in the pathogens [1, 2, 4, 15–18]. A well-known phenomenon is the sensitivity of a plenty of parasites to oxidative stress [5]. That is the reason why production of reactive oxygen species (ROS) or deceleration of endogenous antioxidant enzymes could be a novel impetus for therapeutic approach to design new antiparasitic drugs. Unique highlights emphasize metabolic pathways together with redox enzymes of unicellular (*Plasmodium falciparum*, *T. cruzi*, *T. brucei*, *L. donovani*, *Entamoeba histolytica*, and *Trichomonas vaginalis*) and multicellular parasites (*Schistosoma mansoni*), which could be applied to endorse ROS mediated toxicity. Potential objectives for drug development can be enzymes connected with various vital redox reactions. The pinpointing of redox-active antiparasitic drugs together with their mechanism of action will play a key role in designing novel drugs in the future. The nearly ubiquitous glutathione redox system is replaced in trypanosomatids by the trypanothione system [1, 2, 4, 15–18]. Whereas the glutathione processing is carried out by the flavoenzyme glutathione reductase in mostly all eukaryotes and prokaryotes, in trypanosomatids this role is played by trypanothione as the dominant low molecular-mass thiol which is maintained in its reduced form by trypanothione reductase. This unique trypanothione system defends the parasites from oxidative stress. Thus, enzymes responsible for trypanothione processing could serve as selective targets for antiprotozoal drugs [15–17, 19, 20]. Peroxidases including peroxiredoxins are major players protecting *T. cruzi* against macrophage- and endogenously-derived peroxynitrite [21–23]. The protozoan parasite *T. cruzi* and fungi display similarities that concern their sterol

lipid biosynthesis, because ergosterol and other 24-alkylated sterols are its primary endogenous sterols. The sterol metabolism can therefore serve as a potential drug target for the therapy of Chagas disease [24]. Lipoamide dehydrogenase is not specific for parazites; however some selective inhibitors can be also developed [16]. Another enzyme critical for parasite (*P. falciparum*) survival is L-lactate dehydrogenase (PfLDH) [4, 25–27]. PfLDH is a key enzyme of the anaerobic glucose fermentation and serves in the last step of glycolysis, where it assists in reduction of pyruvate to lactate with NADH. Since enzyme kinetics and structure of PfLDH very much differ from its human LDH analogues, PfLDH is an attractive drug target for the treatment of malaria [4, 28–30].

Acridines serve as privileged structures enhancing the drug discovery efficacy in the area of protozoan diseases [4, 31] due to cost-effective approaches and less demanding intellectual property concerns. In addition, owing to their inherent affinity for many targets, privileged structures serve as starting points for the discoveries of novel multi-target compounds. Moreover, acridines belong to the most potent antiprotozoal drugs due to selective interactions with protozoal enzymes and processes. History of antimalarial drug development has been reviewed. From the series of more than 300 acridines, the quinacrine was one of the best drugs [4, 32–35]. Synthesis of quinacrine was covered by patent [36]. Quinacrine possesses strong trypanocidal activity against *T. cruzi* and suppresses the transfer of Chagas disease by blood transfusion. Quinacrine selectively inhibits *T. cruzi* trypanothione reductase without effect on human glutathione reductase. Specific pair-wise interactions between functional groups of the drug and the trypanothione reductase [37] concern those residues that are not conserved in human enzyme [38] (Fig. 5.5).

The synthetic access to many inhibitors of parasitic cysteine protease and trypanothione reductase was reviewed [4, 39]. Development of new anti-trypanosomal agents together with new drug design strategies and also a deep insight into the understanding of the mechanisms of action of new trypanocidal agents were reviewed [15]. Despite of only few cases of acridine presented, the reviews [17, 39, 40] could serve as an inspiration for design of acridine carriers for antiparasitic drugs.

The structure of the complex between trypanothione reductase from *T. cruzi* and quinacrine was solved at 2.9 Å resolution [41]. However, the structure is not available via RCSB PDB database. The database contains only the structure of quinacrine-mustard with the enzyme (see also Sect. 5.3). Four amino acid residues in the disulfide substrate-binding site of trypanothione reductase Glu^{18}, Trp^{21}, Ser^{109}, and Met^{113} are of key importance for inhibitor binding. The acridine ring of quinacrine is located at the active site in proximity to the hydrophobic wall created by Trp^{21} and Met^{113}.

The combination of two structural features of known trypanothione reductase inhibitors — quinacrine and diaryl sulfides — leads to cooperative interactions with two hydrophobic patches from the active site [2]. The cooperative binding was proposed by molecular docking study of compound **150** with trypanothione reductase (Fig. 7.1). According to molecular docking, the acridine ring is located in the quinacrine binding site, and the thiophenyl residue aims toward the hydrophobic area around Cys^{52} and Tyr^{110}. Both NH groups of **150** are involved in hydrogen bonds with Glu^{18}, and the protonated piperazine nitrogen interacts with $Glu^{465'}$ and

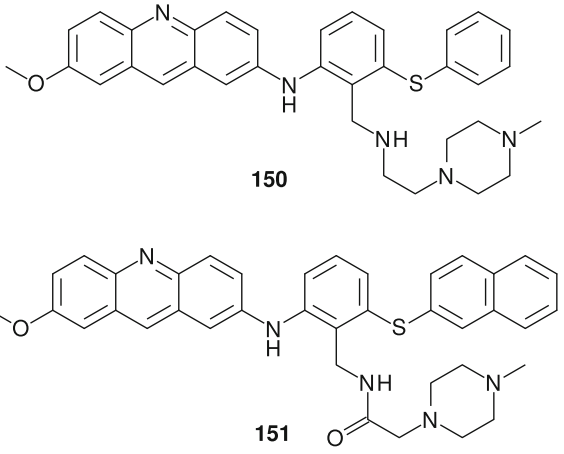


Fig. 7.1 Powerful inhibitors of trypanothione reductase. Compound **150** could be a suitable drug lead, whereas compound **151** served for staining of *T. b. rhodesiense* in fluorescence microscopy [2]

$Glu^{466'}$. In comparison with the corresponding parent inhibitors, the binding efficacy of these conjugates is enhanced. They inhibited the parasite enzyme with K_{ic} down to $0.9 \pm 0.1\ \mu M$ and manifested high selectivity for trypanothione reductase in comparison with human glutathione reductase. Regardless of their significant molecular mass and permanent positive charges of some compounds, in vitro experiments demonstrated IC_{50} in the low micromolar to sub-micromolar range against *T. b. rhodesiense* and *T. cruzi*, as well as the malaria parasite *P. falciparum*, which lacks trypanothione metabolism [42] and instead, parasite specific glutathione reductase is involved. The inhibitors show strong fluorescence because of their aminoacridine moiety [2]. The high accumulation of **151** (Fig. 7.1) was visible by fluorescence microscopy even after rapid exposure.

Trypanothione reductase from *T. cruzi*, the causative agent of Chagas disease is competitively and irreversibly inhibited by 9-aminoacridines and (terpyridine) platinum(II) complexes, respectively [1]. Syntheses of four chimeric compounds with 2-methoxy-6-chloro-9-aminoacridine bound to the (2-hydroxyethanethiolate) (2,2'; 6',2"-terpyridine)platinum(II) complex were described and investigated as inhibitors of trypanothione reductase. The compounds varied by the nature and/or the size of the linker joining the two aromatic systems. All four derivatives served as μM inhibitors of trypanothione reductase. The conclusion that the connection of a competitive and an irreversible inhibitor could create reversible mixed type inhibitors emphasizes the problems related to inhibitor design originating from available crystallographic and kinetic data on inhibitors of trypanothione reductase.

A group of 9-amino and 9-thioacridine analogues resembling quinacrine have been synthesized and tested as inhibitors of trypanothione reductase from *T. cruzi* [43]. The 9-aminoacridines played a role of competitive inhibitors of trypanothione reductase with apparent K_i in range between 5 and 43 μM. The best inhibitors were those with the chloro and methoxy groups of quinacrine and amino or $NHCH(CH_3)(CH_2)_4N(Et)_2$ at C9 with K_i 7 and 5.5 μM, respectively. Detailed kinetic

data show that multiple 9-aminoacridines can bind to the enzyme. In contrary, the 9-thioacridines inhibit trypanothione reductase with mixed-type kinetics. Due to allosteric inhibition of the enzymes, structurally very similar acridines can have totally different inhibition mechanisms, thus molecular design of new inhibitors is very challenging.

Inhibitors of *T. cruzi* trypanothione reductase were designed as bis(2-aminodiphenylsulfides) with three side chains [44]. The most active inhibitor contained the myristoyl moiety (IC_{50} 200 nM). The derivative 2-(*N*-4-[*N*-(acridin-9-oyl)]aminobutanoylimino)-*N,N'*-(3-2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanylphenyl)diacetylamide, where the myristoic acid was replaced with acridine-9-carboxylic acid possessed almost the same activity (IC_{50} 250 nM) and provided fluorescence for biological investigations. Furthermore, the compound had EC_{50} 17 μM, 1.8 μM, and >12.5 μM against *T. cruzi*, *T. brucei* and *L. infantum*, respectively. Cytotoxicity towards mouse peritoneal macrophages CC_{50} was >25 μM. Interestingly, correlation between inhibition of trypanothione reductase and the in vitro trypanocidal effect was not observed. The acridine derivative was not cytotoxic and manifested the greatest activity against *T. brucei*. The acridine compound served for uptake and cellular localization studies into *T. cruzi* epimastigotes.

Quinacrine mustard served as a lead structure for identification of potent inhibitors of Chagas disease based on combination of structural data with molecular docking [20].

10-Allyl-6-chloro-2-fluoro-9(*10H*)-acridinone was prepared by Ullmann–Goldberg reaction [45] and tested for its in vitro trypanocidal activity [46]. The EC_{50} 50 μM was not sufficient for potential trypanocidal drug. Nevertheless, the new mechanism causing *T. cruzi* death via targeting of the parasite's mitochondrion selects the compound as a lead for further drug development.

Twenty-four acridine derivatives were tested for trypanocidal activity in *T. brucei* [47]. Among them some azidoacridine derivatives could prolong survival of infected mice under conditions of photodynamic therapy. The most effective 3-amino-6-azido-10-methylacridinium chloride demonstrated substantial trypanocidal activity at 0.1 μM concentration i.e. prolonged survival from 4.4 days of control to 30.0 days in the treated mice under irradiation. The effect practically disappeared without irradiation i.e. the survival was prolonged only to 6.4 days. A disadvantage is photochemical instability of azidoacridines, i.e. preexposure with light suppressed their activities. The acridines most effective as trypanocides were compared with those most potent in production of mitochondrial damage in yeast. These acridines were the same. Therefore, probable site of acridine trypanocidal activity could be the large specialized mitochondrion of the trypanosome which is named the kinetoplast.

Antiparasitic activity of synthetic dicationic acridone derivatives was determined. Acridones manifested in vitro nanomolar IC_{50} values against *T.b. rhodesiense* with selectivity indices >1000 [48]. The activity of compounds **153**, **154**, and **155** (Fig. 7.2) was fully comparable with the reference drug – melarsoprol. The activities observed against wild-type (NF54) and resistant (K1) strains of *P. falciparum*, were

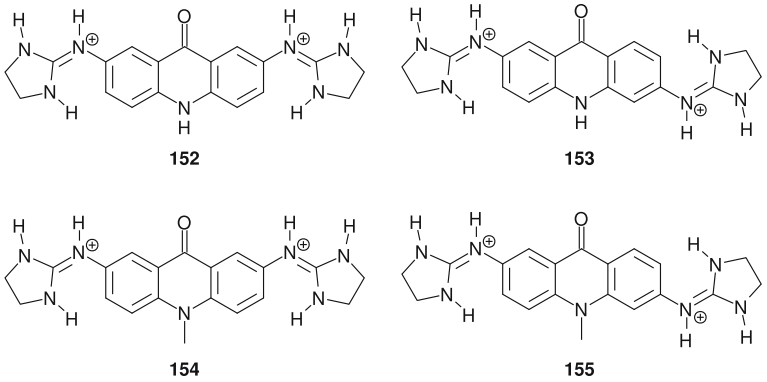


Fig. 7.2 Dicationic acridones **152**, **153**, **154**, and **155** active against *T. b. rhodesiense* [48] with IC_{50} 69, 7, 7, and 8 nM, respectively

in the submicromolar-range. No significant activity was detected against *T. cruzi* and *L. donovani*. Derivatives **152** and **153** were curative in the STIB900 mouse model for human African trypanosomiasis. UV spectrophotometric titrations and circular dichroism (CD) experiments with fish sperm DNA showed that these compounds form complexes with DNA with binding affinities in the 10^4 M^{-1} range. Both biological and biophysical data show that DNA binding, antiparasitic activity and toxicity of this compounds are driven by the relative position of both imidazolium cations on the heterocyclic scaffold. E.g. activities of the derivatives against *T. b. rhodesiense* decrease in the order of 2,6 > 2,7 ≫ 3,6 substituents.

Many amphiphilic cationic drugs inhibited growth of *T. cruzi* at concentrations below 1 mM with similar activity against epimastigote and trypomastigote forms [49]. Epimastigotes promptly accumulate acridines at the beginning in discrete subcellular organelles. An incorporation of the drug during 15 min of incubation is satisfactory for following lysis of both trypomastigotes and epimastigotes. Trypanocidal activity depended on drug exposure time and the extracellular pH (optimum ≧ 8) but not on the temperature, red blood cell density, and serum dilution. The exposure to 3.7 μM quinacrine only lysed trypomastigotes after 168 h. The problem of parasite survival in stored blood was emphasized.

Prepared 6-chloro-2-methoxy-*N*-[4-(3-nitro-*1H*-1,2,4-triazol-1-yl)-butyl]acridin-9-amine was active against *T. cruzi* and *T. b. rhodesiense* with IC_{50} 151 and 134 nM, respectively [50]. Selectivity indices were above 66 for reference cell line of rat skeletal myoblasts (L6 cells). In the series of 3-nitrotriazole with aromatic amines, their potency decreases as follows: acridines ≧ quinolines > 1,5-naphthyridines > quinazolines.

During the growth of intracellular malaria parasite in host erythrocyte, host cytosolic proteins are ingested in "digestive vacuole". It is believed that antimalarial drugs are accumulated in the digestive vacuole [51]. The parasite resistance towards antimalarial drugs was attributed to expression of various proteins such as Pgh1 and

PfCRT in the digestive vacuole membrane as well as to the changes of pH in digestive vacuole [52–54]. However, in a model of *P. falciparum*-infected human erythrocytes stained with AO, the fluorescence was localized in the parasite cytosol and not in the parasite's digestive vacuole [55].

Arylacridin-9-yl sulfones show an antimalarial activity on *P. falciparum* [11]. This action was explained by structural similarity with dapsone and sulfonamides. The evidence is that the activities are independent to the presence of *p*-aminobenzoic acid (PABA) unlike to dapsone and sulfonamides, which are inhibitors of PABA uptake. Second, even if sulfonyl group is significant for the activity, there is no correlation with either pK_{HB} [56] or charge density on oxygen atoms. Finally a good correlation was observed between the activity and fission of the *S–C9* bond. The influence of pK_a of the benzene sulfinic acids created after the cleavage is smaller. The conclusion is that acridine ring contributes to a specific activity of arylsulfones. The most active compound was 2-methoxy-6-chloro-9-[(4'-*N*,*N*-dimethylamino-methyleneamino)-phenylsulfonyl)]-acridine with IC_{50} 4 and 6.5 μM against 3D7 and W2 strains, respectively.

Undoubtedly some mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT) modify the parasite's sensitivity to diverse drugs. Complexation of PfCRT with tricyclic anti-malarial compounds were studied [57]. Chloroquine resistance-providing isoforms of PfCRT decreased the sensitivity of the *P. falciparum* to quinacrine and AO. The mutant (PfCRTDd2) and wild-type (PfCRTD10) variants of the transporter were assessed. In chloroquine-resistant parasites, AO and quinacrine augmented the parasite's accumulation of, and predisposition to, chloroquine. All compounds bound to PfCRTDd2, and the transport of quinacrine with this protein was saturated and inhibited by the known chloroquine resistance-reverser verapamil. The study demonstrated that the PfCRTDd2-mediated transport of tricyclic antimalarials decreased the parasite's sensitivity to these drugs.

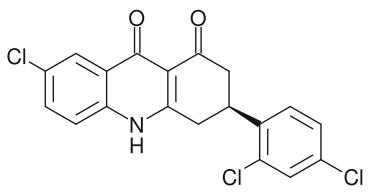
Because of its low expansion in most regions of the world, *Plasmodium malariae*, the cause of quartan malaria, has attracted somewhat little attention in the experimental chemotherapy of malaria. Infection caused by *P. malariae* is clinically and epidemiologically manifested less than those caused by *P. falciparum* or *P. vivax*. On the other hand, the *P. malariae* in the peripheral blood stream persists to the drugs longer than the other malaria parasites. The effects of quinacrine, quinine, and chloroquine, on this species of malaria parasite were compared [58]. Treatment with chloroquine led to the first negative blood after the first day of treatment, whereas quinacrine provided the same effect on the fourth day. The quinine was even less effective and the treatment resulted to the parasite removal on the fifth day. Inspite of the fact that the percentage of parasites removed in the first 24 and 48 h in comparison with those of *P. vivax* looks favorably, it lasted longer to clear the blood stream of the few remaining parasites. The activities (IC_{50}) of drugs against the *P. falciparum* strain 3D7 were 46, 56, and 62 nM for chloroquine, quinacrine, and quinine, respectively [59]. There is significant tolerance of other *P. falciparum* strains for chloroquine e.g. for K1 strain, chloroquine and quinacrine have IC_{50} 569 and 100 nM, respectively. Quinacrine thus can serve as a cure for these resistant strains.

