

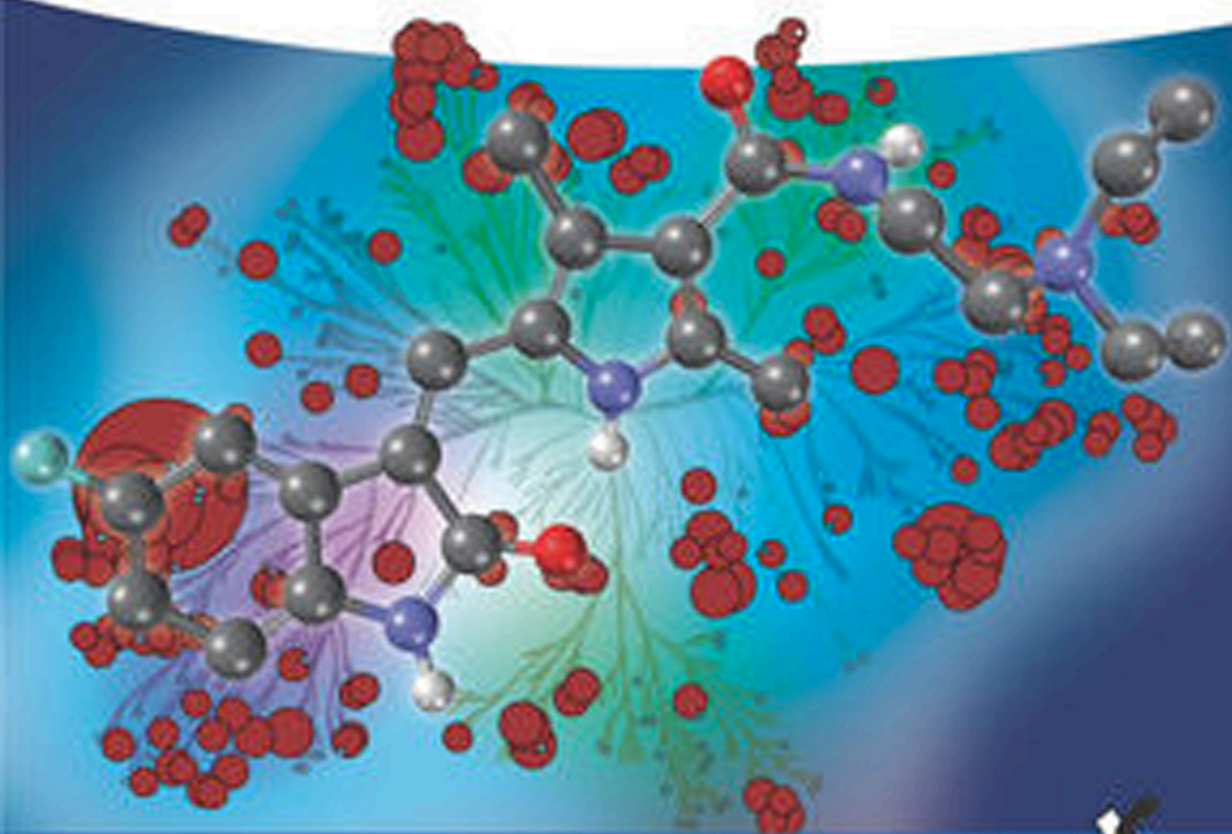
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Norbert Handler and Helmut Buschmann

Drug Selectivity

An Evolving Concept in Medicinal Chemistry

Volume 72

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R. Mannhold, H. Buschmann,
J. Holenz



Methods and Principles in Medicinal Chemistry



*Edited by Norbert Handler
and Helmut Buschmann*

Drug Selectivity

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Drug Selectivity

An Evolving Concept in Medicinal Chemistry

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Series Editors

Prof. Dr. Raimund Mannhold

Rosenweg 7
40489 Düsseldorf
Germany
mannhold@uni-duesseldorf.de

Dr. Helmut Buschmann

RD&C Research, Development &
Consulting GmbH
Ludwigsallee 21
52062 Aachen
Germany
hbuschmann@gmail.com

Dr. Jörg Holenz

GSK
R&D Neuroscience TAU
1250 S. Collegeville Road, Collegeville
Collegeville, Pennsylvania, 19426-0989
United States
joerg.x.holenz@gsk.com

Editors

Dr. Norbert Handler

RD&C Research, Development &
Consulting GmbH
Neuwaldegger Strasse 35/2/3
1170 Wien
Austria
n.handler@rdc-concepts.com

Dr. Helmut Buschmann

RD&C Research, Development &
Consulting GmbH
Ludwigsallee 21
52062 Aachen
Germany
hbuschmann@gmail.com

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Preface

A successful drug needs to be highly efficient and safe at the same time, and both attributes are strongly linked to the concept of drug selectivity.

Drug selectivity can be defined in different ways, and its simplest one is using the drug's ability to preferentially produce a desired versus a non-desired effect. However, with many diseases – especially those in the neuroscience space – being very complex, this definition has evolved, particular when looking at the molecular biological target level. In order to find meaningful medicines for diseases, the concept of polypharmacology [1] has been developed for drugs paying tribute to the idea to cure or at least positively modify a complex disease by hitting more than one biological target with a single drug [2]. In fact, a very large number of empirically found as well as designed drugs on the market act via multiple molecular targets, for example, most of the standard-of-care antidepressants, antipsychotics, or anxiolytics. Alternatively, when combating diseases like cancer, tuberculosis, and malaria, bacterial infections, and viruses like HIV or hepatitis C, approaches combining several drugs in one medicine have been tremendously successful. While the importance of the two concepts “polypharmacology” and “selective drugs hitting one molecular target” has been controversially discussed over decades, there is no doubt that both have their place in modern drug discovery, have generated an impressive track record of success, and need to be mastered by medicinal chemists.

In contrast to “desired” secondary targets a chemical compound can modify in order to boost efficacy, medicinal chemists need to make sure that the drug does not engage with “undesired” targets (often termed off-targets), leading to safety-relevant findings or side effects in the clinic. There is an increased demand from patient organizations, payers, and prescribers to provide “patient quality of life” data when a new drug is introduced to the market, and new drugs should restore a normal quality of life as much as possible. Knowledge of potential off-targets and strategies on how to measure and generate selectivity early on in a drug discovery program is key to avoid the pitfalls associated and to avoid attrition in the clinic [3].