Synthesis of forty bis(9-amino-6-chloro-2-methoxyacridines) and examinations of their biological activities against the erythrocytic stage of *P. falciparum*, trypomastigote stage of *T. brucei*, and amastigote stage of *T. cruzi* and *Leishmania infantum* were carried out [60]. The influence of the linker on biological activities was investigated. The data obtained were compared with their cytotoxic effects upon MRC-5 cells. Many tested compounds were without cytotoxicity to MRC-5 cells at 25 μM, one had IC_{50} values ranging from 8 to 18 nM against various *P. falciparum* strains whereas three others completely inhibited *T. brucei* at 1.56 μM. Bridge with the piperazine scaffold had unique properties such as strong and selective effect to *Plasmodium*, a localization outside of the food vacuole, binding mainly to the parasite nucleus, and an absence of cytotoxicity to MRC-5 cells and murine macrophages. Polar groups in the side chain of the linker switched off antimalarial activity whereas it switched on the activity against *T. brucei*. Four compounds were identified as safe and cheap leads against *P. falciparum* and *T. brucei*.

1-(2-Dimethylaminoethylamino)-9(*10H*)-thioacridone fitted into the chloroquine binding site of PfLDH and was potent inhibitor of *P. falciparum* with IC_{50} 1.4 μM [61].

Dihydroacridinediones serve as selective antimalarial drugs, which inhibit parasite mitochondrial bc1 complex [62]. Inhibition of the bc1 complex causes a lost of the mitochondrial membrane potential and subsequent cell death. The most powerful compound **156** (Fig. 7.3) killed the *P. falciparum* with IC_{50} 15 nM and inhibition constant of *P. falciparum* bc1 enzyme was 0.3 nM. Noteworhty, therapeutic index of **156** is higher than 4600. The compound is more powerful than racemic floxacrine **157**.

Pynacrine is an acridine analog of the schizontocidal antimalarial drug, pyronaridine. The hematin-targeting properties of pynacrine were tested to explain the role of the benzonaphthyridine moiety [63] (Fig. 7.4). Pynacrine (**158**) and pyronaridine (**159**) had the same activity in inhibiting glutathione-induced hematin degradation and in enhancing hematin-mediated membrane lysis. Pynacrine created a 1:2 complex with hematin; however it was 50-fold less potent inhibitor of β-hematin formation. Nevertheless, pynacrine and pyronaridine are equal inhibitors of intraerythrocytic *P. falciparum* growth in culture.



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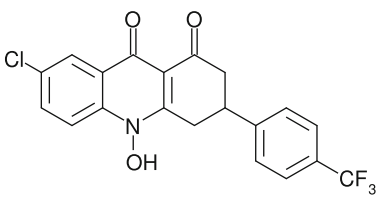

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Fig. 7.3 *(S)*-Acridine dione **156** and racemic floxacrine **157** are powerful inhibitors of parasite mitochondrial bc1 complex [62]

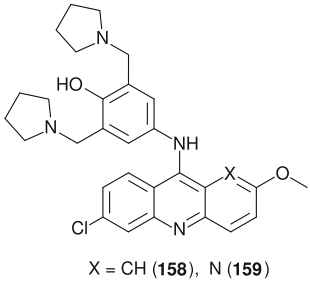

Fig. 7.4 Structures of pynacrine (**158**) and pyronaridine (**159**) [63]

9-Aminoacridine and artemisinin–acridine conjugates were synthesized and tested in vitro for antimalarial activity against both the chloroquine sensitive and resistant strains of *P. falciparum* [64]. The hybrids were prepared by a microwave-assisted method. The prepared compounds were found active against both the *Plasmodium* strains and displayed superb selective toxicity in relation to the parasitic cells. Conjugate containing ethylenediamine linker was the most active of all of the synthesized compounds. Its anti-gametocytocidal activity was seven-fold higher in comparison with chloroquine and was also seven-fold more potent than chloroquine against the Dd2 strain, with very selective action towards the parasitic cells. This conjugate also demonstrated favorable anticancer activity against the HeLa cells, three- and eight-fold higher than those of chloroquine and melphalan, respectively. That's why this conjugate may serve as drug candidate in the search for efficient drugs against malaria and cervical cancer.

The antimalarial activities and physical properties of derivatives of 1,2,3,4-tetrahydroacridin-9(*10H*)-one were studied by QSAR [65]. The models have suggested that parameters MLOGP, LP1 and JGI6 are three common important features for activity against W2 and TM90 strains.

Design of antiparasitic drugs is challenging. Small variations of substituents led to totally different activities. E.g. 3-amino-9-((4'-aminophenyl)amino)acridine has in vitro antimalarial activity with IC_{50} 0.1 μM, whereas it is inactive against *T. lewisi* at concentrations of up to 10 μM [66]. It can be more generalized that 9-anilinoacridines with NH_2 groups at C3 and C6 were inactive against *T. lewisi*, but they had strong activity against *P. falciparum*.

Five acridine derivatives were synthesized and tested in CD1 mouse on *P. berghei* [67]. The best derivative, 9-(2,6-dioxopiperidin-1-yl)-1,2,3,4-tetrahydroacridine healed the mices at 50 μmol/kg. The highest tolerated concentration was between 100 and 150 μmol/kg.

The capability of several acridine derivatives and other heterocyclic compounds to prevent incorporation of radioactivity from exogenous AMP-8-^{3}H to the DNA and RNA of erythrocyte-free malarial parasites (*Plasmodium berghei*) was explored [68]. Acriflavine was the most potent inhibitor preventing the 50% incorporation at 1–10 μM, whereas AO achieved the same effect at nearly 100 μM. Non-acridine dye – acridine red was even less effective leading to the same effect at 1 mM concentration.

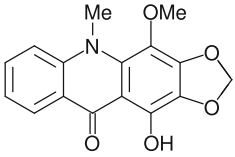

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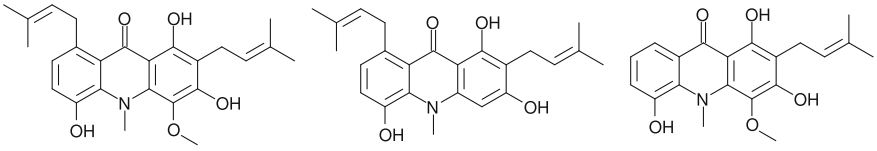
Fig. 7.5 Normelicopidine, an acridone alkaloid with anticancer and antiprotozoal activities [69]



161 162 163

Fig. 7.6 Acridone alkaloids from *Swinglea glutinosa* (Bl.) Merr. **161**, **162**, and **163** with activity against *P. falciparum* 3D7 with IC_{50} 2.6, 2.6, and 0.3 μM, respectively

A member of the Rutaceae family, *Zanthoxylum simulans* Hance, is a popular natural spice. It belongs to the most used herbs in traditional Chinese medicine. The methanolic root bark extract of *Z. simulans* Hance afforded besides 4-methoxybenzoic acid, daucosterol, β-sitosterol, also five acridone alkaloids: normelicopidine, normelicopine, melicopine, melicopidine, and melicopicine [69]. All five acridone alkaloids exhibited cytotoxic and antimalarial activities in vitro. From the alkaloids tested, normelicopidine (Fig. 7.5) was the most potent against two prostate cancer cell lines PC-3M and LNCaP, and Dd2 strain of *P. falciparum* with IC_{50} values of 12.5, 21.1, and 18.9 μg/mL, respectively.

Many acridone alkaloids separated from *Swinglea glutinosa* (Bl.) Merr. were tested for in vitro activity against chloroquine-sensitive *P. falciparum* 3D7, *T. brucei rhodesiense* STIB900 and *L. donovani* L82 [70] (Fig. 7.6). Nine of them had IC_{50} values ranging from 0.3 to 11.6 μM against *P. falciparum*. In contrast, only a small number of compounds demonstrated significant activity against *T. brucei rhodesiense*. None had activity against *L. donovani*.

The life cycle of protozoan parasites from kinetoplastidae requires the diversity of genetic events, in which topoisomerases play a crucial role in cellular processes influencing the topology and organization of intracellular DNA [6]. Thus, topoisomerases serve as important targets owing to their indispensable function in cell biology.

The derivatives of 9-anilino-3,6-diaminoacridine were evaluated for their activity against a multidrug resistant K1 strain of the malaria parasite *P. falciparum* in erythrocyte suspensions [71]. 3,6-Diamino substitution on the acridine ring resulted in lower mammalian cell cytotoxicity and higher antiparasitic activity than other substitution patterns. These compounds were potent inhibitors of the DNA strand-passing activity of human topoisomerase II and the DNA decatenation activity of the

corresponding parasite enzyme. In contrast, the compounds without the 3,6-diamino substitution were inactive in both assays up to 100 pM. Overall, there was a positive relationship between the ability of the drugs to inhibit parasite growth in culture and their ability to inhibit parasite topoisomerase II activity in an isolated enzyme assay.

N-Alkylaminoacridine derivatives linked to nitrogen heterocycles served as inhibitors of heme biocrystallization of *P. falciparum* [72]. This heme removal is crucial for detoxification process of the parasite, which is essential for *P. falciparum* survival. These compounds were also inhibitors of *Sulfolobus shibatae* topoisomerase VI. Heme biomineralisation can be prevented also by pyrrolidine-acridine hybrids [73].

A set of 9-anilinoacridine topoisomerase II inhibitors with anilino substituents was active against the amastigote and promastigote forms of *Leishmania major* [74]. A 3,6-diNMe$_2$ substitution type on the acridine greatly enhanced toxicity to *L. major* without significant mammalian toxicity. This increased the therapeutic index over that of the lead structure. The 2-OMe, 6-Cl acridine decoration typical for antimalarial quinacrine also provided antileishmanial activity and high therapeutic indices. Subsets of the most active derivatives against *L. major* were also tested against *L. donovani*, *T. cruzi*, and *T. brucei*; however, no consistent SAR was observed in these models. The most active compound against *L. major* was 3,6-bis(dimethylamino)-9-(4-phenylamino)acridine. Interestingly, it had also the highest therapeutic index. The second most active was 3,6-bis(dimethylamino)-9-[[4-(*N*-hexyl-*N*-methylamino)-phenyl]amino]acridine.

9-Chloro and 9-amino-2-methoxyacridines bearing different substituents in position 7, together with their corresponding dimeric and tetrameric derivatives had strong in vitro antiproliferative properties against *L. infantum* [75]. Their effects on DNA synthesis raised the hypothesis that DNA metabolism constituted their main target in *Leishmania* promastigotes.

Quinacrine is also a powerful inhibitor of parasitic protozoa *Giardia lamblia* (IC_{50} 0.19 μM) and *E. histolytica* (IC_{50} 1.1 μM) [76]. *G. lamblia* colonizes the upper small intestine [77], whereas *E. histolytica* normally occupies the large intestine [78].

A screening of focused compound library with 18 members for bioactivity against seven protozoan and one helminth parasite species served as a powerful tool for identification of potentially effective antiparasitic compounds [79]. This strategy selected many bis-acridine derivatives with bioactivity against more than one parasite and relatively low cytotoxicity toward mammalian cells. Interestingly, for bioactivity of polyamine-connected bis-acridines a minimum linker length of approximately 10 Å is required. The "magic" distance is similar for effective bis-intercalation [80, 81], as well as, for powerful antiprion activity [82]. However, it also caused increased cytotoxicity toward mammalian cells [79]. The compounds obtained can be useful candidates for drug discovery. For example, compound bearing two azoacridine units from pyronaridine linked with spermidine is very active against *P. falciparum* W2 resistant toward chloroquine with EC_{50} 34 nM and activity against *T. b. brucei* had EC_{50} 1.4 nM [79].

One of the approaches how to accelerate drug discovery is to scrutinize new applications for present approved drugs [83]. This process is called 'drug repositioning'

or ‘drug repurposing’. In recent years, this process has become more attractive. The literature data analysis affords many instances of FDA-approved drugs with activity against multiple targets (by other words promiscuous) which can serve also as therapeutic advantage for repurposing for many neglected and rare diseases. This method was also applied for several acridine based drugs such as quinacrine, acrisorcine, and tacrine [83].

One of the possible approaches to combat infectious diseases is photodynamic therapy, which applies a non-toxic dye, so called a photosensitizer, and visible light [84]. In the presence of oxygen, this combination led to production of cytotoxic species.

For use of acridines for staining and detection of parasites in blood see also Chap. 10.

7.2 Cancer Treatment

Selective toxicity of acridines was also applied for treatment of cancer and tumor associated diseases. Some aspects of these uses were already discussed in sections about interactions of acridines with nucleic acids 4 and nucleic acid processing proteins 5.1. See also other reviews [4, 18, 85–87].

Traditional antitumor treatment is often hampered by the emergence of the so-called cancer stem cells (CSCs). Their features are low metabolic rates and high resilience to nearly all current therapies. CSCs are the reason of many problems of clinical oncology and a low efficiency of current treatments [88, 89]. The development of new compounds capable of removing both fast proliferating tumor cells and standard treatment-resistant CSCs are therefore of utmost importance. Thus, synergistic action of acridines is required to at least three fundamental molecular cascades necessary for tumor progress such as the p53, NF-κB, and HSF1 metabolic pathways. Moreover, some acridines inhibited FACT (facilitates chromatin transcription protein complex).

Renal cell carcinomas (RCC) usually maintain wild-type but functionally inactive p53, which is suppressed by a different mechanism [88]. From the library, 9-aminoacridine, amsacrine and quinacrine were selected. They strongly induced p53 in RCC and various cancer cells. Evocation of p53 by these compounds does not result in genotoxic stress and is provided by suppression of NF-κB. In the contrary to compounds that target IκB kinase 2, 9-aminoacridine and quinacrine efficiently repress both basal and inducible functions of NF-κB. Thus, they serve as inhibitors that switch NF-κB from a transactivator into a transrepressor. p53 function in RCC was restarted by both ectopic expression of a superrepressor of IκB or by aminoacridines. The complete or partial repression of p53 in many tumors could be caused by activation of NF-κB. This indicates anticancer use for the well known antimalaria drug quinacrine.

AO has unique biological activities. It is a useful fluorescent dye specific for DNA and RNA, antitumor and antimalarial drug, a pH indicator, photosensitizer, and

detector of bacteria and parasites, apoptosis and sperm mobility [90]. AO accumulates in musculoskeletal sarcomas and after irradiation with low-dose X-rays, or lighting of the tumors with visible light the dye quickly produces selective cytocidal effect against the sarcoma cells. That's why surgery combined with radio- or photodynamic therapy with AO has been used to human musculoskeletal sarcomas [90]. It was explained that AO, depending on the acidity, accumulates in acidic organelles or structures, especially lysosomes of cancer cells. Application of AO in translational research of photodynamic therapy focused to cancer acidity was reviewed [91].

The photochemical anticancer therapy of Ultra Violet-C (UV-C) radiation on the fluorochrome, AO, in human breast cancer cells (MCF-7) was studied [92]. AO under UV-C shows increased dose dependent cytotoxicity with efficient ROS generation at higher doses in comparison with light and dark toxicity. Apoptotic studies display that increased exposure to UV-C of intracellular AO greatly elevates the Sub-G1 fraction and morphological modifications were distinct in comparison with the control cells. UV-C was an optimal light delivery system for AO mediated photo-chemotherapy of cancer.

Ultrastructural changes caused by (*Z*)-2-(acridin-9-ylmethylene)-*N*-phenylhydrazinecarbothioamide [93] treatment on human breast adenocarcinoma cancer cells MCF-7 were investigated, together with the assessment of phosphatidylserine externalization and DNA splitting in treated cells [94]. Analysis of cell viability demonstrated concentration and time dependent cytotoxicity. Treated MCF-7 cells did not expose phosphatidylserine residues to the outer plasma membrane surface and DNA fragmentation was not observed. Vacuolization occurred after this treatment with 60 μM acridine derivative. Electron microscopies detected characteristic features of autophagy, particularly the presence of membrane blebbing and autophagosomes, besides shriveled cells and cell debris in treated MCF-7 cells.

The photo effect of new proflavine derivatives, 3,6-bis((1-alkyl-5-oxoimidazolidin-2-yliden)imino)acridine hydrochlorides (AcrDIMs), with antitumor and DNA-binding activities, was tested as a possible photosensitizer for photodynamic antitumor therapy [95]. In accord with EPR measurements, superoxide radical anion and singlet oxygen were produced after irradiation of AcrDIMs with UV-A light (>300 nm) in the presence of oxygen. This shows that AcrDIMs can act as photosensitizers. Most active were pentyl-AcrDIM and hexyl-AcrDIM. They displayed photocytotoxic effect toward the lymphocytic leukemia cell line L1210 in mouse and human ovarian cancer cells A2780. Irradiation of A2780 cells (365 nm, 1.05 J/cm^2) increased antitumor activity of pentyl-AcrDIM about 12 times (72 h incubation). The photocytotoxicity appears to be connected with oxidative stress. Study of intracellular localization of pentyl-AcrDIM proved its partial accumulation in mitochondria and lysosomes.

Anaplastic thyroid cancer (ATC) accounts for more than one third of thyroid cancer–related casualties. ATC is often resistant to traditional therapy, and NF-κB signaling was suggested as a feature of the disease. An activity of the antimalarial drug quinacrine, which is known to target NF-κB signaling, was evaluated together with the clinically relevant kinase inhibitor sorafenib in ATC cells [96]. Increased expression of NF-κB–p65/RELA and Mcl-1 was observed in the nucleus of a set of

ATC in comparison with nonneoplastic thyroid. The combination of quinacrine and sorafenib is well tolerated in mice. At the molecular level, these compounds suppressed expression of prosurvival Mcl-1, pSTAT3, and lowered NF-κB signaling. Median survival times of the mouse with ATC were more than doubled when combination of sorafenib and quinacrine was used in comparison to the case when sorafenib or quinacrine were applied separately. Thus, the synergistic effect of sorafenib and quinacrine is evident. This simultaneous application had lowered toxicity in mice and triggers potent antitumor reaction.

The reaction of 9-aminoacridine derivatives with 9,10-dihydroanthracene-9,10-α, β-succinic anhydride led to various imine products at room temperature [97]. The similar imine formation was also achieved with phthalic and succinic anhydride under microwave-assisted condensation. Substances *N*-(3-methylacridin-9-yl)-9,10-dihydro-9,10-ethanoanthracene-11,12-dicarboximide, *N*-(2-methoxyacridin-9-yl) -9,10-dihydro-9,10-ethanoanthracene-11,12-dicarboximide, 2-(3-methylacridin-9-yl)isoindoline-1,3-dione, and 1-(2-methylacridin-9-yl)-1*H*-pyrrole-2,5-dione were active against various cancer cell lines: breast T47D, lung NCl H-522, liver Hep G2, and colon HCT-15 with IC_{50} values 5.4, 4.2, 4.5, and 2.4 μM, respectively.

Biological activities of an anticancer drug nitracrine (**8**) and its congeners were reviewed [98]. Its potency was ascribed to cytostatic effects through the interactions of the drug with DNA. It was suggested that the reduction of the nitro group of nitracrine is one of the activation steps leading to the drug covalent binding to DNA and proteins both in subcellular systems and in the cell. DNA–drug non-covalent interactions and covalent conjugates were examined in several model systems and compared with the properties of a number of derivatives with programmed structural changes. The DNA–protein crosslinks and interstrand crosslinks were detected in the cells following exposition to the drug. Nitracrine exhibited selective toxicity and radiosensitization effects to hypoxic mammalian cells.

1-Nitroacridines such as nitracrine and 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine display good antitumor properties [35]. They are in clinical trials against prostate cancer.

The synthesis of three bis-acridines with antitumor activity against L1210 murine leukemia was described [99]. Two acridine rings were linked via dicyclohexylmethylene tether. The most active isomer was *cis-cis*.

Acridine–thiazolidinone derivatives have been prepared and their mutual influence to calf thymus DNA and cancer cell lines such as leukemic cells HL-60, L1210, and human epithelial ovarian cancer A2780 were examined [100, 101]. The compounds tested had high affinity to calf thymus DNA with binding constants in the range of $1.37 \times 10^6 - 5.89 \times 10^6\,M^{-1}$. All derivatives tested were strongly cytotoxic in vitro. The highest activity in cytotoxic assays was encountered for methyl 2-[2-(acridin-9-ylimino)-3-(4-bromophenyl)-4-oxo-1,3-thiazolan-5-yliden]acetate with $IC_{50} = 1.3 \pm 0.2\,\mu M$ (HL-60), $3.1 \pm 0.4\,\mu M$ (L1210), and $7.7 \pm 0.5\,\mu M$ (A2780) after 72 h incubation. The acridine derivatives were very fast accumulated into the cancer cells. The incorporation led to significant changes of the glutathione concentrations. The compounds served as inhibitors of cell proliferation

and inductors of an arrest of the cell cycle and cell death. Their activity was explained by their reactivity with thiols and binding to DNA.

Antiproliferative activity and mechanisms of the action of several aminoacridines were tested [102]. Six diverse tumor cell lines were applied to assess the cytotoxic effect of prepared aminoacridines. MTT test was used for cell bioavailability assay. Moreover, the potential cytotoxic effect of the tested derivatives on non-cancer cells was examined in rat skeletal muscle myotubes (L6) and in bovine aortic smooth muscle cells. The DNA binding studied by CD and DNA T4 ligase assay was correlated with cytotoxic effects. The mutagenic activity of the lead compound – 2-((acridin-9-ylamino)methyl)benzoic acid – was examined. The DNA binding mode of the lead compound is different than that of the parent one. The lead compound is more cytotoxic than *m*-amsacrine; however, it is not cytotoxic to cancer free cells. At similar concentrations, 9-aminoacridine and amsacrine are very toxic for normal cells.

The synthesis, biological activity and biochemical properties of a series of 9-substituted acridine derivatives having a reactive alkene moiety were delineated [103]: 9-[*(E)*-2-phenylethenyl] acridine and methyl (*2E*)-3-(acridin-9-yl)-prop-2-enoate. Calf thymus DNA binding constants were determined in the range of 1.9×10^5 to 7.1×10^5 M^{-1} for these DNA intercalators. These ligands inhibit topoisomerase I at a concentration of 5 μM and are cytostatic for leukemia cell line L1210, and ovarian carcinoma cell line A2780. Conjugation with Cys may suppress the cytotoxicity. Methyl (*2E*)-3-(acridin-9-yl)-prop-2-enoate is more reactive with thiols than 9-[*(E)*-2-phenylethenyl]acridine. This is another proof of the pivotal role of polarity in the interaction with detoxification agents such as glutathione or cysteine.

9-Arylmethyleneaminoacridines were prepared [104] and tested for antiproliferative activity against K562 and HepG2 cell lines. *N*-((Pyridin-2-yl)methyl)acridin-9-amine possessed good antitumor activity against both cell lines, DNA binding and topoisomerase I inhibition. Further chemical optimization can lead to compounds with better solubility and bioavailability.

The 9-aminoacridine (**4**) at low concentrations (1–10 μM) specifically suppressed growth and induced apoptosis in human melanoma A375 cells and AT-2 rat prostate cancer cells, but did not influence the survival and growth of normal human skin fibroblasts in cell culture [105]. Molecular mechanisms responsible for the different responses of normal and cancer cells to compound **4** at low concentrations need to be explained. Thus, possible powerful anticancer drugs are overlooked. Moreover, acridine **4** can be principally delivered into the cells using magnetic iron oxide/mesoporous silica core/shell nanoparticles [106].

The toxicity of *N*-acridin-9-yl caproyl amide [107] was tested on human oral squamous carcinoma cell line SAS. It arrested mitosis in G2/M phase at 5.3–10.6 μM and induced polyploidy at 21.2 μM. The compound showed synergy with X-irradiation to suppress clonogenic survival of SAS cells.

Acridinediones with three aromatic substituents acted as inhibitors of HepG2, whereas HeLa cells were not affected in submillimolar range [108].

Bis-acridine derivatives have been synthesized by condensation of 9-chloro-2, 4-(un)substituted acridines or 9-isothiocyanato-2, 4-(un)substituted acridines with two different diamines [109]. These bis-acridines were tested in vitro for activity against

a group of human cancer cell lines of lung (NCI H-522), ovary (PA1), breast (T47D), colon (HCT-15), and liver (HepG2). Several bis-acridines have good anticancer activity against diverse cancer cell lines. Of these, (2-methoxyacridin-9-yl)-[3-(9-{3-[(2-methoxyacridin-9-ylamino)]-propyl}-2,4,8,10-tetraoxa-spiro[5,5]undec-3-yl)-propyl]-amine showed good anticancer activity against all cancer cell lines evaluated with exception of liver (HepG2) cell line. Moreover, these compounds were tested for anti-inflammatory activity at a dose of 50 mg/kg p.o. Derivative (2-methyl-acridin-9-yl)-[3-(9-{3-[(2-methylacridin-9-ylamino)]-propyl}-2,4,8,10-tetraoxa-spiro[5,5]undec-3-yl)-propyl]-amine exhibited 41% anti-inflammatory activity, which is slightly better than the most ordinarily used standard drug ibuprofen, which demonstrated 39% anti-inflammatory (at 50 mg/kg p. o.) activity.

Derivatives of 5-methylacridine-4-carboxylic acid were synthesized and tested for antiproliferative activity [110]. The DNA binding characteristics of the synthetic acridines were analyzed by competitive dialysis and compared with the antiproliferative activities. Whereas the most active 5-methylacridine-4-carboxamide derivatives had high affinity for DNA but showed poor specificity, the inactive acridine derivatives possessed high selectivity for G-quadruplex structures. Connection of two units of the 5-methylacridine-4-carboxamide via a threoninol phosphate backbone afforded the most active compound. Interestingly, the binding activity was not suppressed by the negative phosphate backbone. These results show that connection of several intercalating units having a negative backbone opened new horizons for design of DNA intercalating drugs, because the DNA binding affinities are not suppressed and the compounds have higher solubility in biological fluids.

Acridine drugs can be combined with bioactive compounds such as DNA repair protein inhibitors to suppress tumor resistance and enhance outcomes [35].

Some acridinone derivatives with antitumor activity were examined by principal component analysis to study correlation between structural descriptors and lipophilicity and their antileukemia activity. Principal component analysis provided two main factors describing more than 90% of simulated property based on lipophilicity [111]. Interestingly, distribution of tested drugs on the chart determined by two and three principal components showed patterns in good agreement with chemical structures and antitumor activity.

Quantitative structure-activity relationships (QSAR) studies of antitumor activity of acridinone derivatives were studied by multivariate adaptive regression splines (MARSplines) [112]. Molecular modeling studies were carried out with HyperChem and Dragon software. First, geometries of the compounds were preoptimized with the molecular mechanics and semi-empirical AM1 method in the HyperChem. Second, the geometries were evaluated with Dragon software. Several molecular descriptors of acridones were selected as predictor (independent) variables in the MARS model setup. Principal component analysis was applied for the training and test sets. The optimal MARS setup possesses 28 basis for description of acridones antitumor activity. It is characterized by high correlation between predicted and experimental antitumor activities.

Acridinone derivatives like triazoloacridones and imidazoacridones represent new potent antitumor agents with diverse mechanisms of action related to their ability to

interact with DNA. QSAR and QSRR (Quantitative Structure- Retention Relationship) models for prediction of the biological activity of acridones were evaluated [113]. They showed stabilization of the secondary structure of DNA (ΔT) predicted from structural parameters and chromatographic retention data. Therefore, 20 acridinone derivatives underwent chromatographic analyses and molecular modeling, followed by statistical analyses using multiple linear regression method (MLR). As an innovation aspect, the chromatographic parameters were obtained by RP-HPLC and HILIC-HPLC. Convincingly, the conjunction of QSAR and QSRR models in silico predicts interactions of acridones with DNA without the need of any biological assays both in vitro and in vivo. Later, the chromatography system was improved using combination of column with stationary phases: phosphatidylcholine (IAM) and α1-glycoprotein (AGP) [114]. The obtained retention parameter $logk$ describes binding affinity of acridinones to phospholipids or proteins. Appropriate correlation of retention data with structural parameters allows for quite reliable prediction of acridone crosslinking of interstrand DNA.

Radiolabeled acridines were prepared for treatment of melanoma [115]. Two ^{125}I-radiolabeled acridine derivatives (*N*-(2-diethylaminoethyl)-9,10-dihydro-7-iodo-9-oxoacridine-4-carboxamide hydrochloride salt and *N*-(2-diethylaminoethyl)-5-iodoacridine-4-carboxamide dihydrochloride salt) were successfully tested for Auger-electron radionuclide therapy of melanoma [116]. Acridines with a higher nuclear localization were better candidates for application in targeted radionuclide therapy using ^{125}I [117].

Pentyl- and hexyl derivatives of 3,6-bis(3-alkylguanidino)acridines were tested for cytotoxicity against three cell lines: a human neuroblastoma cell line SH-SY5Y, a human ovarian carcinoma cell line A2780, and a murine immortalized fibroblast cell line NIH-3T3 [118]. These acridines were cytotoxic to NIH-3T3 and A2780 lines but they demonstrated only a negligible cytotoxicity to SH-SY5Y cells. Selective toxicity of the acridines was explained by difference in cellular localization. Whereas the acridines are localized in nuclei of NIH-3T3 and A2780 cells, they cannot reach the nucleus of SH-SY5Y cells. AChE activity in the SH-SY5Y cells diminished after incubation with the acridines. According to kinetic study, these acridines inhibited AChE by the same way as tacrine. A low cytotoxicity of the acridines to SH-SY5Y cells can be explained by their binding to AChE which is abundant mainly at the plasma membrane.

N-Cinnamoylated analogues of quinacrine, which were previously identified as powerful dual-stage antimalarial leads, can be used as well for combating cancer [119]. These compounds possessed antiproliferative activities and targeted cancer cells over healthy ones more than quinacrine. E.g., 2-methoxy-6-chloro-9-(4-(4-fluorocinnamoylamino)-butyl)aminoacridine was selective against the gastric cancer MKN-28 cell line. This compound was extensively trapped by MKN-28 cell monolayers.

One of the essential but not sufficient requirements for successful cancer therapy is overcoming drug resistance. Tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) is a powerful therapeutic as an activator of apoptosis, especially in tumor but not in healthy cells. Nevertheless, its efficiency is restricted by the resilience

of tumors to the therapeutic drug. This limitation was overcome by the design of a controlled release system [120]. The system was based on matrix-metalloproteinase (MMP)-sensitive and Arg-Gly-Asp-Ser peptide derivatized poly (ethylene-glycol) (PEG) particles. The synthesis proceeds via visible-light-induced water-in-water emulsion polymerization. TRAIL sensitizer drug quinacrine was incorporated into the particles and the role of this nanodevice in the induction of apoptosis in malignant type of brain cancer, glioblastoma multiforme (GBM) was studied. MMP-sensitive hydrogels were superior to accelerate TRAIL-induced apoptosis in GBM cells when loaded with quinacrine. Synergistic apoptotic inducing behavior was observed. It was enhanced when quinacrine was loaded into gel particles. This synergistic behavior also stimulated different apoptotic pathways which leads to new possibilities for treatment of resistant GBM cells with dual targeting strategy.

Acridine **173** was capable to penetrate blood–brain barrier and to cure malignant glioma cells [121].

3-(4-[4-(Acridin-9-ylamino)phenylthio]phenyl(3-hydroxypropyl)amino)propan-1-ol (CK0403) was cytotoxic to human breast cancer cells due to induced G2/M arrest [122–124]. The growth inhibition of breast cancer cell lines with CK0403 was in the following order: SKBR3≧MDA-MB-231>BT-474≧MCF-7 with range of LC_{50} from 0.07 up to 1.06 μM. Whereas non-cancerous human breast cell line MCF-10A had improved survival with LC_{50} 4.25 μM. In previous study, similar compound 2-(4-[4-(acridin-9-ylamino)phenylthio]phenyl(2-hydroxyethyl)amino)ethan-1-ol (CK0402) caused apoptosis and autophagy in SKBR-3 cells [123]. Induction of autophagy proceeded via a variety of metabolic stresses such as hypoxia and oxidative stress. Thus, normoxic and hypoxic conditions were evaluated. Activity of CK0403 under hypoxic conditions was lower than that at normoxia [122]. CK0403 was better than CK0402 for treatment of ER-negative and HER2-overexpressing breast cancers. Moreover, the growth of HER2-overexpressing breast cancer cells was further suppressed by synergy with herceptin. Both CK0403 and CK0402 were more powerful than amsacrine [125].

Epigenetic inhibition of tumor–suppressor and various regulatory genes is a key factor in carcinogenesis [126–129]. Transcriptional silencing is usually regulated by DNA methyl transferase (DNMT)-mediated hypermethylation of CpG motifs in promoter DNA. Nucleoside derivatives such as azacytidine and decitabine served as inhibitors of DNMT and re-activators of genes, and are used in medicine [130]. Their use is limited by a short half-life and a slow onset of the effect. Thus, identification of specific and fast-acting gene unsilencing agents is important. Tricyclic heterocycles such as quinacrine and acridin-9-yl-[4-(4-methyl-piperazin-1-yl)-phenyl]-amine caused re-expression of epigenetically silenced genes implicated in carcinogenesis [131]. Among unsilenced genes are e.g.: p16, TFPI2, the cadherins E-cadherin and CDH13, and the secreted frizzle-related proteins (SFRPs) SFRP1 and SFRP5 in cancer cell lines. These acridines started rapid re-expression within 12–24 h. During re-activation of silenced genes, depletion of DNMT1 at the promoters of activated genes and demethylation of DNA occurred. Acridines are capable to inhibit DNMT1 in vitro.

7.3 Antibacterial Drugs

In the past, acridines were also used for treatment of bacterial infections [4, 18, 35]; however, the more effective antibiotics such as penicillin were discovered later. Some of acridines can still serve as local antiseptics. Currently, with increasing resistance of microorganisms to common antibiotics, some acridines may return to the use.

Friedländer reaction was used for the synthesis of twelve acridine analogues and polycyclic acridine derivatives [132]. The one-pot reactions of 2-amino-5-chloro or 5-nitrobenzophenones and a variety of cyclic ketones were performed under microwaves with TFA catalysis in good yields. 7-Nitro-9-phenyl-1,2,3,4-tetrahydroacridine was identified as an inhibitor of *Mycobacterium tuberculosis* H(37)Rv (Mtb) with remarkable MIC values against the rifampin resistant strain.

4-Chloro-*N*-(4-((2-methylacridin-9-yl)amino)phenyl)benzenesulphonamide served as a selective inhibitor of *M. tuberculosis* DNA gyrase [133].

3,6-Diamino-10-methylacridan, a product of borohydride reduction of 3, 6-diamino-10-methylacridinium chloride, served as its uncharged and masked precursor, which can easily penetrate through cell membranes [134]. After membrane trafficking, the methylacridan is oxidized by cells to toxic methylacridinium. The methylacridan is toxic to Gram-negative bacteria (*Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) ca 5 times more and over 15 times more than the corresponding methylacridinium. It was also active against viral infection such as *Herpes simplex* type-1 virus.

The antimicrobial activities of many 9-acridinones and 9-thioalkylacridines towards *E. coli*, *S. aureus*, *Mycobacterium smegmatis* and *Candida albicans* were tested [135]. The acridines were weakly antimicrobial with the most active substance having nearly the same toxicity as proflavine and acriflavine. Nevertheless, the amazing susceptibility of *M. smegmatis* to almost all the acridines was observed. The toxicity against *M. smegmatis* was enhanced by a methoxy group at position 4 on the aromatic ring. Intercalation into DNA did not correlate with biological activities.