Norbert Handler and Helmut Buschmann focus this book around the different concepts of drug selectivity and provide the reader with strategic knowledge and methods as well as with hands-on examples on how to successfully master this challenging field. The book is well structured in three major chapters around

“selectivity of marketed drugs,” and “therapeutic areas for designed multiple ligands” and provides the reader with numerous “real-life” opportunities to learn from the leading experts in the field and to transfer these learnings into own successful projects and drugs.

The series editors thank Norbert Handler and Helmut Buschmann for organizing this volume, as well as Frank Weinreich and Waltraud Wuest from Wiley-VCH for their important contributions to this project.

July 2017

Raimund Mannhold, Düsseldorf

Helmut Buschmann, Aachen

Jörg Holenz, Collegeville, Pennsylvania, USA

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- 3 Papoian, T., Chiu, H.-J., Elayan, I., Jagadeesh, G., Khan, I., Laniyonu, A.A., Li, C.X., Saulnier, M., Simpson, N., and Yang, B. (2015) Secondary pharmacology data to assess potential off-target activity of new drugs: a regulatory perspective. *Nat. Rev. Drug Discovery*, **14**, 294–297.

A Personal Foreword

Drug selectivity is one of the most discussed “hot topics” in medicinal chemistry that will require additional detailed work and joint efforts of the scientific community. This book is trying to fill an essential information gap and give a highly relevant overview on the current status of the science in the field especially for medicinal chemists.

The book comprises several chapters including basic information on the concept of polypharmacology, discovery techniques for finding and developing multiple ligands, and many examples on former and current molecules with multiple target profile classified by medical indication.

In more detail, an introductory chapter on polypharmacology describes the pharmacological principle behind the paradigm of multiple ligands including several examples and discusses advantages and disadvantages of this approach. Additionally, a general chapter deals with the latest trend in pharmaceutical and medical science to individualize the medication according to the individual human genotype and phenotype, which is a highly complex project being just at the very beginning of the development.

Another series of chapters highlights the selectivity of marketed drugs, where the drug class of kinase inhibitors is analyzed as a highly relevant family of drugs. Additionally, the possibilities to find new indication for marketed drugs based on their selectivity profile are described in detail, and on the other hand discovery technologies for new indications and drug discovery techniques for lead generation are elaborated.

A highly relevant area of topics deals with various techniques for discovery and development of multiple ligands as new chemical entities providing a desired binding pattern. They include the designing-in approach, where molecules with a certain existing binding pattern gain additional binding properties through variation of the structure; the linker approach to develop drug conjugates, where certain structural elements are connected via linkers; the design of merged multiple ligands, where certain binding properties of different molecules are merged into a single molecular structure; and finally, the development of relevant pharmacophore models to define common structural patterns for binding to multiple targets. To complement this medchem-driven section, a chapter dealing with the development of cellular assays for multiple ligands was included.

Finally, an overview on current therapeutic indications, where development of multiple ligands has already been successful or is currently ongoing,

respectively, was added dealing with the serotonin transporter system and the angiotensin system, additionally describing multiple ligands at peroxisome proliferator-activated receptor (PPAR) receptors and latest developments in antibiotic research and finally highlighting the role of multiple ligands in neurodegenerative diseases.

Overall, we are very happy that so many authors accepted our invitation to write a chapter for the present book and would like to cordially thank everyone who contributed to make this project possible.

July 2017

Norbert Handler, Wien

Helmut Buschmann, Aachen

Part I

Introduction

1

Polypharmacology in Drug Discovery

Oscar Méndez-Lucio¹, J. Jesús Naveja^{2,3}, Hugo Vite-Caritino²,
Fernando D. Prieto-Martínez², and José L. Medina-Franco²

¹ University of Cambridge, Centre for Molecular Informatics, Department of Chemistry, Lensfield Road, Cambridge CB2 1EW, UK

² Universidad Nacional Autónoma de México, Facultad de Química, Departamento de Farmacia, Avenida Universidad 3000, 04510 Mexico City, Mexico

³ Universidad Nacional Autónoma de México, Facultad de Medicina, PCEM, Department of Pharmacy, Avenida Universidad 3000, México, D.F. 04510, Mexico

1.1 Polypharmacology

Currently, the main paradigm in drug discovery is the development of target-specific inhibitors. This also implies molecules with high-fold potency and selectivity toward one isoform. This mainstream view has its origins in the so-called magic bullet as enunciated by Paul Ehrlich over 150 years ago. Indeed, such concept was engraved in the mind of many health professionals and researchers as the top achievement in drug discovery. However, as years came by, this has proven to be a disappointment mainly because of the off-target responses, which may involve toxicological concerns or side effects. For example, considering the wide array of enzymatic systems, classes, and isoforms identified in biology, it is no wonder that many target-specific agents had been developed via trial-and-error approaches [1].

Recent statistics show that pharmaceutical industry is struggling as many promising drugs fail during the early stages of drug development along with the associated significant economic disadvantages [2]. This shows we have reached an impasse: just between 1996 and 2001, a large number of drugs were withdrawn from the market because of similar reasons [3]. Furthermore, even selective drugs are not exempt of drug–drug interactions that also represent a drawback, especially for chronic therapies. After reaching this point, we must ask ourselves if this mainstream view needs refinement or a drastic change of perspective. So if target-based drug discovery has not lived up to expectations, what choices we have left? What if the so-called side effects are not “failures” after all? In the right context, multitarget modulation is desired or perhaps mandatory for successful therapies [4].

What exactly does polypharmacology mean? Strictly speaking, polypharmacology refers to molecules that are recognized by different molecular targets.

The affinity shown toward the targets may vary, but as previously mentioned, such compounds may be discarded fearing this promiscuity may trigger off-target effects [5]. Thus, we are walking a fine line between positive and negative connotations. For that matter polypharmacology usually associates with positive outcomes. It involves the search of “master key compounds” to tackle chronic diseases, for example, CNS disorders share multifactorial processes that ultimately lead to degeneration, physiologically speaking. Therefore, a single-target inhibition is of no use here as complex processes require integral approaches [6].