Acriflavine is an effective inhibitor of parasitic infections. Acriflavine caused both apoptosis and necrosis in the yeast *Candida utilis* at 30–180 μM concentration [136]. Fluorescence measurements indicated a proportional flux of acriflavine into the cells. Acriflavine caused a reduction of cell number, a growth in trypan blue-positive cells, and a diminution in cell viability. Cells grown in the presence of acriflavine displayed an alteration in their respiratory control ratio and a drop in their cytochrome content. AO staining revealed apoptotic cells in cultures intoxicated with acriflavine. In addition, quantitative measurements showed that in acriflavine-treated cells the amount of cytochromes b, c, and cytochrome c oxidase declined. The cytochrome c oxidase diminution was more severe than that of cytochromes b and c. The order of cytochrome biosynthesis sensitivity to acriflavine was cytochrome c oxidase>cytochrome c>cytochrome b. Both apoptosis and necrosis were proven by electron microscopy. Apoptosis was less common than necrosis. Apoptosis and necrosis by acriflavine in *C. utilis* was attributed to the result of multiple factors among them the effects on DNA and proteins. Nevertheless, the most crucial factor

of cell death was the uncoupling of mitochondria, followed by a collapse of the electrochemical proton gradient, subsequent acidification of the cytoplasm, and cell death.

Acridine–chromene conjugates were examined for antimicrobial and anticancer activities [137]. 4-(Acridine-9-ylmethyl)-*2H*-(6-chlorochromen)-2-one was the most active against *P. aeruginosa* (MIC 25 μg/mL), whereas mixture of 4-(acridin-9-ylmethyl)-*2H*-(5-chlorochromen)-2-one and 4-(acridin-9-ylmethyl)-*2H*-(7-chlorochromen)-2-one was most active against *S. aureus* (MIC 50 μg/mL). These three mentioned chromenes were active against cancer cell line HL-60 at ca 27–29 μM level.

Many of prepared derivatives of 1,8-acridinediones were inhibitors of *E. coli*, *P. aeruginosa*, *Salmonella enteritidis*, and *S. aureus* with activities comparable to reference erythromycin [138]. Moreover, 1,8-acridinediones were inhibitors of *Vibrio* spp. [139].

Benzothiazoles conjugated with 9-aminoacridine via glycine hydrazide were evaluated for antimicrobial activity by recording of inhibition zone [140]. 4-Chlorobenzothiazole conjugate was the most active against *Klebsiella pneumoniae* with activity of 107% of reference streptomycin.

Charge-transfer complexes between antiseptic acriflavine and 4 aromatic systems were studied for their antimicrobial and antimycotic activities [141]. The complex with stoichiometric ratio acriflavine:hydroquinone 1:2 was slightly active against *S. aureus* (73% of tetracycline) and *C. albicans* (89% of amphotericin B).

Conjugation of 9-aminoacridine via lysine side chains to a nuclear localization sequence (NLS) led to construction of dendrimeric carrier linked via disulfide bridge [142]. The construct has formula [H-Lys(Acr)-Lys(Acr)-Lys(Acr)-Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys()-OH]$_2$. These hexa-acridines were significantly more toxic towards *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* than 9-chloroacridine and the NLS peptide. The MICs were 8 μM with an exception of that for *P. aeruginosa* (16 μM). On the other hand, *N*-terminal monoacridinylation of antimicrobial peptides from social wasps *Polistes major major* did not lead to improvement of either their activity or therapeutic indices [143].

Acriflavine was incorporated into polymeric drug, from which it was slowly liberated by *B. subtilis*, *E. coli*, and *S. aureus* in suicidal mission [144, 145].

1-Nitro-4-(2-aminoethyl)-9-acridon inhibited *E. coli*, *S. aureus*, and *M. smegmatis* with MIC 50 μg/ml [146].

7.4 Antiviral Drugs

Inhibition of HIV viruses by interaction of acridines with RNA is described in Sect. 4.3.

Dercitin, an antiviral/antitumor marine alkaloid, was employed as a starting structure for design of anti-HIV and anti-tumor compounds. The structural motives responsible for cytotoxicity were removed from initial structure. This provides

analogues having lowered T-lymphocyte toxicities [147]. (10-Cyclopropyl-9,10-dihydro-9-oxoacridin-2-yl)thioacetic acid provided total protection against HIV-1 at 12.5 μg/mL. No T-cell toxicity was detected up to 400 μg/mL. The tested compound prevented HIV-1 interaction with H-9 lymphocytes. The antiviral activity most probably proceeds by a unique dual extra- and intracellular pathways which prevent both viral binding to lymphocytes as well as insertion into viral nucleic acid. The 2-thio-9-acridones stand for a new structural motif with activity against HIV and can serve as potential anti-AIDS agents.

Molecular simulations were carried out in order to describe stereo-electronic properties needed for optimal antiviral activity. Quinacrine was identified as inhibitor of dengue virus [148]. Quinacrine antiviral activities in the DENV2 expressing cells was EC_{50} 5 ± 2 μM. The infectivity tests showed the EC_{50} value of 7 ± 2 μM. Quinacrine acts through inhibition of autophagy, thereby influencing DENV2 replication. Furthermore, quinacrine also demonstrated antiviral activity against the rapidly disseminating Zika virus with EC_{50} 2.3 ± 0.2 μM.

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Chapter 8
Pharmacokinetics and Metabolism of Acridine Drugs

Abstract Pharmacokinetic reasons have strong influence on applications of acridine drugs. Balancing of processes such as absorption, distribution, metabolism and excretion (ADME) is necessary for their proper action. For treatment of CNS tumor or neurodegenerative diseases, the ability of acridines to penetrate blood–brain barrier is important. Pharmacokinetic profiles of acridine drugs can be improved by conjugation with various nanoobjects such as liposomes or dendrimers. Despite pharmacokinetic limitation for transport of intercalators, the design of acridines with efficient diffusion to tumor tissue is possible. During the metabolism, usually, acridines are converted to more hydrophilic species by oxidation. However, a formation of practically insoluble acridones can also appear. In tumor cells, the reductive environment can provide partially hydrogenated acridines. Rapid metabolism of some acridines can lead to failure of the drugs in clinical trials.

Pharmacokinetic reasons have strong influence on applications of acridine drugs. Balancing of processes such as absorption, distribution, metabolism and excretion (ADME) is necessary for their proper action. Generally, pharmacokinetic parameters are obtained by determination of drug concentration in blood or plasma as a total drug concentration. However, many acridines bind plasma proteins, thus free drug levels strongly differ from the total ones [1–3]. The free drug concentration can be determined by many methods such as dialysis [4], or ultrafiltration [1]. Binding of acridines to human serum albumin can be studied by affinity chromatography using HPLC [5].

For treatment of CNS tumor or neurodegenerative diseases, the ability of acridines to penetrate blood–brain barrier is important. DACA was strongly uptaken by brain capillaries ca 90% extraction by one transit, whereas amsacrine and asulacrine were retained only by 15 and 20%, respectively [6]. For brain extraction the optimal $log\,P$ was in range between 0.70 and 0.85 [6]. DACA exhibits high activity against solid tumors [7]. Dose-limiting neurotoxicity was observed in mice after IV administration of DACA, which is thought to be due to the high uptake of DACA and/or DACA metabolites into normal brain [6, 8]. Some pharmacokinetic parameters of DACA were evaluated in phase I study for doses in range from 9–800 $mg.m^{-2}$ [9] (Table 8.1). Most data correlated well with a two-compartment model. Linear responses between

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dose and area under the curve (AUC), and dose and peak plasma concentrations were observed. However, no reliable fit between dose and either clearance or apparent volume of distribution at steady state was obtained. Independently of dose, there were no significant differences between 1st and 3rd day DACA parameters. Although the pharmacokinetic parameters of DACA were promising, DACA development was stopped due to a lack of efficacy in patients suffering from advanced colorectal cancer, advanced ovarian cancer, glioblastoma multiforme, and non-small cell lung cancer [10–13].

Efficient tumor tissue penetration limited action of DNA intercalating drugs. Combination of multicellular layer experimental model with theoretical modeling evaluated the hypothesis whether DACA possessed favorable extravascular transport properties [14]. DACA was accumulated by various cells less than the analogous C9 substituted acridine – 9-[3-(dimethylamino)propylamino]acridine (DAPA). DACA was metabolized to corresponding 9-acridan by V79 but not EMT6 cells. The flux of DACA through cell layer was significantly faster than that of DAPA. Theoretical modeling of the system based on diffusion–reaction model (metabolism and reversible binding) revealed that the faster flux of DACA was caused by a 3-fold higher free drug diffusion coefficient and 10-fold lower binding site density. AUC did not depend on the distance from blood vessels. Thus, principally, the design of DNA intercalators with efficient diffusion to tumor tissue is possible.

Administration of [^{3}H]-DACA IP to mice in an optimal plasma dose to cure advanced Lewis tumors (410 μmol/kg) enabled to estimate a pharmacokinetics, tissue distribution and toxicity of DACA [15, 16]. The drug is rapidly absorbed with C_{max} at 5 min. Contrary to IV administration, no reduction in bioavailability was observed, but the shape of plasma concentration–time profile was considerably different. The high, but variable tissue uptake of DACA was recorded with time from 0.08 to 8 h in plasma versus tissues in brain, liver, kidney and heart. Comparison of the pharmacokinetics, tissue distribution and toxicity values after [^{3}H]-DACA IP administration suggests that the reduction of acute toxicity is not due to reduced exposure of the brain to DACA, but may be associated with its lower C_{max} value or the slower rate of entry into the brain after IP administration.

After the IV administration of [^{3}H]-DACA (121 μmol/kg), high radioactivity levels were observed in all tissues especially in the brain and kidney [7]. The plasma radioactivity has decreased gradually as drug had been eliminated, with 26 and 47% of the delivered drug being excreted by the urinary and faecal routes, respectively. Starting dose of [^{3}H]-DACA was well tolerated by mice, with sedation as the only side effect. No significant alterations of hematological parameters were recorded. An increase of the dose from 152 to 182 μmol/kg has led gradually to clonic seizures, sedation and finally resulted in death after DACA application [7].

The pharmacokinetic data collected for DACA, using an IV administration of [^{3}H]-DACA in rats (18, 55 and 81 μmol/kg), were employed for extrapolation of corresponding kinetic data to human application [17]. Taking into account the species differences in the unbound DACA fraction in plasma (mouse, 16.3%; rat, 14.8%; human, 3.4%), allometric equations were developed from rat and mouse pharmacokinetic data for prediction of total drug concentrations in humans. The mean

model-independent pharmacokinetic parameters, like clearance, steady-state volume of distribution, mean residence time, and terminal elimination half-life obtained in the rats, were estimated.

Aldehyde oxidase causes a detoxification of DACA in the rat and mouse due to its oxidation to 9(*10H*)acridone, followed by ring hydroxylation and glucuronidation [18]. The acridone formation in both the cytosolic and enzyme-enriched fractions is highly sensitive to the classical cytochrome P450 inhibitor SKF525A with mixed noncompetitive inhibition. The major urinary metabolite (34%) of DACA was DACA-*N*-oxide-9(*10H*)acridone [19] (Fig. 8.1). The minor metabolites were these: desmethyl-DACA-9(*10H*)acridone (2%), DACA-9(*10H*)acridone (∼3%), desmethyl-DACA (<1%), and DACA-*N*-oxide (<1%).

Catalytic inhibitors of topoisomerase II outperforming DACA were designed [20, 21] (Fig. 8.2), which caused a G1/S phase arrest. The arrest of cell cycle is consistent with the role of topoisomerase II in DNA unwinding during the beginning of replication. With one oral dose per day, acridine **173** prolonged survival of glioma-bearing mice [1].

Compounds **172** and **173** were stable in human liver microsomes for 120 min without formation of any significant metabolites even formation of glucuronide ones was not detected [1]. Both acridines easily permeated MDCK cells. The acridine–indole conjugate (**172**) is rapidly excreted via P-gp and BCRP efflux transporters. Both acridines were substrates for organic cation transporter. They bound with more than 95% to plasma proteins. Interestingly, whereas concentration profile of acridine **172** followed one-compartment model, that of acridine **173** fitted to two-compartment model having both distributional and elimination phases [1]. Response to one 60 mg/kg oral dose of acridines **172** and **173**, the C_{max}'s in brain were ca 0.2 and 0.6 μM, respectively. The ratios between C_{max}'s in plasma vs brain were ca 9 and 33 for compounds **172** and **173**, respectively (Table 8.1). With the plasma half-life 22 h, the compound **173** remained in the body ca 5 times longer than the compound **172**. Combination of long half-life (22 h), high volume of distribution (0.27 L), and high bioavailability (84%) for compound **173** led to very slow clearance 0.039 L/h. When oral and IV administration of compound **173** were compared, the 4 times lower IV dose provided 2.8 times higher brain C_{max} than the corresponding oral dose.

Asulacrine is a weakly basic and highly lipophilic drug, which combines the substructures of *m*-amsacrine and DACA [22]. In contrast to amsacrine, asulacrine possesses broader scope of applications against leukemia, Lewis lung tumors, and many solid tumors. Asulacrine metabolic pathway is different from that of amsacrine demonstrating different side effects, hepatotoxicity and excretion. Asulacrine is probably failing in phase II of clinical trials due to its toxicity especially phlebitis. Nonlinear pharmacokinetics of asulacrine during intravenous administration to mouse correlates with one-compartment model. Asulacrine half-life and AUC are 1.4 times longer and 1.8 fold larger in tumor in comparison with amsacrine. Asulacrine was loaded to plasma proteins in ca 80% of dose, when it is treated with mouse blood [23]. The drug was excreted in feces in 72 h after intravenous administration [24].

A combination of HPLC and field desorption MS enabled an identification of the metabolic products of the *m*-amsacrine thiolysis after administration of the

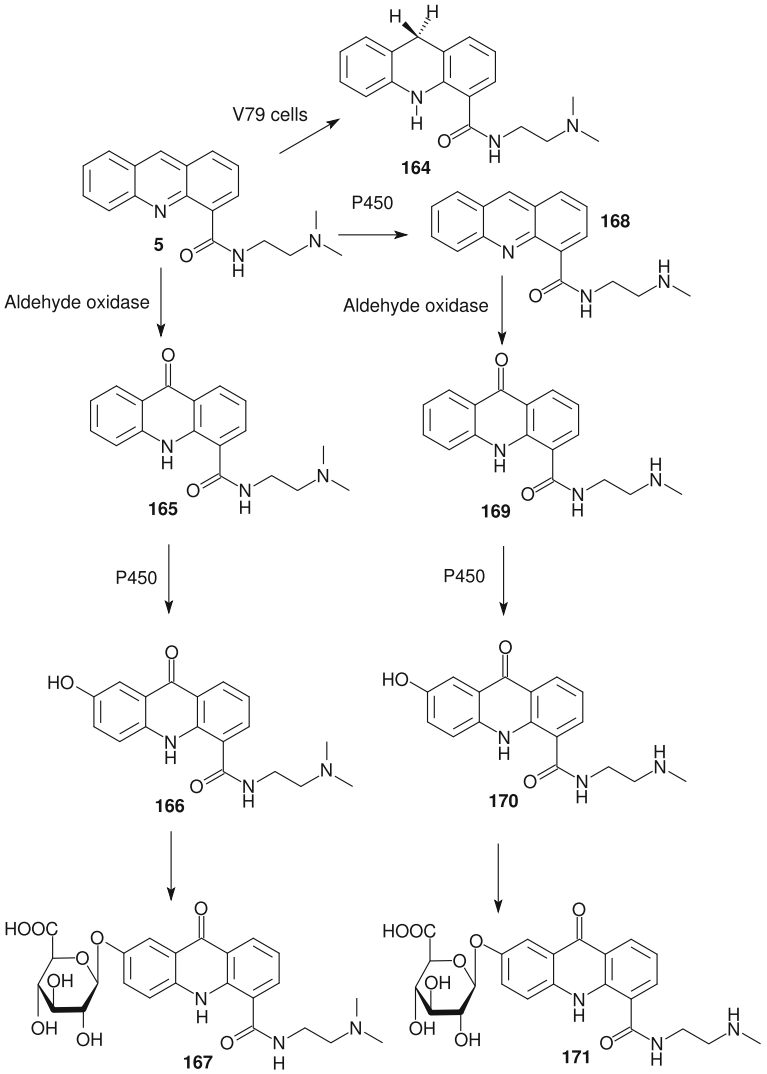

Fig. 8.1 DACA is metabolized to glucuronides with involvement of aldehyde oxidase and cytochrome P450 [19]. However, in rare cells such as V79, reductive metabolism of DACA can also occur [14]

drug therapeutic dose in rat bile and incubation with glutathione, cysteine and *N*-acetylcysteine in sodium phosphate buffer [25]. The conjugation products thioether linkage were formed spontaneously. The thiolytic route resulted in the 4-amino-3-methoxymethanesulfonanilide formation, which was monitored in all in vitro experiments and in rat serum after IV injection of *m*-amsacrine. The minor products of

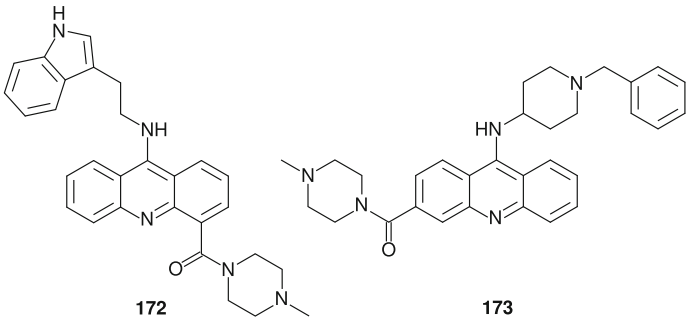

Fig. 8.2 Inhibitors of topoisomerase II with improved pharmacokinetic profiles [1, 21]

the thiolytic cleavage in vitro 9(*10H*)-acridone and 9-aminoacridine were identified by field desorption and partially by high resolution electron impact MS.

Amsacrine and asulacrine are strongly processed in liver [4, 26]. The conjugates of asulacrine and amsacrine at 5'- and 6'- position with glutathione were formed. Moreover, the asulacrine was also conjugated at C9 and formation of 5-hydroxymethyl derivative was observed [26, 27]. In literature [27], wrong numbering of acridine ring was used, and the derivative was called 4-hydroxymethyl. The 5-hydroxymethyl derivative was further eliminated via aglycone or glucuronide pathways ca 56% within 3.5 h. When the concentration in bile of mouse and rat models were compared, C9 conjugate decreased from 24 down to 2%, whereas 5-hydroxymethyl increased from 10 to 90%, respectively [27]. In contrast to amsacrine, the asulacrine reacted poorly with myeloperoxidase enzyme [26], and formation of reactive quinonediimines was suppressed. Probably, an alternative way of asulacrine metabolism with insignificant levels of quinonediimines can lead to lower hepatotoxicity than that of amsacrine.

9-[^{14}C]*m*-amsacrine was utilized in a clinical study on treatment of human disseminated neoplasms [28]. The plasma half-lives of *m*-amsacrine were prolonged in patients with severe liver disease. Hepatic metabolism and biliary excretion were the most important routes for amsacrine elimination. Renal elimination is significant in patients with severe kidney dysfunction. The initial 40% dose reduction is suggested for patients with pharmacologically documented impaired drug clearance.

Metabolism of *m*-amsacrine depends on a hepatic microsomal enzyme system based on liver microsomes, a reduced NADH-generating system, cytosolic protein (or glutathione) and oxygen [29]. An omission of any one of them, or incubation under inert atmosphere, caused inhibition of the metabolic reaction. Moreover, the inhibitors of microsomal metabolism such as α-naphthoflavone, metyrapone, or SKF525A decreased amsacrine metabolism. On the other hand, microsomes activated with phenobarbital or 3-methylcholanthrene accelerated amsacrine metabolism.

Elacridar (**14**) serves as a powerful inhibitor of P-gp and breast cancer resistance protein and served for studies of the influence of these efflux transporters on drug distribution to brain [30]. The half-life of elacridar was ca 4 h after IP and IV dosing

Table 8.1 Selected pharmacokinetic parameters of some acridines

Compound (administration)	Dose (mg)	$t_{\frac{1}{2}}$ (h)	AUC (ng.h.mL^{-1})	V_D (L)	CL_{Plasma} (L.min^{-1})	Bioavailability (%)	C_{max} (ng.mL^{-1})	Reference
Tacrine (12)	25	1.6	29					[49]
(human, oral)	50	2.1	83					
	50(rep.)	2.9	143					
(human, IV)	3	1.2		68	0.63	2.4		
	5	2.2		168	0.87	3.1		
(human, oral)	25	1.4	24			17	60	[53]
(human, IV)	30	1.6	231	349	2.4		222	
(human, IV)	25	2.8	126	14	2.6	30	17	[51]
(human, oral)	50	3.2	78	49	9.3			
(rat, IV)	1× kg^{-1}	3.4	125	40	0.13× kg^{-1}	28		[90]
(rat, oral)	2× kg^{-1}	1.5	89					
(rat, oral)	4× kg^{-1}	1.2	109				72	[91]
Velnacrine (13)	25	3.5	60				222	[49]
(human, oral)	50	4.1	143					
	50(rep.)	5	200					
(rat, oral)	4× kg^{-1}	2	785				303	[91]
OHA (181)	2	2.7	2.1	5	0.33		0.7	[55]
(young,	4	2.9	4	5	0.35		1.5	
human, oral)	8	2.7	5	7	0.51		2.0	
(old, human, oral)	4	3	4				1.4	
T6FA (rat, oral)	40× kg^{-1}	2.0	127				94	[52]

(continued)

Table 8.1 (continued)

Compound (administration)	Dose (mg)	$t_{\frac{1}{2}}$ (h)	AUC (ng.h.mL^{-1})	V_D (L)	CL_{Plasma} (L.min^{-1})	Bioavailability (%)	C_{max} (ng.mL^{-1})	Reference
Quinacrine (11)	$2 \times kg^{-1}$	26	1.3		$2.3 \times kg^{-1}$	100	633	[69]
(rabbit, IV)								
(rabbit, IP)	$10 \times kg^{-1}$	26				100	865	
MBAA	$2 \times kg^{-1}$	0.9	369	$8 \times kg^{-1}$	$1.6 \times kg^{-1}$		554	[34]
(rat, IV)								
acridine 173	$60 \times kg^{-1}$		3×10^4		5.4×10^{-4}	84	1×10^5	[1]
(mice, oral)								
(mice, IV)	$15 \times kg^{-1}$		9×10^3	0.266	6.6×10^{-4}		4×10^3	
DACA (5)	$40 \times kg^{-1}$	1.4	1.8×10^3	11.8	$0.35 \times kg^{-1}$		6×10^3	[7]
(mouse, IV)								
(mouse, IP)	$120\times kg^{-1}$			$14.1\times kg^{-1}$	$0.29 \times kg^{-1}$		6×10^3	[15]
plasma		2.7	6.7×10^3					
brain		2.2	5.8×10^4					
liver		1.3	1.4×10^5					
kidney		1.3	1.3×10^5					
heart		1.6	3.9×10^4					
(rat, IV)	$5.3 \times kg^{-1}$	2.1	1.1×10^3	5.0	$0.09 \times kg^{-1}$		1.8×10^3	[17]
	$16.1 \times kg^{-1}$	2.4	3.2×10^3	9.4	$0.09 \times kg^{-1}$		3.8×10^3	
	$23.8 \times kg^{-1}$	1.9	4.4×10^3	9.0	$0.09 \times kg^{-1}$		6.2×10^3	

(continued)

Table 8.1 (continued)

Compound (administration)	Dose (mg)	$t_{\frac{1}{2}}$ (h)	AUC ($ng.h.mL^{-1}$)	V_D (L)	CL_{Plasma} ($L.min^{-1}$)	Bioavailability (%)	C_{max} ($ng.mL^{-1}$)	Reference
Asulacrine (7)	$2.9 \times kg^{-1}$	1.7	1.7×10^4	0.32	$3.1 \times 10^{-3} \times kg^{-1}$		8.2×10^4	[92, 93]
(rabbit, IV)	$5.9 \times kg^{-1}$	1.8	2.0×10^4	0.32	$2.7 \times 10^{-3} \times kg^{-1}$		1.6×10^5	
	$11.8 \times kg^{-1}$	2.4	2.4×10^4	0.41	$2.2 \times 10^{-3} \times kg^{-1}$		3.2×10^5	
AC04	$1.5 \times kg^{-1}$	45	788	138	$0.06 \times kg^{-1}$			[35]
(rat, IV)								
lung		156	798				18	
spleen		371	263				8	
liver		24	304				12	
kidney		49	15				502	
heart		8	6.1×10^3				376	
fat			23				404	
brain		19	403				50	
1-Oxo-AC04	$1.5 \times kg^{-1}$	23	89				4	[35]

and almost 20 h after oral administration. The brain-to-plasma distribution ratio of elacridar is proportional to plasma exposure. This might be explained by saturation of the efflux transporters at BBB. The brain–plasma ratio was 0.82, 0.43, and 4.31 after IV, IP, and oral administrations, respectively.

The elacridar was applied both in vitro and in vivo as an inhibitor of P-gp to understand the role of transporters in the disposition of various compounds [31]. Pharmacokinetic properties of elacridar in the mouse, rat, dog, and monkey and its in vivo efficacy of absorption modulation in the monkey was explored. Elacridar has shown acceptable absorption and systemic exposure in all species; however, in rodents, dose of 300 mg/kg provided a considerably less than linear increase in systemic exposure in comparison with dose 30 mg/kg. According to intestinal and hepatic exposure and potency against P-gp, elacridar has shown significant modulation of erythromycin systemic exposure in the monkey, while propranolol (a negative control molecule) was unaffected. Elacridar did not show strong inhibition of various human cytochrome P450 enzymes.

In order to improve some pharmacokinetic parameters of acridine drugs, they can be loaded into conventional liposomes or even better into long circulating liposomes or immunoliposomes. E.g. half life of drug elacridar is increased ca 2–3 times when loaded to long circulating liposomes (LCL) or immunoliposomes [32]. Despite the half lives of elacridar loaded to LCL and immunoliposomes is approximately the same, the clearance of the immunoliposomes is ca 2–3 times higher than that of LCL. Immunoliposomes provided the highest possible concentrations of elacridar in the brain at the same dose.

Graphite rod laser desorption vacuum-ultraviolet post-ionization mass spectrometry served as suitable technique for detection and quantification of 9-phenylacridine drug [33].

To explore pharmacokinetics and metabolism of VEGFR-2 and Src dual inhibitor, 6-chloro-2-methoxy-*N*-(2-methoxybenzyl)acridin-9-amine (MBAA), a simple, rapid, and accurate LC-MS method was developed and validated for analyses in the rat plasma, urine and bile [34]. After IV administration of 2.0 mg/kg to male rats at a single dose, MBAA was rapidly eliminated. Four metabolites could be then monitored and characterized using the parent compound and various fragments of MBAA. The demethylation at acridine or benzene ring occurred. Corresponding phenolic hydroxyl was then conjugated with glucuronic acid. Furthermore, *N*-hydroxylation can be detected.

Plasma pharmacokinetics and tissue distribution of 5-(acridin-9-yl-methylidene)-3-(4-methyl-benzyl)-thiazolidine-2,4-dione (AC04) and its 1-oxo-AC04 metabolite were investigated in rats [35]. Protein binding 98 ± 2% was determined by ultrafiltration. AC04 showed high penetration into the lung, spleen and liver. The individual AC04 concentration–time profiles fitted best to the two-compartment model. The 1-oxo-AC04 metabolite represented 10% of AC04 plasma concentration. All results indicate that AC04 is potentially a good antitumour candidate due to extensive penetration in most tissues with maintenance of high concentrations for a long time.

Acriflavine and proflavine plasma concentration–time profiles fitted to three- and two-compartment pharmacokinetic models after intravascular dosing, respectively

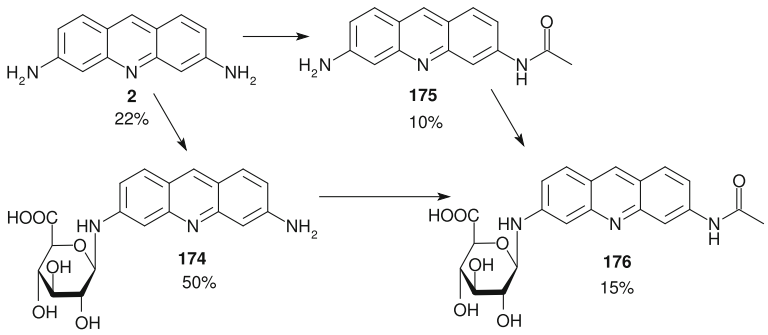

Fig. 8.3 Proflavine **2** is metabolized at amino group in liver to glucuronide **174**, acetamide **175**, or both **176** [36]. The distribution of individual species is depicted with percentage

[36]. Their elimination half-lives in plasma were 11 and 8.7 h, respectively. Drugs were more distributed to the excretory organs and poorly to muscle, fat and plasma. With proflavine, residues in the liver and trunk kidney consisted mainly of glucuronosyl and acetyl conjugates (Fig. 8.3), whereas it is almost untouched in muscles (>95%). On the other hand, acriflavine was kept almost constant in all tissues (>90%). Absorbtion of both acriflavine and proflavine was poor in catfish during waterborne exposure. The distribution was quite well, however, the muscular concentration was below of 1% of that in water.

Tacrine was approved in the US, Canada and parts of Europe in 1993 for the suppression of cognitive symptoms of AD [37, 38] (Pfizer and Warner-Lambert, New York, NY). Tacrine is significantly metabolized by cytochrome P450 to at least three products. The major metabolite, 1-hydroxytacrine possesses the tacrine activity [39]. Roughly 20% of tacrine-treated patients could show improvement, but its use is limited due to the severe side effects e.g., hepatotoxicity and gastrointestinal antagonism [40–42]. The toxicity could be caused by reactive metabolites in a two-step process starting with 7-hydroxylation of tacrine, followed by a 2-electron oxidation providing a chemically reactive quinone methide [43, 44] (Fig. 8.4). Quinone methide can interact with essential cellular macromolecules, induce futile redox cycling and lipid peroxidation, generate free radicals, and deplete cellular cofactors such as glutathione. Tacrine hepatic oxidative damage can be blocked with liquiritigenin via GSK3*beta* inhibition [45]. The mono- and dihydroxylated tacrine and glucuronide conjugates were identified in a urine, which is the primary route of excretion with an elimination half-life of tacrine to be 1.5–2.5 h after single oral and IV doses and 2.9–3.6 h after multiple oral doses [46].

Oral administrations of tacrine were rapidly and well absorbed with peak plasma concentrations achieved in the kidney, liver, adrenal gland within 0.5–3 h (after a single dose of 20–50 mg). Tacrine appears to have a wide tissue distribution, which is reflected by its large volume of distribution. It has a low bioavailability due to extensive first-pass metabolism [46]. Bioavailability can be increased upon rectal

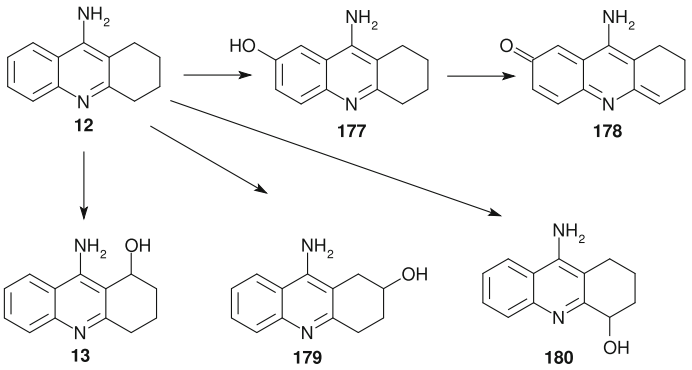

Fig. 8.4 Tacrine (**12**) is metabolized by cytochrome P450 to reactive and non-reactive metabolites [44]. The reactive quinone methide (**178**) can be responsible for its hepatotoxicity. The non-reactive one can serve as drugs with improved profiles [47]

administration and drug dose may be reduced by almost 50% compared to oral application [48].

In several patients with AD, the clinical pharmacokinetics of tacrine hydrochloride have been characterized using serum concentrations after 25 and 50 mg oral doses or a small IV dose. Its metabolite was monitored in urinary excretion for 24 h. The serum half-lives varied from 1.6 to 2.9 h depending on way of administration and dose. After IV administration, clearance was above 600 mL $\cdot$ min^{-1} and oral bioavailability was calculated at below 5% (see Table 8.1). The low bioavailability of tacrine hydrochloride was partly attributed to presystemic metabolism [49].

During the 12–31 month treatment of AD patients, an average steady-state concentration 1.1–30 μg $\cdot$ L^{-1} of tacrine in plasma was obtained with daily doses of 40–160 mg [50]. The mean elimination $t_{\frac{1}{2}}$ was 5–7 h without any change at increased dose of tacrine. Treatment with 160 mg daily dose of tacrine resulted in a 40% inhibition of plasma cholinesterase activity and 60% inhibition of AChE activity in red blood cells. The low bioavailability of the oral formulation in range 9.9–36.4% and not altered plasma half-life indicate that the tacrine elimination occurs by a 1st-order process [51].

A rapid, specific, and precise quantification of tacrine conjugate with ferulic acid and hexamethylenediamine linker (T6FA) in rat samples using LC-MS/MS method with electrospray ionization was established [52]. This method was also successfully applied for the pharmacokinetic measurement of T6FA with an oral administration of 40 mg/kg to rats. T6FA was absorbed quickly with a mean T_{max} of 0.10 ± 0.04 h and eliminated slowly with a mean k_e of 0.4 ± 0.1 h^{-1}.

In patients with amyotrophic lateral sclerosis (ALS) a clinical trial involved IV administration of tacrine (30 mg) followed by the first and last oral doses (25 mg) for 7 weeks. Plasma concentrations of tacrine and velnacrine were assayed using HPLC and they fitted to the two-compartment model (see Table 8.1). Absorption $t_{\frac{1}{2}}$ and distribution $t_{\frac{1}{2}}$ of tacrine were 21 ± 15 min and 1.8 ± 0.8 min, respectively. After

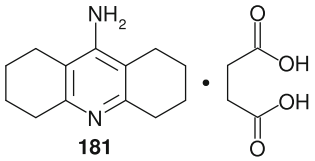

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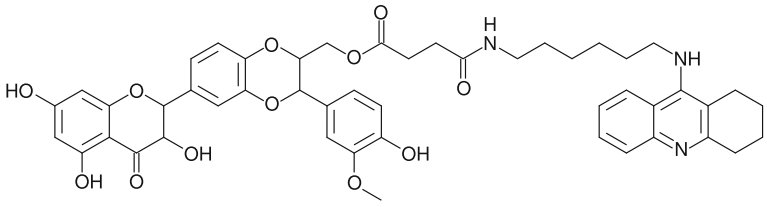
Fig. 8.5 OHA, a potential substitute for tacrine [55]



182

Fig. 8.6 Tacrine–silibinin codrug with improved pharmacological profile [56]

seven weeks of treatment, the concentrations of velnacrine in plasma were higher than those of tacrine, the application of which was associated with side effects in the majority of the patients [53].