Compound promiscuity is a concept closely related to polypharmacology. This of course tells us about a molecule that interacts with many proteins or receptors. Promiscuity is usually related to negative connotations, for example, it is conceptualized as unwanted characteristic such as toxic effects due to off-target interactions. In turn, compound promiscuity is related to the pan assay interference compounds (PAINS). These molecules appear to be a jack-of-all-trades with potent binding and activity, while the truth is they are a master of none. Baell and Waters first warned about these “con artists” as they lure naïve chemists or biologists who waste valuable resources with a lost cause [7]. Of note, PAINS are not always promiscuous. They can be flagged as active because they produce metal chelation, chemical aggregation, redox activity, compound fluorescence, cysteine oxidation, or other kinds of interference. Putting briefly these concepts together, the pressing matter here is to understand and give the right context to “polypharmacology,” meaning that while related to “chemical promiscuity,” we cannot put them on the same basket any longer.

Polypharmacy is one more concept related to polypharmacology. Polypharmacy “can mean the prescribing of either many drugs (appropriately) or too many drugs (inappropriately). The term is usually used in the second of these senses, and pejoratively. However, when talking about polypharmacy, it would be wise to qualify it as appropriate or inappropriate” [8].

As of 2014 the number of articles citing “polypharmacology” as part of its title and/or as a keyword has increased significantly, with almost 200 articles published in the past three years only [9]. So a multitarget approach is gaining adepts at steady pace. While this shows more promise in the grand scheme of drug discovery, we must be careful and correctly assess the opportunities and challenges of this transition era. We should not instantly accept polypharmacology as a panacea of sorts, but only time and advances in current knowledge will determine the success of such paradigm change; we must conserve an objective view on the subject with realistic expectations.

Although the road ahead in polypharmacology drug discovery may seem blurry or difficult to achieve, the development of polydrugs is currently possible. As discussed in this chapter, the development and application of computational methods and tools for *in silico* drug discovery should be a starting point and compass to navigate the “chemical wilderness.” Computational approaches include, but are not limited to, chemoinformatics, molecular similarity, docking, molecular dynamics, virtual screening, and quantitative structure–activity relationship (QSAR).

This chapter is organized in six sections. After this introduction, general aspects of multitarget versus target-specific drugs are discussed including the

rationale, the “master key compound” concept, and the safety panels to address the possible unwanted effects of drug multitargeting. The next part elaborates on the relationship between polypharmacology and other major concepts in drug discovery, including drug repurposing, combination of drugs, and *in vivo* testing. The section after that describes briefly examples of applications of polypharmacology and polypharmacy to the development of epi-drugs and antiviral compounds, respectively. It follows a discussion on different modern approaches to study systematically polypharmacological relationships and design multitarget drugs. A special emphasis is made on the concept of chemogenomics. The last part of the chapter presents summary conclusions.

1.2 Multitarget versus Target-Specific Drugs

As discussed before, the increasing awareness of the large complexity of systems biology is shifting the paradigm in drug discovery from a single-target to a multitarget approach [10]. Despite the fact that the latter approach is significantly more complicated than the one-drug–one-target strategy (largely influenced by a reductionist perspective of systems biology) [11], it may lead to drugs that are more effective in the clinic. However, it has to be considered that multitarget drug design, and polypharmacology in general, highly depends on the dose to deliver an overall clinical benefit [12]. For instance, a drug may have a positive effect at therapeutic doses because of the interaction with multitargets. However, the interaction of the same compound with antitargets at higher doses will lead to undesirable side effects [12]. Thus, similar to the appropriate or inappropriate polypharmacy discussed by Aronson [8], polypharmacology can also lead to desirable or undesirable (e.g., unwanted promiscuity) multitarget drug interactions that will depend not only on the nature of the structures of the drugs and targets but also on the compound concentrations. The “dual face” of multitarget drugs is schematically illustrated in Figure 1.1.

1.2.1 “Master Key Compounds”

A “master key compound” (luckily “master key drug”) is a molecule that binds to a given number of targets that produce a desirable clinical effect without hitting (or with a minimum effect) off-targets that are related to undesirable secondary effects [10]. In a simple analogy with a master key, a “master key molecule” should have the ability to operate on a group/set of selected targets (doors) but not on any “doors,” in particular those antitargets that lead to undesirable side effects. Table 1.1 illustrates examples of master key drugs that are used in the market.

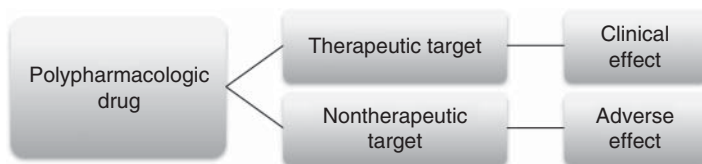
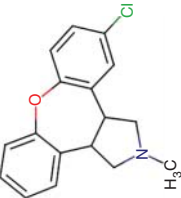
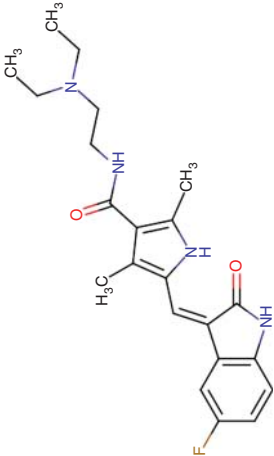
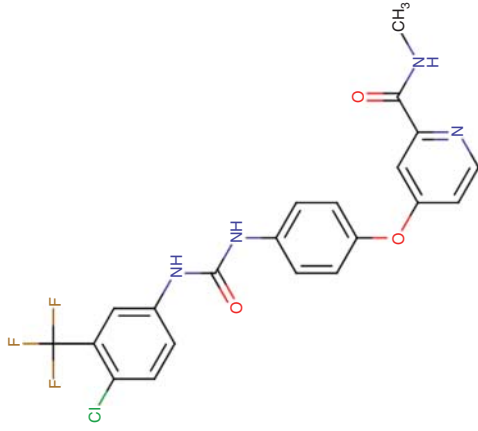


Figure 1.1 The “dual face” of multitarget compounds and relationship with “master key drugs.”

Table 1.1 Examples of master key drugs approved for clinical use.