Bis-tacrines linked with oligomethylene bridges between 9-amino groups, as well as, mono-tacrines were hydroxylated in liver on saturated ring [54].

The pharmacokinetics of tacrine analogue - 1, 2, 3, 4, 5, 6, 7, 8-octahydroacridin-9-amine (OHA) succinate (Fig. 8.5) - indicate that OHA might be a promising drug for AD therapy. The study was performed in healthy Chinese subjects with 16 male and 14 female (aged 18–45 years), treated with doses of 2, 4 and 8 mg, further with 10 participants that were given another single dose of 4 mg OHA and with 7 male and 5 female in elderly (65–75 years) healthy subjects. In all model entities, following a single- and multiple-dose, the OHA was rapidly absorbed. There was no evidence of a dose-related increase in adverse events with OHA administration [55].

The tacrine–silibinin codrug exhibits pharmacological efficiency superior to an equimolar mixture of tacrine and silibinin [56] (Fig. 8.6). In culture medium, the codrug remained stable over an incubation period of 24 h, whereas exposition to microsomal enzymes led to rapid cleavage of the ester bond to form silibinin and a tacrine hemisuccinamide, in which no effects were observed with regard to cell viability and mitochondrial impairment. The improved efficiency and no toxicity of the tacrine–silibinin codrug supply an important information for further development of the codrug "in vivo".

Quinacrine, a powerful drug against protozoal infections (see also Sect. 7.1) was also examined for treatment of prion diseases (see also Sect. 6.3). However, it failed in clinical trials for antiprion activity. There are many hypotheses for the failure: one correlated it with clearance of quinacrine from the brain [57]. Even though quinacrine can achieve a brain concentration corresponding to 10-fold of EC_{50} of in vitro assay

[58], quinacrine was quickly cleared by P-gp efflux transporter [57]. MDR0/0 mice, deficient in P-gp multi-drug resistance (MDR) transporter, can increase the concentration of quinacrine in the brain up to 100 μM. In in vitro experiments, when total concentration of quinacrine in neuroblastoma cell culture was set up to 300 nM (EC_{50} for antiprion effect), the real extracellular and intracellular concentrations were 120 nM and 6.7 μM, respectively [59]. After administration of therapeutic and toxic quinacrine doses at healthy ewes, cerebrospinal fluid concentrations reached 11 and 55 nM, respectively. The concentrations were not sufficient for desired antiprion effect [59]. Another pharmacokinetic reason is based on clearance and/or accumulation of quinacrine via metabolism with proteins or glutathione [60–63]. Other hypotheses are based on pharmacodynamic origin as limited formation of PrP^{Sc} [64], as the selection of quinacrine-resistant prions during the treatment [65, 66], and as a reset of the susceptibility of the cells to prion infection [63]. Quinacrine is also metabolized to *N*-desethyl and *O*-desmethyl derivatives [67]. Human cytochrome P450 isoforms CYP3A4/5 caused the mono desethylation (Fig. 8.7).

Pharmacokinetics of quinacrine after administration of 30 mg to cynomolgus monkeys via the intrauterine route and via intravascular injection was compared [68]. The rapid transfer of the drug from the uterine to the vascular compartment and uptake by almost all tissues examined was observed. Although plasma concentrations disappeared within 24 h, levels could be detected in most tissues for at least 1 week following intrauterine injection.

The uptake of quinacrine from the pleural space was rapid and complete after intrapleural instillation or IV infusion to rabbits [69]. The mean absorption half-life was approximately 7 min and the mean bioavailability was slightly in excess of 100%. Due to the high bioavailability and the large doses used for pleural sclerosing (pleurodesis) in patients, neurological disease and psychiatric disturbances that predispose to CNS toxicity should be considered as contraindications to intrapleural quinacrine.

Free radical metabolism of the quinacrine and 9-aminoacridine was investigated using horseradish peroxidase–H_2O_2 and prostaglandin–arachidonic acid systems [70]. Only quinacrine rapidly formed a free radical intermediate. The incubation of the acridines resulted in covalent, NADPH-dependent binding to microsomal membranes.

A tentative pathway of anaerobic acridine transformation began with oxidation of either the benzene or the pyridine ring and thereafter followed the common degradation pathway for oxidized aromatic compounds: oxidation, reduction, decarboxylation, ring cleavage, and the breakdown of aliphatic products [71].

Biodegradation of heterocyclic aromatic compounds including acridine and its derivatives occurs under both aerobic and anaerobic conditions, depending upon the environmental conditions, different types of bacteria, fungi, and enzymes. By using ^{14}C-labeled substrates, it was possible to show that ring fission of a specific heterocyclic compound occurs at a specific position of the ring. In studies involving ^{18}O labeling, as well as, the use of cofactors, it was possible to prove that specific enzymes (e.g. mono- or dioxygenases) are involved in a particular degradation step. By using $H_2[^{18}O]$, it could be shown that in certain transformation reactions, entire

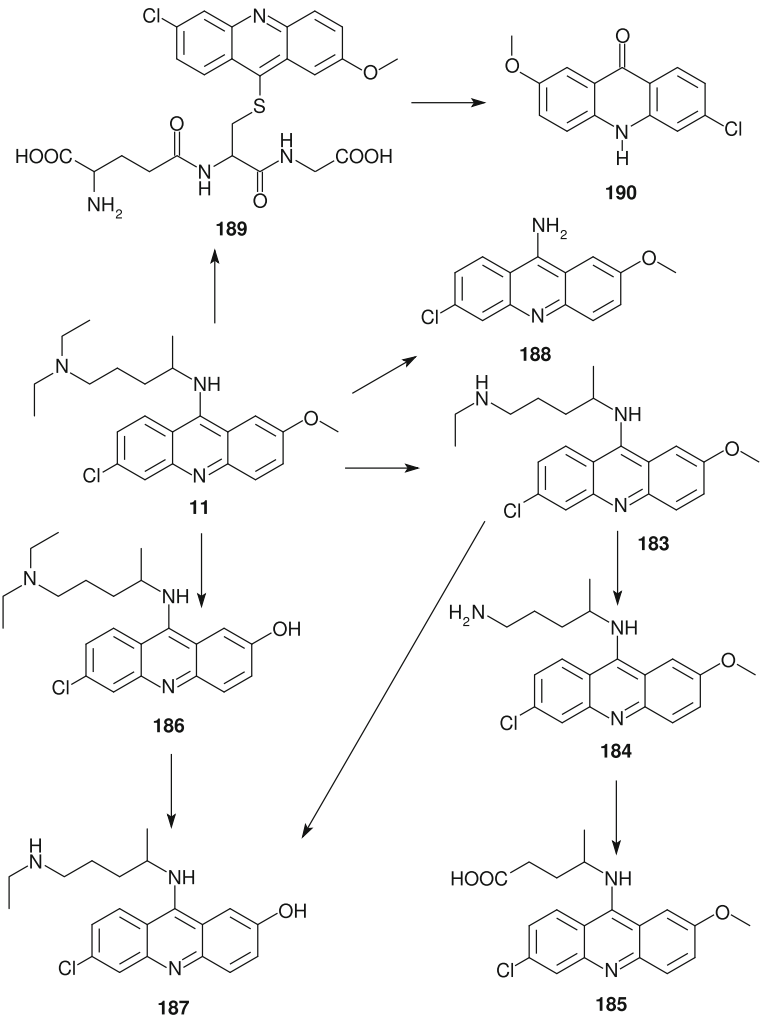


Fig. 8.7 Quinacrine (**11**) is metabolized by Human cytochrome P450 isoforms CYP3A4/5 to mono desethyl analogue. Other metabolites can be also detected [67]. In the presence of glutathione, acridine ring transfer occurred which led to precipitation of acridone [61–63]

oxygen was derived from water and that these reactions might also occur under anaerobic conditions [72].

An extensive metabolic potential of acridine implies difficulties in complete description of acridine mutagenicity. Acridine has been reported to be mutagenic to *Salmonella typhimurium* in the absence of PCB-induced activating enzymes. The metabolic products, acridine-1,2-dihydrodiol or acridine-3,4-dihydrodiol, were presumably derived from an epoxide, which is produced by the corresponding PCB-induced liver enzymes. During "in vitro" transformation of acridine to 9-acridone by

aldehyde oxidase neither atmospheric oxygen nor NADPH are required. Acridines produce chromosomal aberrations in cultured Chinese hamster cells both with and without enzymatic activation [73].

Acridines are quite strong DNA and protein binders (see also Chaps. 4 and 5). Tight and non-specific binding of biopolymers severely limits the extravascular distributive properties of drugs, as diffusion is driven by the free drug concentration [63, 74, 75]. For these compounds, the effective diffusion coefficient (D_{eff}) that governs the rate of extravascular diffusion, is significantly diminished by DNA binding and correlates with dissociation constant. It obeys the equation $D_{eff} = \frac{D}{1+\frac{S_f}{K_D}}$, where D represents the diffusion coefficient, K_D stands for the biopolymer dissociation constant and S_f is the concentration of available biopolymer-binding sites [63]. For a total biopolymer-binding site concentration of about 1 mM and non-specific binder (about 10% of the sites in chromatin), the lower limit of K_D to keep the diffusion time useful on the pharmacological timescale corresponds to 100 μM [63, 74]. Thus, even quite moderate biopolymer binding suppresses diffusion of would-be drug. For DNA–9-aminoacridines, the typical dissociation constant is 10 μM, thus acridine bioavailability is very low. The conjugation of 9-aminoacridines with peptides can improve acridine bioavailability by reduction of their DNA binding [75]. However, when glutathione gets acridine moiety from quinacrine, the DNA binding increases ca 5-folds [63].

Another player influencing bioavailability of drugs in a body is distribution coefficient $log\,P$ between n-octanol–water [76], where n-octanol can be considered as a model of biological membranes. For polyelectrolytes such as many biopolymers, $log\,D_{pH7.4}$ serves as the more accurate measure of permeability. During measurement the pure water is substituted with the aqueous physiological buffer with pH 7.4 [77]. Most common drugs have $log\,D_{pH=7.4}$ in the range −0.5–3 [63, 78–81], thus they possess balanced membrane permeability and aqueous solubility. Assays using colon Caco-2 cells serve as more accurate screening method for understanding drug absorption and bioavailability [77, 80, 82]. However, other cancer cell lines (gastric MKN-28) can be used as well for monitoring of acridine absorption [83]. In Caco-2 model, bis-tacrines absorptive P_{app} were ca two orders of magnitude lower than that of tacrine [84]. Secretive permeabilities were much higher than those of absorptive. Tacrine efflux ratio was 0.87, whereas for bis-tacrines the ratios were in the range from 3 to 37. With increasing pH values, bis-tacrines started to permeate the Caco-2 cell layer from pH 5 [54]. Close to pH 7 maximal permeability was achieved. Bis-tacrines were strongly retained by Caco-2 cells i.e. 56 and 29% were trapped by cells during absorptive and secretive transport, respectively. Compounds were investigated also by even better permeation model using rat in situ intestinal perfusion [54]. Bis-tacrines permeabilities are ca an order of magnitude worse than that of glucose.

Pharmacokinetic parameters of BRACO19 – powerful inhibitor of telomerase and quadruplex DNA binder [85, 86] (see also Sect. 4.2) – were investigated [5]. BRACO19 had good and quick solubility (ca 2 mg.mL^{-1}) in water in a pH range of 2.8–7.4. BRACO19 belongs to weak-to-medium binders of human serum albumin

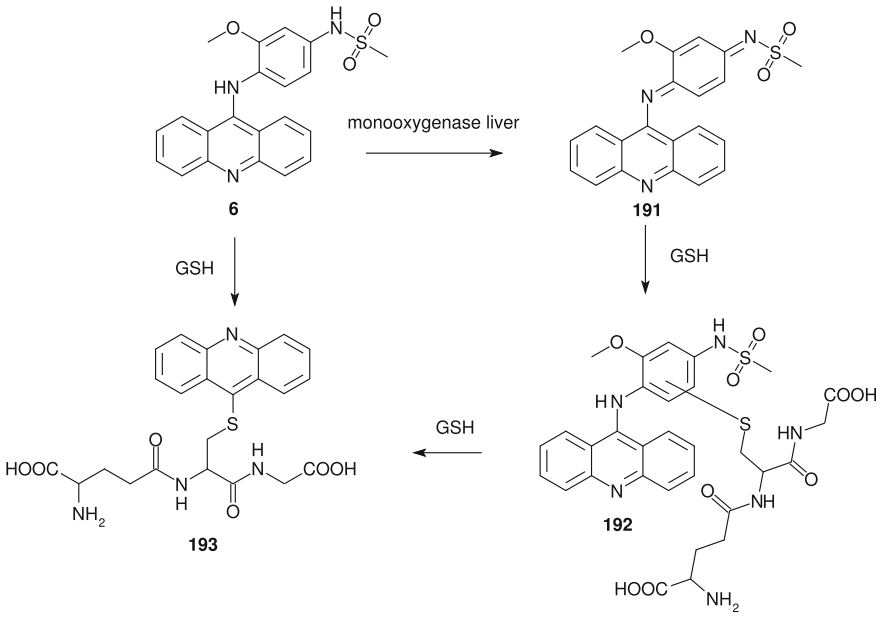


Fig. 8.8 Redox metabolism of *m*-amsacrine [24, 27, 89]

and can poorly overcome barriers like phospholipid membranes [5]. Thus, BRACO19 did not permeated the Caco-2 cells in absorptive direction. In secretive direction, it is worse permeator than fluorescein.

Generally, 9-aminoacridines can lead to depletion of glutathione [24, 87] and interaction with redox milieu in the living systems. The depletion is probably caused by chemoselective reaction of C9 acridine unit with peptide/protein thiols [61, 88]. By this way, many acridine drugs such as quinacrine [88], amsacrine, and asulacrine [23] can react with glutathione. In some cases, the reaction may be facilitated by glutathione transferases and other enzymes [24, 27]. However, the enzyme catalyzed reaction may lead also to conjugation of glutathione at various positions of aniline ring of amsacrine and asulacrine [24, 27]. The primary microsomal oxidation product of *m*-amsacrine (**6**), led to diimine (**191**), which reacted with equimolar glutathione, cysteine, *N*-acetylcysteine and *N*-acetylcysteine methyl ester to form *m*-amsacrine-(5')-thiol conjugates linked at the aniline ring, as major products (**192**) [89] (Fig. 8.8). The reaction of **192** with increased molar amounts of thiol, and with cysteamine as a bifunctional nucleophile at equimolar amounts afforded the concurrent formation of conjugation products (e.g. **193**) by nucleophilic displacement at the acridine moiety of **6**. Acridine–glutathione conjugates can undergo autocatalytic decomposition in which glutathione is regenerated and corresponding sparingly soluble acridone released [62, 63] (Fig. 8.7). Acridines can also react covalently with thiol containing proteins such as bovine serum albumin and prion protein [23, 24, 61].

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Chapter 9
Acridine on Dendrimeric Carriers

Abstract Dendrimers are synthetic monodisperse polymers with a repeatedly branched, highly structured architecture adopting globular three-dimensional shape. Applications of dendrimers are very diverse. They can be used in physics, organic chemistry, biomedical applications, catalysis, and as solubilizing agents. Acridine-loaded dendrimers can serve as inhibitors of topoisomerase II and proteasome. Due to enhanced solubility and prolonged half-lives, they are superior than corresponding monomeric acridines in treatment of various cancers. Moreover, multiple presentation of acridines significantly increases biological activity of dendrimeric drugs much more than corresponds to the increase of acridine concentration. Brush-like dendrimers containing acridine units are suitable for delivery of gene across the blood–brain barrier.

Dendrimers are synthetic monodisperse polymers with a repeatedly branched, highly structured architecture adopting globular three-dimensional shape. These highly branched macromolecules have a core, interior region shells, and an exterior grooved surface, with a high surface area-to-volume ratio. The higher generation of dendrimer leads to exponentially growing number of end groups, and the properties of the dendrimer are more influenced by the character of the surface end groups [1–9].

Applications of dendrimers are very diverse. They can be used in physics, organic chemistry, biomedical applications, catalysis, and as solubilizing agents. Their biocompatibility and toxicity and their applications in nanoscience, nanotechnology, drug delivery, and gene delivery are also important [1–5, 8, 10–13]. In this section, examples of dendrimers conjugated with acridines are given.

Starting from *o*-bromobenzoic acids and *o*-methylanilines, 4-methylacridines were prepared, which were brominated at methyl group with 1,3-dibromo-5, 5-dimethylhydantoin [14]. These building blocks were used for tetra-acridinylation of alkane-α, ω-diamines providing dendrimeric scaffolds [15]. The dendrimers served as inhibitors of topoisomerase II and proteasome.

Bis- and tetra-acridine derivatives (dendrimers) were used [16] to study their affinity to DNA and their antitumor potencies were compared with the appropriate monomeric DACA analogues in a battery of cell lines, including wild-type (JLC) and

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mutant (JLA and JLD) forms of human Jurkat leukemia. These dimeric analogues of DACA were superior compared with the monomeric one [17].

Peptide dendrimers with 2 to 3 Acr bound to Lys side chains were used to study amphiphilic helical peptides. Thanks to "multivalency" effect [1], picomolar affinity toward HIV-1 RRE and TAR was achieved [18]. For more details see Sect. 4.3.