Name	Structure	Clinical use	Associated receptors
Asenapine Schering-Plough (2009)		Antipsychotic	D ₁ -D ₄ dopaminergic; 5-HT _{1A} , 5-HT _{1B} , 5-HT _{2A-C} , 5-HT _{5A} , 5-HT ₆ , and 5-HT ₇ serotonergic; α ₁ and α _{2A-C} adrenergic; and H ₁ and H ₂ histaminic
Sunitinib Pfizer (2006)		Anticancer	Vascular endothelial growth factor (VEGFR), PDGFR, c-Kit, Flt-3, resonance energy transfer (RET), colony-stimulating factor 1 receptor (CSF-1R)

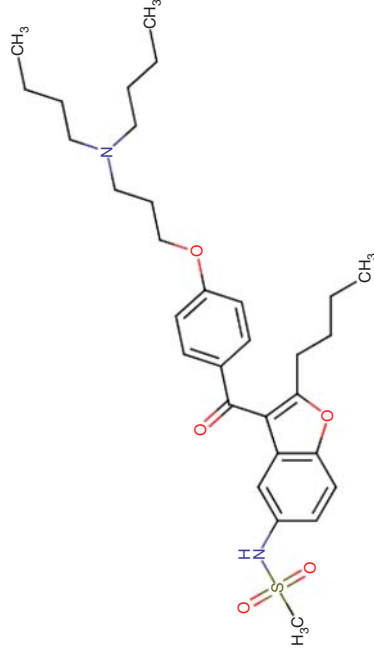
Sorafenib
Bayer
(2005)



Anticancer

B-Raf, VEGFR, PDGFR, c-Kit,
Fms-like tyrosine kinase 3
(Flt-3), RET

Dronedarone
Sanofi-Aventis
(2009)



Antiarrhythmic

Sodium, calcium, and
potassium channels; α_1 and β_1
adrenergic

The name and targets are indicated.

The table summarizes the name, chemical structure, clinical use, and the associated molecular target receptors.

Kinase inhibitors are representative yet controversial examples of master key compounds used in the clinic. Despite the fact there are differences in the kinase domains, the binding site of ATP is highly conserved across all the kinases. Since the ATP site is targeted by a large number of kinase inhibitors, there are selectivity issues, and there is a significant challenge to develop master key inhibitors of kinases (Figure 1.2). Several efforts in the pharmaceutical industry and academia have been dedicated to develop selective kinase inhibitors in order to reduce side effects. However, it is also noteworthy that some extremely promiscuous kinase inhibitors have shown good clinical performance, even when treating unrelated tumors, for instance, dasatinib, which binds to at least 159 kinases [15]. This has not been overlooked by the scientific community, which is already working on the idea of designing multitarget protein kinase inhibitors [16, 17]. Currently, there are two strategies to exploit polypharmacology against kinases, that is, combination of selective compounds and design of “selectively nonselective,” that is, master key kinase inhibitors (Figure 1.3) [10, 16]. The former refers to the simultaneous administration of two selective compounds designed to inhibit different kinases in order to achieve an enhanced phenotypic effect. The latter, and the most difficult, consists in merging the inhibitory activity against two or more kinases in one single compound with none (or only few) off-targets. The key factor in either of the mentioned strategies is to identify the targets that should be inhibited simultaneously to produce a selective phenotypic effect against the tumor. Kinase inhibitors are discussed in detail in Section 2.4.2 of this book.

1.2.2 Safety Panels

Many of the adverse drug reactions (ADRs) are caused by unintentional interaction of a drug with a nontherapeutic target to which is given the name “antitarget.” The most frequently found antitargets are already well studied and characterized. Examples of these receptors are shown in Table 1.2.

Animal toxicity models are not practical to predict the adverse effects caused by antitargets in humans due to differences between species. For instance, human ion channels differ greatly from their rodent orthologs. Therefore, the International Conference on Harmonization (ICH) guideline S7A for security studies recommend performing antitarget screening tests. These tests are ligand binding assays using enzymatic methods to obtain data and provide suggestions concerning potential adverse effects of the molecule under study. Antitarget screening tests also protect the early volunteers in clinical studies from developing ADRs as it is estimated that the toxicity in the preclinical stage causes that 30% of the compounds do not advance to the clinical stage.

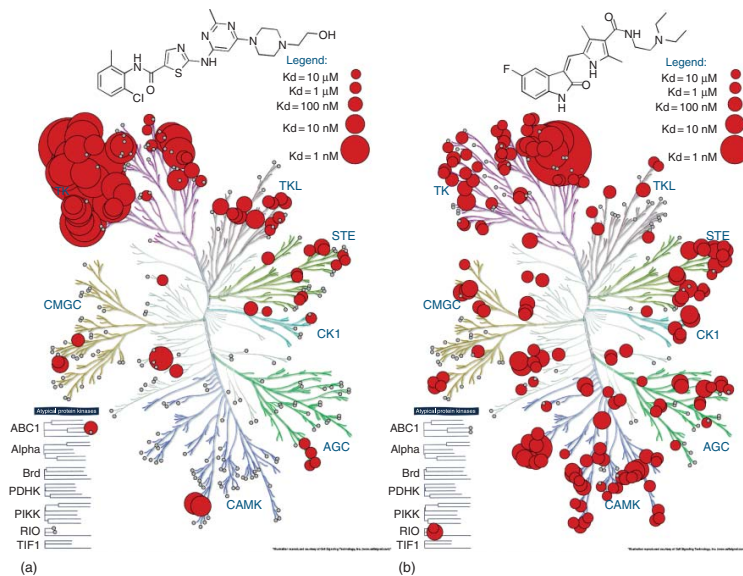


Figure 1.2 Examples of poor selectivity of kinase inhibitors. This figure shows the cross-reactivity of dasatinib (a) and sunitinib (b) across the kinome. (Data was obtained from Karaman *et al.* [13] and the figure was generated using Kinome Render [14].)