Synthesis of peptide dendrimers containing central fluorescent groups was done by Fmoc solid phase peptide synthesis [19] using Acd building block for introduction of acridinone moiety [20]. Specific sequences of miscellaneous fluorescent-peptide dendrimers were prepared using this method. These dendrimers were highly water soluble because of the presence of branched amino acids containing dicarboxylic acid Asp in a dendrimeric form (together 32 free COOH groups). Fluorescent probes are often used for detection of bioorganic compounds both *in vitro* and *in vivo*. Therefore, water-soluble fluorescent-peptide dendrimers are potentially very useful as probes. The fluorescence intensity of the fluorescent groups in the dendrimers was rising by growing degree of branching and the number of branched amino acids. Based on HPLC and fluorescence spectroscopy, the authors [19] determined that the fluorescent groups are in the core of the dendrimer and the terminal carboxylic acids are located on the outside. Fluorescent-peptide dendrimers containing a combination of three fluorophores were also prepared. The occurrence of three-color FRET among them was confirmed thanks to the observed conversion of shorter wavelength light to a longer wavelength.

Peptide Lys-Lys was employed as a multiple drug carrier of three different drugs at once and tested for drug delivery and drug release [21]. However, the bioassays related to drugs containing acridine unit (3-(9-acridinylamino)-5-hydroxymethylaniline – AHMA) have not been revealed yet.

For other dendrimeric structures with acridine and their application in treatment of neurodegenerative and protozoan diseases see [22]. For more data about acridine dendrimers see [16].

9.1 Application for Drug and Gene Delivery

Nucleic acids are delivered to desired targets by many of viral and nonviral delivery systems, both of which have advantages and disadvantages [1, 2, 23–25]. In comparison with viral vectors, the nonviral carriers provide the advantage of safety and flexibility. This is paid by lower efficiency. Efficient protection and nuclear delivery of nucleic acids represent the key requirements for enhancing the transfection.

The blood–brain barrier (BBB) is a firm barrier of cells which isolates the circulating blood from the CNS. The capillaries in the cell walls of BBB are composed of endothelial cells, which form tight intersections [26, 27]. Integral membrane proteins of these intersections serve as a seal between adjacent endothelial cells. The BBB is Janus-faced. BBB is indispensable for maintaining CNS function and homeostasis. On the other site it represents a major hurdle in the treatment of various CNS diseases. The limited permeability of different drugs and supply systems across the

BBB is primarily caused by tight junctions, lack of capillary fenestrations and presence of efflux transporters. More than 98% of CNS drugs can be blocked by BBB. Therefore quest for finding new opportunities for delivery of drugs to the CNS safely and effectively is of fundamental importance.

Free amino groups of polypropylenimine dendrimers after PEGylation were acridinylated using acridine-9-isothiocyanate [28, 29]. These dendrimers are promising components for design of systemic nonviral transfecting strategy, which can penetrate through the BBB and modulate BBB drug permeability.

Brush-like dendrimers containing acridine units are suitable for delivery of gene across the BBB, which is challenging for nonviral transfection [28, 29]. As the model of BBB, brain capillary endothelial cells bEnd.3 were used. The dendrimer was conjugated with PEG and some peptides. The dendrimer–DNA complexes were condensed by Lipofectamine2000 or Exgen500 into nanosized particles. When the peptide was nuclear localization signal (NLS), polyplexes with 5–6 NLS per DNA molecule significantly increased the number of transfected cells [28]. Acridine did not increased toxicity of polyplexes. In another approach, the polyplexes were formed using three different peptidic sequences connected to the same acridine system [29]. The NLS, ApoE, and TAT were used. The polyplexes were condensed with biodegradable pLPEI polyamine and used for delivery of anti-MRP4 siRNA-producing vectors. They were 25-fold more effective in transfection of bEnd.3 cells than ExGen500. The transfection led to transient down-regulation of MRP4 and enhanced uptake of AZT. Thus, the possible gene therapy can modulate the transmissibility of BBB.

Influence of number of acridine units on binding with dsDNA was investigated [30]. The 6-(9-acridinylamino) hexanoic acid was used for derivatization of ε-amino groups of lysines as long distance spacer allowing for polyintercalation mode. Intercalators serve as protection against quick degradation of genes during delivery. Polyintercalators with 3–5 acridine units were superior to mono and bis-intercalators. These polyintercalators with DNA formed polyplexes with negative surface charge and size 200 nm. This negative charge allowed for rapid delivery of gene during electroporation.

Brush dendrimers containing up to 6 acridine units formed stable polyplexes and served for gene delivery and hydrodynamically stimulated expression [31].

Nonviral gene delivery of dendriplexes composed of pGL3 gene, PEG, and oligoacridine lysine brush dendrimers was studied including a delayed hydrodynamic stimulation [32]. It was shown, that the type of chemical bond connecting PEG to the oligoacridine lysine dendrimer and the PEG length have a great impact on the in vivo gene transfer efficiency. Important relationships between PEGylated oligoacridine lysine dendrimer structure, physical properties, in vivo metabolism, PK and biodistribution were determined. This lead to optimal PEG length and linkage that results in a robust hydrodynamic stimulated gene expression in mice. PEG with 5 kDa was found as an optimal linker for attachment to a *C* or *N* terminal Cys through a maleimide bond ([H-Lys(Acr)-Lys_4]$_3$-Lys(Acr)-Lys-Cys(maleinimide-βAla-NH-mPEG5000)-OH). The resulting pharmacokinetics and biodistribution properties led to maximal hydrodynamic stimulation when used at up to 4–5 h following post-DNA delivery. The described relationships between pharmacokinetics, biodistribution, metabolism

and gene expression can be used for optimization of other PEGylated DNA and RNAi delivery systems.

Ethyl *N*-Boc-aminoethylglycinate was conjugated with acridin-9-yl-6-aminohexanoic acid [33]. The branched structure was used for synthesis of peptide brush dendrimers (for the definition see [1, 2, 34]) containing up to 3 acridine units. Principally, it expands the synthetic routes towards acridine–peptide conjugates via building block approach (see also Sect. 3.2). The prepared dendrimeric bis- and tris-acridine NLS conjugates [33], owing to their high affinity for dsDNA, can be used as auxiliary agents for improvement of nuclear localization of cellularly transfected DNA vectors, thereby enhancing their expression. The enhancing effect is confined to a narrow N:P ratio of transfection agent to DNA. The effect can be optimized using other transfection reagents and by further optimization of the NLS conjugates. The new acridine monomer can be applied in constructing other peptides and ligands with strong binding to dsDNA by intercalation.

From nucleic acids and polyacridine Arg-rich peptides conjugated with melittin via disulfide bridge, polyplexes were obtained [35]. These polyplexes were efficient in gene delivery and endosomal escape.

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Chapter 10
Acridines Used for Staining

Abstract Acridines are applied for staining of cations, anions, small molecules, nucleic acids, proteins, lipids, viruses, protozoa, and cancer cells in optical and fluorescence microscopies. Acridinium esters are broadly used for clinical application due to strong chemiluminescence which can provide detection limits down to 1000 molecules. Encapsulation of acridines into nanoparticles can provide versatile devices for detection of various biologically important targets.

Since many acridines serve as characteristic chromophores and fluorophores [1, 2], they are used for staining of nucleic acids [3–9], proteins [2, 9–16], lipids [17], and also for detection of low molecular weight drugs such as ceftriaxone, fluvoxamine, and isoniazid [18]. Interactions of acridines with hydrophobic regions of macromolecules usually led to increased fluorescence, which is useful for various staining protocols [19]. Conjugation of acridines with biotin led to construction of various fluorophores [20].

Acridinium esters are broadly used for clinical application due to strong chemiluminescence [14]. Acridinium esters served as sonochemiluminescent probes for labeling of proteins [16]. Dimethylphenyl esters of acridinium salts are highly sensitive chemiluminescent tags that are applied in clinical diagnostics [21, 22]. These acridinium esters, when attached to an antibody with a neutral pI displayed faster emission kinetics, reduced non-specific binding and improved chemiluminescence stability in comparison with labels containing an N-sulfopropyl acridinium ring. Their light emission is switched on with alkaline peroxide in the presence of the cationic surfactant cetyltrimethylammonium chloride (CTAC) [22, 23]. CTAC has a key role in the process of chemiluminescence by both increasing light yield from acridinium esters and accelerating emission kinetics. CTAC pushes down their emission times to <5 seconds and also improves overall light yield 3–4 times [23]. Nevertheless, cationic surfactants like CTAC are toxic to aquatic life. Thus, their replacement with less toxic and degradable cationic surfactants with carbonate or amide linkages was designed [22]. The observed improvement in acridinium ester chemiluminescence is sensitive to the polarity of the micellar interface. The synthesis of new acridinium ester labels with fluorous chains of varying fluorine content is reported [23] together with their chemiluminescence in the presence of various micelles. Introduction of

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fluorinated chains led to increase of chemiluminescence stability compared with nonfluorinated systems.

Regardless the fact that acridinium esters have been extensively used for decades as chemiluminescent labels, theoretical guidelines for design of acridinium ester with improved quality over the basic structure are inaccessible. Instead, a more empirical approach was used to propose new acridinium esters with enhanced stability, fast light emission, quantum yield, low non-specific binding and improved immunoassay output [24]. The effect of branching of alkoxy substituents connected to C2 and C7 of the acridinium ring on various chemiluminescence parameters was studied. The branched acridinium esters outperformed the unbranched ones.

A highly sensitive chemiluminescent assay for H_2O_2 was developed [25] using 10-methyl-9-(phenoxycarbonyl) acridinium fluorosulfonate. The chemiluminescence was produced at neutral pH and was used to an enzyme immunoassay. The detection limits of H_2O_2 and β-D-galactosidase were 1 pmol and 0.062 amol, respectively.

Reaction of an acridinium ester alkaline solution with Mn(II) leads to a strong chemiluminescence. Mn(II) increases the chemiluminescence more than 100-times in comparison with other tested metals [26]. The data obtained indicated that oxygen is involved in the reaction and the primary product in the system was Mn(IV). Intensity of chemiluminescence depends linearly on Mn(II) concentration: it is increased for concentration below 0.01 m; however, at higher concentrations, the precipitation of $Mn(OH)_2$ occurred and caused decrease of chemiluminescence. The limit of the detection was 3.0×10^{-13} mol/L. Perhaps, some acridine systems may be useful for photodynamic therapy [27].

A very sensitive chemiluminescence technique for the determination of sympathomimetic drugs in urine was elaborated by an easy precolumn derivatization with acridinium ester [28].

The thermodynamically captured luminescence intermediate **196** can serve as a dye, the luminescence of which turns-on by gentle heating [29] (Fig. 10.1). The compound **196** can serve for labeling of biological samples with ultrasensitive detection. The concept was applied for synthesis of organically modified silica nanoparticles with amino groups and the dye **196** [30]. If antibody and 1000 molecules of the dye are attached to one particle, the signal can be amplified to the level comparable with enzyme catalyzed reactions.

Acridine orange (AO) was obtained in a form of extract from coal tar over 100 years ago [31]. It is a weak basic dye useful for staining of nucleic acids and its derivates are explored as stains not only for nucleic acids [3], but also for cells, and other particles [4, 32]. E.g. AO can stain *Bacillus anthracis* by green and orange fluorescence for dormant spores and transcriptionally active vegetative cells, respectively [33]. However, this method is limited by requirement of microspectrofluorimetry of microscopic image. Another example, AO staining can lead to naked-eye detection of amplified spleen and kidney necrosis virus [34]. Interestingly, films formed from DNA and AO can serve for time-dependent holographic recording [35]. AO formed dimers in SDS, which were used for determination of proteins [36]. Radioactive AO analogues can serve as staining of amyloidal plaques in the brain [37]. Time resolved confocal microscopy and molecular dynamic simulations provided conclusions that

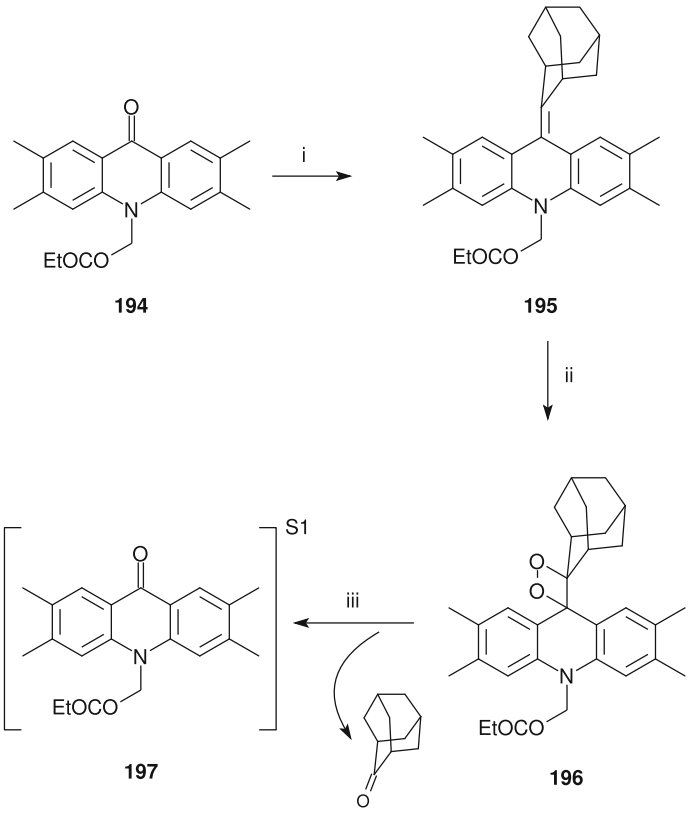

Fig. 10.1 Synthesis and luminescence of thermoluminescent dye **196** [29]. Gentle heating lead to decomposition and formation of excited acridone **197**. *i* adamantan-2-one, $TiCl_3/LiAlH_4$, Et_3N, THF, reflux. *ii* polymer-bound Bengal rose or methylene blue, hν, O_2, CH_2Cl_2, 0 °C. *iii* heating below 100 °C

AO forms a dimer in acidic organelles of non-cancer and lung cancer cells [38], which can be used for mitochondrial targeting [39].

AO can serve for staining of RNA selectively incorporated into prion particles [40] or into AD neurofibrillary tangles and senile plaques [41].

A fluorescence resonance energy transfer between AO and silver nanoparticles was elaborated as a sensitive turn-on fluorescence assay for methimazole [42]. A detection limit is 5.5 nM.

AO can be used for detection of trypanosomal or plasmodial infections in blood using fluorescence microscopy [43–45]. AO stains DNA of parasite inside hosting erythrocytes. However, simplified methods for parasite detection using AO during quantitative buffy coat techniques were also developed [46–49]. Field compatible approach using LED powered fluorescence microscopy for detection of *T. brucei* in smears was described [50]. When fluorescence microscopy is not possible, and only

conventional microscope will be available, AO can be substituted with methylene blue [51]. Optical configuration of microscope is crucial for sensitivity of *P. falciparum* detection with AO [52]. Even lower magnification can lead to better sensitivity. Stability of AO stain is paramount and is improved by storage for a month or more at 4 °C in the dark before use.

Scope and limitations of AO application in detection of malaria and other tropical parasitic diseases were discussed [53]. On the basis of experience obtained, WHO Malaria Working Group proposed AO staining for the detection and identification of malaria parasite.

Two-color analysis of cultures of *P. falciparum* stained by AO indicated similarities to the mammalian cell growth cycle [54]. The way served for tracking of early and late morphological phases in the erythrocytic cycle by evaluating red and green fluorescence, respectively. A lot of red cells can be promptly assessed for DNA and RNA content, and infected cells can be quantified and separated by the sorting power of the flow cytometer. At the common levels of parasitemia in the culture, evaluation of 100,000 red cells generated data on 5–10,000 parasitized cells. Red cells with two or more parasites could bemuse the analysis. This AO staining is accurate when freshly infected animals are tested [55]. In older stages of infection, where anemic animals with enhanced erythropoiesis were used, the augmented concentrations of nucleic acids in early released reticulocytes diminished the differentiation between the infected and non-infected cells. Moreover, reticulocytes with high levels of nucleic acids hampered the comparison between samples with various parasitemia. One has to be aware that previously published techniques for enumeration of *Plasmodium* using flow cytometry with AO are not perfect. The accuracy can be only obtained under specific conditions. *In vitro* inhibition of *P. falciparum* field isolates by drugs was tested by cytometric measurement using alternative staining with thiazole orange [56]. This two-color analysis with AO was used to determine *Plasmodium yoelii* invasion into erythrocytes and reticulocytes of Duffy positive and Duffy knockout mice [57]. Furthermore, there was a good agreement between reticulocytes analyzed with CD71 specific monoclonal antibody bound to FACS, and AO staining.

Accumulation of major classes of trypanocidal drugs in two clones of *Trypanosoma brucei brucei* and *T. b. rhodesiense* was studied by dual laser flow cytofluorimetry [58]. Bloodstream forms of these multidrug-resistant parasites gained smaller intracellular concentrations of acriflavine than the drug sensitive ones at physiological temperature.

AO was applied as a fluorescent marker of reservosomes [59–61]. For necrotic cell death in *Dictyostelium* [61, 62], AO can also be employed to monitor the permeability of acidic vesicles.

Calcium entry towards *T. brucei* bloodstream trypomastigotes is controlled by a signaling pathway including phospholipase A_2-mediated formation of arachidonic acid and boosting a plasma membrane-located Ca^{2+} channel [63]. Ca^{2+} influx in *T. brucei* procyclic trypomastigotes, *L. donovani* promastigotes and *T. cruzi* amastigotes was also enhanced in a dose-dependent manner (50–400 nM) by the amphiphilic peptide melittin [64]. The effect was lost by application

of 3-(4-octadecyl)-benzoylacrylic acid which is a phospholipase A_2 inhibitor. The arachidonic acid induced Ca^{2+} entry in the range of 10–75 μM. The mechanism was sensitive to $LaCl_3$. AO was used for monitoring of some of these processes.

The acidocalcisome, a Ca^{2+} intracellular store, was found in trypanosomatids and stained with AO [65]. Acidocalcisome was susceptible to nigericin. The Ca^{2+}/H^{+}-ATPase system is involved in Ca^{2+} accumulation, which is controlled by a pH gradient formed by ATP- and PPi-dependent proton pumps.