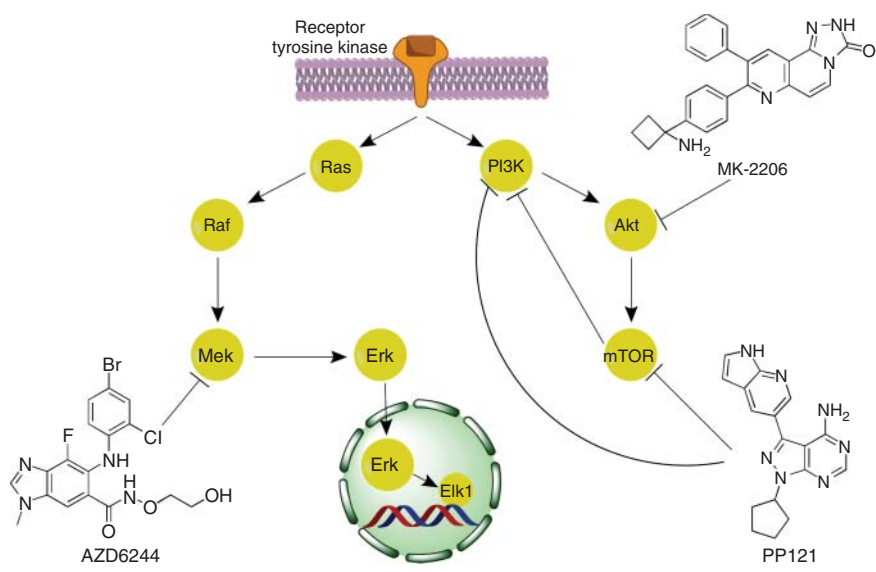


Figure 1.3 Examples of the use of polypharmacology against kinases. Compounds **AZD6244** and **MK-2206** have been used in combination to inhibit the MAPK and PI3K pathways to obtain an enhanced phenotypic effect. Compound **PP121** inhibits both PI3K and mammalian target of rapamycin (mTOR) simultaneously. This dual inhibition has been proposed to be more potent than inhibiting either target individually. The rationale behind this idea is that mTOR activates a negative feedback loop that inhibits PI3K. The inhibition of mTOR alone results in the blockage of the negative feedback loop and in a hyperactivation of PI3K [18].

Table 1.2 Major antitarget receptors.

Antitarget receptor	Hit rate	Adverse drug reaction
Human ether-a-go-go related gene (hERG) channel	—	Arrhythmia
Serotonin 5-HT _{2B}	14	Valvulopathy, pulmonary hypertension
Serotonin 5-HT _{2A}	11	Cognitive impairment, hallucination
α _{1A} Adrenergic	10	Arrhythmia, orthostatic hypotension
Dopamine D ₂	9	Confusion, emesis, orthostatic hypotension
Histamine H ₁	6	Weight gain, sedation, somnolence
α _{2A} Adrenergic	6	Hypotension, sedation
Dopamine D ₁	5	Dyskinesia, tremor
M ₁₋₅ muscarinic	5	Multiple cardiovascular and metabolic adverse effects, cognitive impairment
μ-Opioid	3	Sedation, respiratory depression, abuse potential

1.3 Polypharmacology and Related Concepts in Drug Discovery

The interaction of a compound with multitargets is at the core of several major concepts in current drug discovery [10]. Herein we further elaborate on these relationships.

1.3.1 Drug Repurposing

In general terms, drug repurposing or repositioning is the identification of a new clinical use for compounds that have already proven to be useful to treat a defined medical condition. It can extend only to the conceptual approach or involve the whole process, until the new indication is approved by the respective institutions. Notably, drug repurposing is not by itself a strategy, since it may be a result of different strategies and methods; it can be serendipitous, semi-systematic, or fully systematic, by means of high-throughput screening or *in silico* approaches [19]. The subjacent principles of drug repurposing imply that drugs might have activity against more than one therapeutic target, that is, polypharmacology.

Nonetheless, there are many distinct definitions of drug repurposing, and there are efforts focused on further condensing the different proposals. Recently, Langedijk *et al.* performed a systematic review of the literature in order to unify the otherwise diverse and sometimes discordant definitions of “drug repurposing/repositioning/redirecting/reprofiling/rediscovery” and concluded that the main features of these definitions are regarding [20]:

- a) General concept: strategy, process, and approach
- b) Action performed: identifying, using, or developing
- c) Innovative use: for a different disease, patient population, dosage, or route of administration
- d) The product itself: an existing or abandoned pharmaceutical active ingredient, patent, medicinal product, and so on

In particular, systematic or rational drug repurposing is of current general interest due to the marked advantages of drug repurposing versus *de novo* drug discovery in terms of time, costs, and patients’ safety [21]. More specifically, computational approaches have proven to be cost effective and are viable options under several circumstances, such as finding therapeutic agents against neglected [22–24] or rare diseases [25]. In several recent studies potential alternative activities are being uncovered, and further investigation is underway to see if these compounds can be approved for clinical use for the alternative indication. For example, olsalazine, a drug approved for the treatment of inflammatory bowel disease, was recently identified as a novel hypomethylating agent using a chemoinformatics-based virtual screening approach [26]. Concisely, it is being investigated as a potential epigenetic drug. Following up this successful proof of concept, additional computational studies have been conducted with the aim of repurposing approved drugs as potential epi-drugs [19]. A thorough discussion on drug repurposing is presented on Chapter 4 of this book.

1.3.2 Combination of Drugs

Combinations of drugs are clinically relevant for treating a variety of chronic medical conditions, such as infectious, metabolic, malignant, or neurological diseases [8]. A clear example is the highly active antiretroviral therapy (HAART) used for the treatment of patients infected with the human immunodeficiency virus (HIV) [27]. Combinations of drugs could be used to prevent or attack resistance to single agents and to improve the clinical effect of the treatment. However, this approach often ends with polypharmacy (the intake of five or more drugs), a well-described clinical condition that can lead to increased risks and adverse effects from medications, especially in the elderly or patients with multiple chronic diseases [28, 29].

In the scientific literature, it is generally conceived that the development of polypharmacological agents is the next logical step once it is known that a single chemical compound may affect multiple biological targets (e.g., adverse or off-target effects) and that combinations of drugs that act on different targets might have additive or synergic effects against a disease. Polypharmacology is believed to be a promising feature of drugs that could replace combined drug therapies [30] and thus avoid polypharmacy.

Nonetheless, there is another point of view in which drug combinations are included within polypharmacology approaches following the multitarget paradigm while still recognizing the advantages of multitarget single agents [31]. A third approach was developed recently by Gujral *et al.* after this group identified kinases involved in cellular migration that are specific for cell type. To accomplish this, they tested polypharmacological kinase inhibitors. Their proposal is to exploit polypharmacology of chemical probes to aid in the rational design of more potent and specific drug combinations [32]. This last approach is supported by the finding that combination therapies acting synergistically are also more specific in their pharmacological actions when administered in combination than as single agents [33]. Hence, combination of drugs and polypharmacology does not necessarily imply more severe adverse effects when there is a synergic effect, provided that selectivity is increased in these cases.

Finally, the combination of drugs may be used for preventing adverse effects or severe risks of certain drugs used in monotherapy. For example, Zhao *et al.* discovered that coadministration of exenatide substantially reduces the myocardial infarction risk found in diabetic patients treated with rosiglitazone alone. Both exenatide and rosiglitazone are indicated for the treatment of diabetes mellitus type 2 and they act on different targets [34]. A more detailed discussion of combination of drugs is fully addressed on Chapter 3 of this book.

1.3.3 *In Vivo* Testing

Several drugs have been identified following an *in vivo* screening or natural product mixtures or mixtures of individual compounds. *In vivo* testing is a drug discovery approach that distances itself from the “classical” one-target screening. It has been recognized that *in vivo* screening offers the advantage of an early demonstration that compounds may show activity in disease-relevant models before proceeding with further development. Moreover, despite the limitations

and costs of *in vivo* testing, it allows the rapid selection of molecules that exert their biological effect through the interaction with multiple targets (present in an *in vivo* system). Therefore, this methodology represents an approach to identify “master key compounds” discussed earlier. *In vivo* testing of mixture-based combinatorial libraries has been used as an effective drug discovery approach to rapidly screen hundreds or thousands of compounds efficiently [35]. Moreover, *in vivo* testing of mixture-based combinatorial libraries has enabled to expand the exploration of the chemical space beyond the one populated by currently marketed drugs [10].

1.4 Polypharmacology (and Polypharmacy): Case Studies

In this section, we discuss briefly selected applications of polypharmacology and polypharmacy for the treatment of diseases associated with epigenetic alterations and antiviral infections caused by HIV, respectively. Both types of diseases are different in nature, but both are very complex and represent major challenges to design effective therapeutic treatments.

1.4.1 Polypharmacology in Epigenetics

For diseases with a complex metabolic substrate, such as diabetes, cancer, and autoimmune and neurodegenerative disorders, it becomes increasingly evident that aiming to a single pharmacological target would not be an appropriate strategy. It has been shown to a variable extent for each of the aforementioned diseases that epigenetics (inheritable traits that are not encoded within the genome, e.g., DNA methylation and histone modifications) plays an important role in the establishment and maintenance of the disease [36]. Epigenetic mechanisms are extremely complex, and not yet totally understood, which makes quite difficult to design therapies directed against them. However, epigenetic drugs are appearing in the clinical scenario, mostly for the treatment of malignant or premalignant states, with favorable results [37, 38]. Moreover, there is a current trend for shifting toward epi-polypharmacology drugs against either more than one epigenetic target or combined epigenetic and other targets [4]. Notably, many different epigenetic biological targets share a reduced number of cofactors (e.g., Zn^{+2} , NAD^+ , SAM), and thus it is feasible to guide the design of cofactor inhibitors with polypharmacologic properties [39]. Another approach is the design of hybrid molecules. This strategy has led to the development of pan-demethylase inhibitors by synthesis of hybrid molecules containing inhibitors of histone demethylases LSD1 and JmjC, thus generating compounds that increase H3K4 and H3K9 methylation levels and produce apoptosis selectively to cancer cell lines, with little effect on noncancer cells [40]. In other cases, compounds that are likely to inhibit concise epigenetic targets show polypharmacology against other epigenetic targets. This was the case of the AMI-5 analogs synthesized by Mai *et al.* [41]. AMI-5 was described previously as a small molecule inhibitor of protein arginine and histone lysine

methyltransferases, whereas some of its analogs were able to target multiple epigenetic targets, including protein and histone methyl- and acetyltransferases.

1.4.2 Charting the Epigenetic Relevant Chemical Space

As discussed in the preceding section, epigenetics involves a series of complex phenomena involving different enzymes that work as readers, erasers, and writers. Toward the design of compounds directed to multiple epigenetic targets, we have initiated a first assessment of the epigenetic relevant chemical space (ERCS) focused on DNA methyltransferase (DNMT) inhibitors [42]. To further illustrate this point, Figure 1.4 shows a visual representation of the chemical space obtained by principal component analysis (PCA) of six physicochemical properties of data sets of molecules tested as inhibitors of bromodomains (BRDs), histone deacetylases (HDACs), and DNMTs. As reference, generally recognized as safe (GRAS) molecules were included. The physicochemical properties computed were number of acceptors/donors of hydrogen bonds, number of rotatable bonds, molecular weight, octanol/water partition coefficient, and topological surface area. According to this visualization, different compound

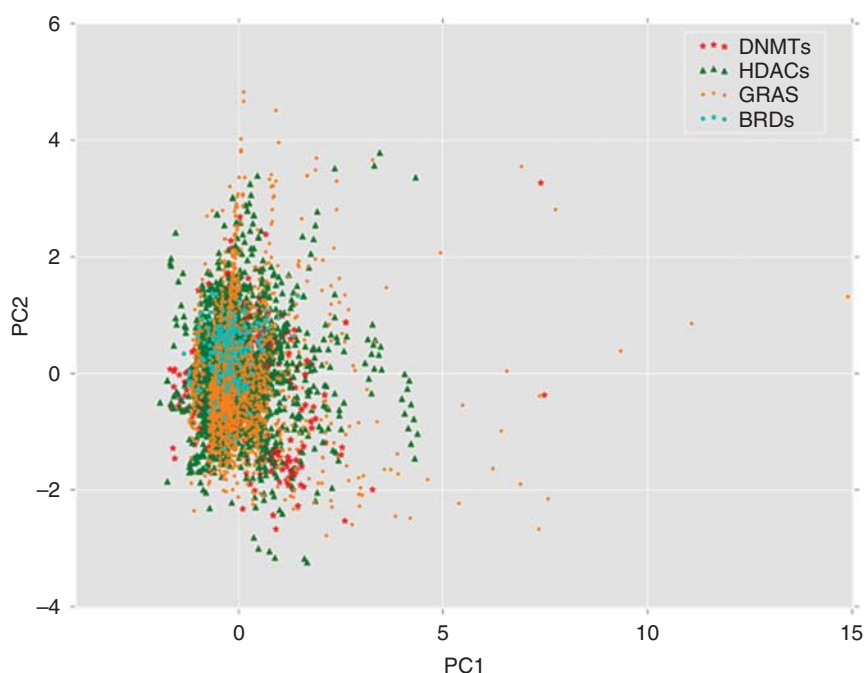


Figure 1.4 Visual representation of the chemical space of inhibitors of histone deacetylases (HDACs), bromodomains (BRDs), DNA methyltransferases (DNMTs), and generally recognized as safe (GRAS) compounds. The principal component analysis was done with six pharmaceutically relevant physicochemical properties. The first two principal components (PCs) are represented in the figure.

data sets populate similar regions in the chemical space since they share similar physicochemical characteristics. From this preliminary analysis, convergence in the chemical space of ERCS can be expected.