Study of Ca^{2+} transport in *Candida parapsilosis* spheroplasts was studied using AO [66].

Quinacrine belongs to the best dyes for long-term imaging of acidic vesicles [67] and its performance is better than that of AO. Long term studies of acidic vesicles open a way to visualize processes such as autophagy, endocytosis, and exocytosis.

To detect beer spoilage *Pectinatus* and *Megasphaera* species, DNA probes were devised [68]. The probes were labeled with acridinium ester and used in a hybridization protection study. The acridinium labeled probes were purified and evaluated for specificity and sensitivity. They were applied to detect brewery isolates of the selected species. This method was compared with contemporary used detection techniques. The described probes detect the species within the range of 0.016–0.0032 pmol.

The conjugation of fluorophores with magnetic nanoparticles leads to magnetic–fluorescent nanoparticles (MFNP) [69], which are better than traditional single-modal nanoparticles. Amino group containing magnetic nanoparticles were activated with glutaraldehyde and AO was attached via N10 as acridinium. These MFNPs are cell-permeant nucleic acid binding dye able to label nucleus and led to separation of DNA. Moreover, MFNPs demonstrated strong intracellular fluorescence after short incubation with 293T cells. The adsorption capacities detected at various concentrations demonstrated enhanced adsorption of double or single stranded DNA in comparison with non-fluorescent ones. These MFNPs have low cytotoxicity towards 293 T cells and can be considered as harmless. Thus, the MFNPs represent a versatile tool for biological applications e.g. cell labeling and DNA adsorption.

Encapsulation of AO into silica using a reverse microemulsion method afforded core-shell fluorescent nanoparticles [70]. All the nanoparticles have spherical shape with a narrow size distribution and can serve as an optical pH sensor. The function of the sensor utilizes the pH-dependence of AO fluorescence at 531 nm during excitation with 480 nm at various pH values. Increasing of the pH value causes decrease of the fluorescence intensity of the AO nanoparticles. Under optimal conditions, fluorescence intensity was linearly dependent on pH between values 8.0 and 10.9. Since the separation of the sensor can be easily done by centrifugation, no risk of environmental pollution compared to the free dyes occurs. In addition, little influence on the determination of pH by changes of ionic strength and co-existing substances were proven.

Photophysical effects of NO and its cysteine conjugate, *S*-nitrosocysteine, on the antibacterial agent AO were reported [71]. The authors suggest that NO-induced resistance to antibiotics is strongly affected by proteins containing cysteine. AO is broadly employed as an intracellular molecular probe. NO, cysteine and their conjugate *S*-nitrosocysteine are generated inside cells and they can extinguish the

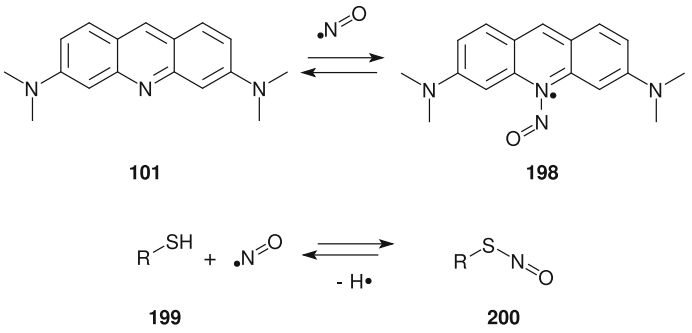

Fig. 10.2 AO fluorescence quenching by NO systems [71]

fluorescence of AO and lead to false fluorescence imaging results (Fig. 10.2). It was proven that cysteine has a principal function in governing NO-induced resistance of bacteria.

The secondary structure of the DNA molecules in the gels can be visualized by AO staining. The green or red fluorescence of AO distinguishes between native (dsDNA) and denatured (ssDNA) [3, 72–76]. Moreover, the AO fluorescence is enhanced by resonance light scattering due to nanoparticle formation with DNA and cetyltrimethyl ammonium bromide [77]. The detection limit was down to 1.8 ng/mL of nucleic acid.

"Turn off–on" phosphorescent biosensors based on quantum dots/AO can be used for detection of DNA [78].

RNA–protein interactions can be visualized by incorporation of fluorescence acridine containing amino acids into interacting protein NCp7, which recognizes RNA [79].

Extensive studies of Gram and AO staining for diagnosis of septic arthritis in different patient populations were performed on 500 episodes of arthritis [80]. AO and Gram stains were found equivalent.

Multi-spectroscopic methods together with parallel factor analysis (PARAFAC) and molecular docking were used to study the binding action of propyzamide with CT-DNA [81]. The positive changes of enthalpy and entropy indicated that DNA association was primarily driven by hydrophobic interactions. Increase of viscosity and melting temperature of CT-DNA and lowering of iodide-quenching effect indicated an intercalative binding mode. The molecular docking revealed that the A-T base pairs of CT-DNA are the principal binding sites for propyzamide. Theoretical prediction was confirmed by infrared spectroscopy. The propyzamide caused the transition of CT-DNA from B- to A-form.

Catalytic solid substrate-room temperature phosphorimetry (SS-RTP) technique for colchicine determination has been developed [82]. The method is based on strong catalytic effect on oxidation of acridine yellow by H_2O_2, which strongly extinguished RTP of acridine yellow. Ultrasensitive, precise, and selective SS-RTP with the limit of quantification 3.1×10^{-13} g . mL^{-1} has been successfully used for colchicine determination in human serum and tea samples. The reaction rate constant and the

activation energy of catalytic reaction were 3.97×10^{-4} s^{-1} and 40.53 kJ . mol^{-1}, respectively.

A selective localization of RNA species to distinct pathological lesions of neurodegenerative disease brains was detected by the study based on identification of the cytoplasmic RNA species within neurofibrillary tangles and senile plaques of AD brain [83]. An AO histofluorescence was employed, alone or in combination with immunohistochemistry and thioflavin S staining, and RNA sequestration was estimated as a common feature of pathological lesions found in progressive neurodegenerative disorders such as AD, ALS, PD, Pick's disease, etc. However, Lewy bodies, Hirano bodies, and cytoplasmic glial inclusions did not contain abundant cytoplasmic RNA species.

For example, quinacrine is suitable for molecular genetic investigation. Despite intercalation into chromosomal DNA without sequence preferences, quinacrine fluoresces brighter in AT-rich region [7]. Thus, positive bands are considered as AT-rich, whereas the negative ones as GC-rich.

Tetrakis-acridinyl peptide Ac-Lys(Qui)-[Lys_2-Lys(Qui)]$_3$-NH_2 was designed for fluorometric analysis of nucleic acids [6, 84]. The signal enhancement is achieved by fluorescence dequenching caused by transition from self-stacking of acridines to bis-intercalative mode, where the acridines are separated by nucleic acid. The magnitude of enhancement was about 1600 folds and detection limit for AT-rich DNA is ca 10 pmol. Interestingly, this effect occurs more for AT-rich regions than the GC rich. Further optimization led to design of peptide Ac-Lys(Qui)-Lys_2-Lys(FITU)-Lys_2-Lys(Qui)-NH_2, which provided AT:GC enhancement ratio of more than 2:1 [85].

Bis-intercalator Ac-Lys(Aol)-Lys-Lys-Lys(Aol)-NH_2 increases its fluorescence 200 times after dsDNA binding irrespective of its sequence [5]. The fluorescence of intercalator is not significantly enhanced by presence of ssDNA. This characteristic property makes the probe a suitable agent for the specific analysis of dsDNA in the presence of ssDNA.

N-4-(2-Methylaminoethylaminocarbonyl)acridin-9-yl)-α-alanine (*N*-(Acr4CA)-α-Ala) was introduced as a fluorescent probe for DNA determination [86]. DNA had the ability to quench the fluorescence of *N*-(Acr4CA)-α-Ala, and the quenched intensity of fluorescence was proportional to the concentration of DNA. It was suggested that the interaction mode between *N*-(Acr4CA)-α-Ala and DNA is an intercalative binding.

Acridines are very bright fluorophores, which can be detected in 1:100,000 dilution by naked-eye [87]. Acd originating from tyrosine was incorporated into calmodulin via protein expression [88] (see also Fig. 3.25). This amino acid can serve as a useful probe for studies of conformational changes of proteins based on quenching interactions with aromatic natural amino acids and/or FRET with common fluorophores. Moreover, the labeled proteins can be easily localized in bioassays.

9-Diarylamino-substituted acridines are donor–acceptor type compounds and their solvatochromic shifts and dependence of the fluorescence quantum yields on the solvent polarity and protonation indicate that these compounds can be used as chemical sensors [89]. These effects were supported by DFT based calculations.

Synthesis of fluorescent probe 9-(4-(1,2-diamine)benzene-*N*-1-phenyl)acridine (DABPA) for detection of nitric oxide (NO) was described [90]. The fluorescence intensity of DABPA was proportional to NO concentration in the interval between 1×10^{-7} to 1.5×10^{-6} mol/L. The detection limit was 1×10^{-8} mol/L. DABPA served as a probe for real-time imaging of NO in PC12 cells.

4,5-Bis(hydroxymethyl)acridine can serve for fluorescence sensing of Pd^{2+} [91]. The reaction of 4,5-bis(bromomethyl)acridine (**114**) with dimethylamine provided selective fluorescence sensor for Zn^{2+} and Cd^{2+} ions [92]. Similar complexes were studied spectroscopically [93]. 4,5-Bis(bromomethyl)acridine (**114**) was conjugated with either *N*-Boc-alaninol or *N*-Boc-phenylalaninol providing compounds L-1 and L-2, respectively [94]. Both compounds can serve as chiral fluorescence sensors of malate anion. They can distinguish the malate not only from the other hydroxy acids but also from its enantiomer in ACN. The same concept was used for design of the sensors based on 4,5-bis(bromomethyl)acridine and methyl esters of amino acids such as Ala, Phe, and Trp [95]. These enantioselective fluorescent sensors were successfully used for recognition of the malate anion in water. Later, use of alaninol and Cu^{2+} provided complexes, which change fluorescence upon binding with amino acids [96]. On the other hand, 2,7-bis(triazolium)acridine can bind to nitrate anion, especially when the interaction is enhanced by host-guest chemistry of rotaxanes [97]. The interaction can be detected by ^{1}H-NMR.

Self-assembly events and conformational changes of polypeptides and proteins are crucial for biological processes. Conformational changes of proteins can be tracked by a fluorescent probe consisting of thiophenephenylanilide–acridinium triad [98]. The triad system was prepared by well known procedure [99, 100]. Triad photophysical characteristic was carried out in the presence of poly-L-glutamic acid (PGA) at various pH values [98]. In aqueous solution, the triad showed negligible emission owing to fast intramolecular electron transfer. Under acidic condition, PGA induced significant emission near 560 nm by the charge shift state. The magnitude of the charge shift state emission was influenced with the concentration, conformation, and size of PGA. Fluorescent microscope snapshots show the shape and size of the supramolecular structures and crystals generated by PGA-triad complexes from acidic solutions.

9-Position of acridines accepts easily and selectively thiol group of proteins providing fluorescently labeled species [2, 101]. *N*-(9-Acridinyl)maleimide is nonfluorescent derivative of acridines, which upon reaction with thiols became strongly fluorescent [11]. This served as selective labeling of protein thiol groups.

10-*N*-Nonyl AO can serve as selective probe for determination of cardiolipin [17].

A facile and effective synthesis of poly(propargyl quinolinium bromide) (PPQB) and poly(propargyl acridinium bromide) (PPAB) was described [102]. Both compounds are conjugated polymers possessing polyacetylene as backbones. They are polyelectrolytes because the side groups are quaternized quinolinium or acridinium.

The molecular masses of the PPQB and PPAB were determined as 180 and 81 kDa, respectively. Both PPQB and PPAB solutions had quite strong fluorescent emissions and their films had good conductivities.

Acridine-1,8-diones can be used for covalent labeling of nucleic acids [103] or directly as sensors for anions [104].

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Chapter 11
Miscellaneous

Abstract This chapter describes curious application of acridines concerning structure and supramolecular chemistry.

Tweezers designed from acenaphthylene-1, 2-diyldi(9-acridine) (**201**) is a unique molecule with extra long C=C with length 1.3789 Å [1] (Fig. 11.1). This bond prolongation was explained by steric repulsion of bulky acridine units. The sp^2-hybridized nature and the lack of electronic perturbation for these pure C=C double bonds is guaranteed by the exclusion of both the conjugation effect and structural deformation.

Previously, a similar strategy was used for construction of compound **202** with longest covalent C–C bond with length around 1.77 Å [2] (Fig. 11.1). The extra long bond is prone to oxidative protonolysis leading to stable dication [3, 4]. However, the bond diradical character is negligible [5]. The crystal showed thermochromism: color changed from almost colorless via yellow to red-brown at -150, 60, and 140 °C, respectively.

Construction of various bis-acridinium scaffolds with extraordinary electronic properties was extensively studied [6]. For instance, chiral fluorescence can be observed which was halochromically shifted as response to the trifluoroacetic acid [7]. Usage of these hindered bis-acridinium systems can lead to on/off switching of fluorescence during the reversible interconversion of long bonds [8]. In future, some of them can have broad applications in staining of various systems (see also Chap. 10).

In the frame of theoretical studies on acridine binding, the structures and relative binding free energies for complexes of pyrazine and pyridine with Rebek's acridine diacid in chloroform have been revisited through Monte Carlo statistical mechanics calculations at 25 °C [9]. The computations were also carried out for quinoxaline. Statistical perturbation theory afforded the associated free energy changes, which combine to yield the relative free energies of binding. The experimentally observed binding order, quinoxaline >pyrazine >pyridine, was reproduced, and structures supporting two-point binding for the diazine guests were found. An explanation for the enhanced binding of quinoxaline that does not include $\pi - \pi$ stacking with the acridine spacer was also provided.

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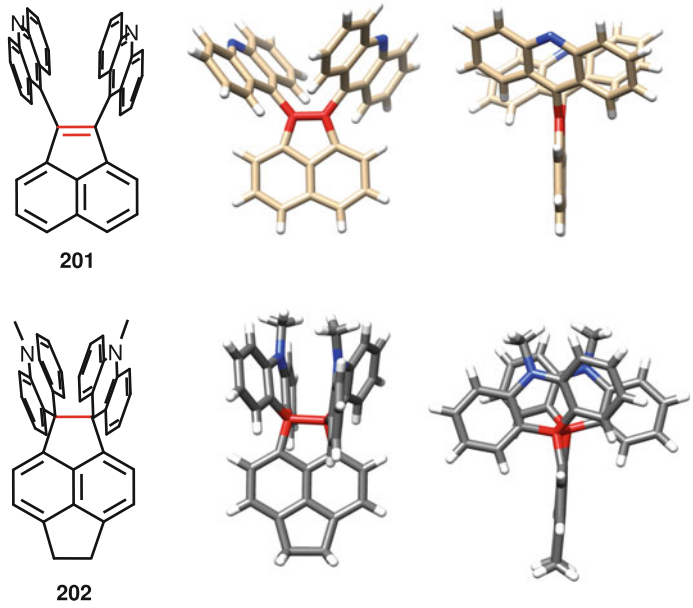
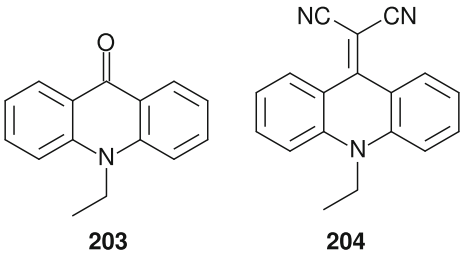


Fig. 11.1 Very long C=C bond (1.3789 Å) of acenaphthylene-1, 2-diyldi(9-acridine) (**201**) and very long C-C bond (1.77 Å) of spiro analogue of pyracene (**202**) are emphasized with *red* color in *front* and lateral views [1, 2]

Fig. 11.2 Crystals of acridines **203** and **204** started to jump, when they were heated [10]



Ethylacridone and dicyanomethylenated acridones were thermosalient structures i.e. they crystals jump upon heating [10] Fig. 11.2. The mechanism of crystal jumping of ethylacridone was attributed to anisotropic dissociation of $\pi - \pi$ stacking in a dimer. On the other hand, the collective fluctuation/flipping motion of a dicyanomethylene unit caused crystal jumping of dicyanomethylenated acridones.

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Chapter 12
Conclusions and Outlook

Abstract Concluding remarks concerning applicability of acridines are presented.

A plenty of potential anticancer agents based on acridines/acridones possess a positive effect on overcoming multidrug resistance. Their efficacy was explained by multi-targeted influence of acridines on nucleic acids and proteins. Direct interactions of acridines with proteins were also responsible for potential treatment of various states of neurodegenerative diseases. Especially, hydrophilic derivatives of tacrine have strong potential for treatment of Alzheimer's disease.

On the other hand, the wide affinity of acridines towards nucleic acids and proteins represents a hurdle for broader application of acridines in medicine. Very careful inspection of possible adverse/side effects is necessary. Thus, new acridine drugs need to be deeply optimized. Especially, hepatotoxicity has to be properly evaluated. The acridines, which do not form reactive quinone methides, have to be selected.

Photophysical properties of acridines opened ways for staining, detection, and photodeactivation of various nucleic acids and proteins. Namely, cleavage of acridinium esters provides intense chemiluminescence suitable for detection of tiny amount of desired objects.

Tuning of acridine substituents and linkers may lead to interesting properties and applications in many fields of sciences such as analytic chemistry, medicine, hygiene, physics, etc. This tuning is also useful for removal of toxicity, increase of bioavailability, selective inhibition of desired enzymes, and shape of UV-Vis and fluorescence spectra.

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