It is expected that the mapping and interpretation of chemical space improves the current knowledge on epigenetics. For example, by assessing the properties of epi-compounds, it may be possible to develop empirical rules to catalog new molecules as epi-modulators and, overall, identify the structural characteristics needed to achieve optimal multiple epi-inhibition [43].

1.4.3 Polypharmacy for the Treatment of HIV Infections

Acquired immune deficiency syndrome (AIDS) is still a major health problem. In 2014 there were 36.9 million people living with HIV. The drugs available today for the treatment of HIV can be classified into several classes: reverse transcriptase inhibitors nucleoside reverse transcriptase inhibitors (nucleotide) (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), HIV protease inhibitors, integrase inhibitors, a fusion inhibitor (to prevent the fusion of the viral envelope with the host cell membrane), and a CCR5 inhibitor (to block the interaction of the virus with one of its receptors at the host cell) [44]. The rapid emergence of resistant strains requires the coadministration of several drugs with different mechanisms of action and hitting different molecular targets. HIV-infected individuals are subject to HAART, where two or more drugs are administered in various combinations and administration schedules. Current treatments require the combination of at least two or three active drugs from at least two different classes. Despite the fact that this polypharmacy approach is able to reduce the viral loads in patients, reducing the incidence of opportunistic infections and deaths in AIDS patients, there are concerns of serious side effects and the eventual fail of a given treatment schedule due to the emergence of resistance. Resistance is primarily due to the development of mutations in RT, integrase, and HIV protease. Moreover, Edelman *et al.* discusses that indeed polypharmacy is the next therapeutic challenge in HIV [29]. Combination of drugs for the treatment of viral infections is discussed in detail in Section 3.5.3 of this book.

1.5 Computational Strategies to Explore Polypharmacology

Since the chemical and biological spaces are huge, the relationship between the two spaces is highly complex. Therefore, in order to describe, understand, and ideally predict the relationship between the two spaces, efficient computer-based methods are necessary. In response to such need, chemogenomics has emerged as a multidisciplinary research field. A number of rich reviews have been published on chemogenomics [45, 46]. In this section, we discuss recent developments on chemogenomics followed by representative and specific computational chemogenomics methods.

1.5.1 Chemogenomics: Intersection of Chemical and Biological Spaces

The concept of polypharmacology is at the interface of the chemical and biological spaces. Both the chemical and biological spaces are intuitive concepts because of its analogy with the cosmic universe [47, 48]. There are several definitions of chemical space. For instance, Virshup *et al.* define chemical space as “an M-dimensional Cartesian space in which compounds are located by a set of M physicochemical and/or chemoinformatic descriptors” [49]. The concept of chemical space has a broad application in drug discovery that can be classified into two major groups: (i) classification of bioactive compounds depending on their therapeutic target or associated pharmaceutical effect and (ii) compound library design and selection. By analogy, biological space can be understood as the set of all possible targets. Some of them, however, are associated with desirable chemical effects, while other are related to off-targets leading to adverse effects, “orphan targets” (for which not compounds/drug have been identified yet), and targets to be identified.

Chemogenomics is a multidisciplinary research field that aims to identify the possible associations of all possible ligands for all possible targets [50]. To achieve this goal a number of *in vitro* and *in silico* approaches are employed [45]. In other words, chemogenomics aims to find the association between the chemical and target spaces or to characterize the intersection between chemical and biological spaces. The concept of chemogenomics is schematically illustrated in Figure 1.5. As discussed in detail elsewhere, chemogenomics is highly

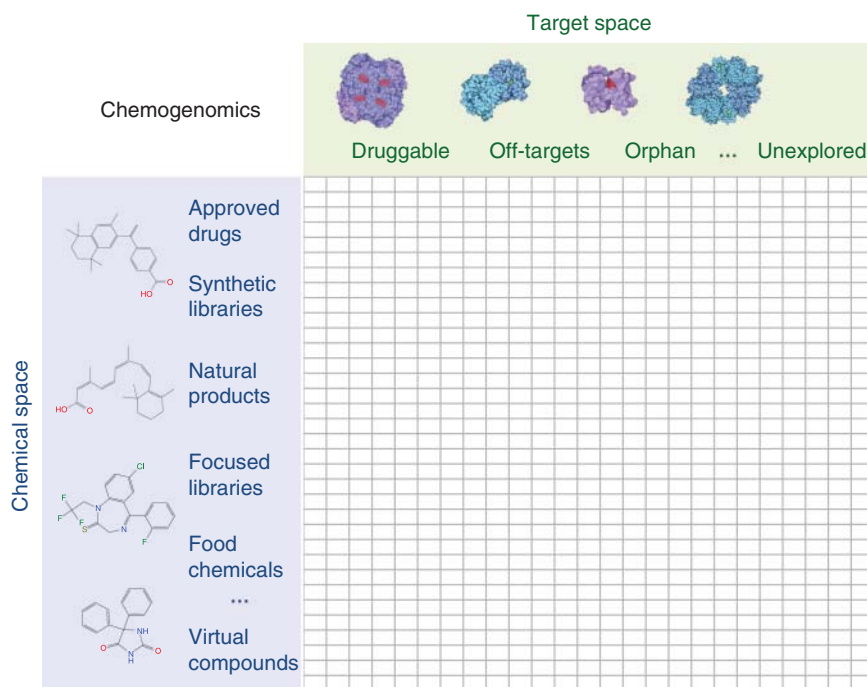


Figure 1.5 Schematic representation of a chemogenomics matrix; the rows represent all possible compounds and the columns represent all possible molecular targets.

Table 1.3 Representative chemogenomics data sets to explore polypharmacology.

Data set	Summary contents	References
ChEMBL	Contains more than 13 million activity data points corresponding to 1,463,270 compounds against 10,774 targets	[51, 52]
PubChem BioAssay	Contains more than 130 million activity outcomes covering more than 5000 protein targets	[53]
Binding Database	Contains more than 1 million binding data for 7302 protein targets and 495,498 small molecules	[54]
MOAD	Collection of 25,771 high-resolution crystal structures, 9141 of them with activity data	[55, 56]
PDBbind	Binding data for 14,260 biomolecular complexes contained in the PDB. Noteworthy, 11,987 correspond to protein–ligand interactions	[57]
EpiDBase	Focused to epigenetic targets. It contains 11,422 activity data corresponding to 5784 ligands against 220 epigenetic targets	[58]
CMAP	More than 7000 gene expression profiles of 1309 compounds in different cell lines	[59]
LINCS L1000	Gene expression signatures of 22,412 unique perturbations (compounds and knockdowns) applied to 56 different cellular contexts including human primary cell lines and cancer cell lines	[60]

associated with concepts such as polypharmacology itself, drug repurposing, *in vivo* high-throughput screening, pharmaceutical profiling, virtual screening, target fishing, and structure–multiple activity relationships (SmARs) (*vide infra*).

Chemogenomics data sets are major resources to conduct systematic studies to find associations between compound–target interactions. Table 1.3 summarizes examples of chemogenomics data sets [51–60]. One of the current limitations of these data sets is that they are still rather incomplete. For instance, in order to analyze drug–target interaction networks, the effect of the lack of data completeness has been analyzed, which has been called the “Achilles heel” of drug–target networks [61]. However, such databases are rich sources of information to describe ligand–target interactions and to uncover new target–ligand relationships. Other major areas of improvement of chemogenomics data sets are the so-called “five I’s”: data may be incomplete, inaccurate, imprecise, incompatible, and/or irreproducible as recently described by Fourches *et al.* [46]. Authors of that work proposed a general workflow to conduct chemical and biological data curation [46]. Figure 1.6 illustrates in a schematic manner examples of computational approaches employed to explore chemogenomics relationships [62–64].

1.5.2 Structure–Multiple Activity Relationships

Drug discovery based on one-molecule–one-target approach gave rise to biological assays where, typically, one compound is associated with one measure of activity. In order to establish the corresponding structure–activity

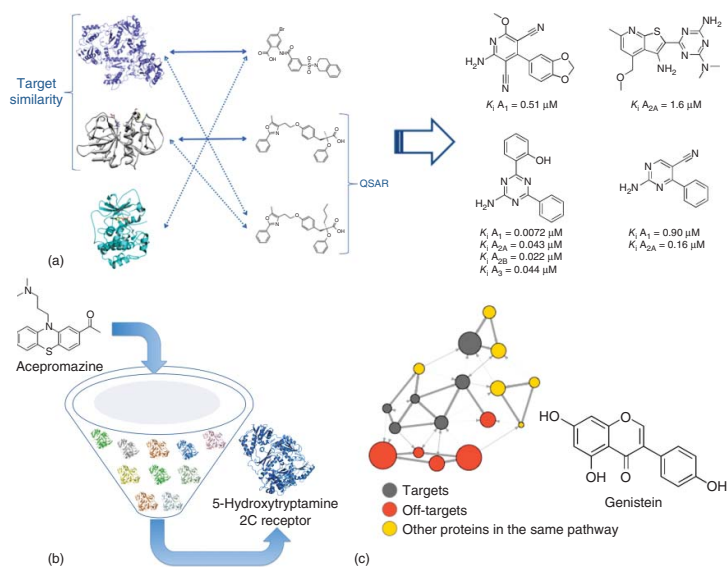


Figure 1.6 Schematic representation of some computational strategies used to explore chemogenomics. (a) An example of proteochemometric model, which combines target and structural similarities to predict activity of new compounds against adenosine receptors [62]. (b) Target fishing, which is commonly used to identify targets of known compounds such as in the case of acepromazine [63]. (c) An example of systems pharmacology that was previously used to identify the anticancer activity of genistein [64].

relationships, several methods have been developed including qualitative and quantitative approaches. Outstanding examples are QSAR [65]. A second more recent approach is the activity landscape modeling (ALM) aimed to identify the relationship between structure similarity (given a set of molecular representation) and activity similarity [66, 67]. Over the last few years, QSAR, ALM, and other computational approaches are being adapted and developed to identify and predict SmARs that emerge when compound data sets are screened across a range of molecular targets [68, 69]. For instance, our research group has reported the SmAR of benchmark data sets screened across multitargets of therapeutic interests and bioassay data obtained from PubChem [68, 69]. In both case studies, structure–activity similarity (SAS) maps, which systematically relate structure similarity with potency difference for each pair of compounds, were adapted to represent multiple activity similarities [67]. Thus, the idea of measuring activity similarity can be applied not only to SAS maps but to basically any other activity landscape model.

1.5.3 Proteochemometric Modeling

Proteochemometric (PCM) modeling can be conceptualized as an extension of QSAR modeling that exploits chemogenomics data by performing a quantitative evaluation of ligand and target structural similarities. As a result, this technique allows the simultaneous navigation, inter- and extrapolation in both chemical space (i.e., ligands) and biological space (i.e., protein target) [70, 71]. By the explicit combination of target and ligand information in a single model, PCM is capable to analyze and predict SmARs of a set of compounds [70, 71]. It has been shown that PCM is better suited for the prediction of SmARs than other methods such as fragment-based models [72] and multitarget QSAR using support vector machines (SVMs) [73]. This technique has been successfully applied to study the SmARs of different target families such as G-protein coupled receptors (GPCRs) [62], cytochrome P450 isoforms [74], and serine proteases [75], among others [76–79]. This method is also well suited for the study of kinase selectivity profiles and has been applied to a large number of data sets [72, 80–83]. In general, predictive models that include ligand and target information represent a step forward for the analysis of multitarget inhibitors as they usually achieve better performance compared with single-target methods. All details of PCM modeling requirements and more applications have been reviewed recently [70, 71].

1.5.4 Target Fishing

The goal of the approach commonly called as “target fishing” is to uncover biomacromolecules or molecular targets that are able to bind to a given ligand (or drug). Many techniques, including computational approaches, can be adapted to carry out the inverse process of traditional virtual screening (Figure 1.5). In other words, the biological target space is interrogated to identify potential targets for (typically) a small molecule.

A typical computational technique employed in target fishing is molecular docking, giving rise to the “inverse docking” strategy [84]. In this technique, introduced by Chen and Zhi [85], a given small molecule is docked across

a database of 3D macromolecular targets. An alternative computational approach used in target fishing is data mining. Other approach is to measure the molecular similarity between the compound of interest and a data set of known ligands of molecular targets (for instance, the structure of co-crystal ligands). These methods and other approaches have been recently reviewed in a rich paper by Cereto-Massagué *et al.* [86]. Of note, this review includes comprehensive lists of molecular databases and web resources useful for *in silico* target fishing.

As in traditional virtual screening, the chemical compounds can have basically any origin such as novel chemical synthesis, commercial libraries, or natural products, to name a few examples. Natural products are, perhaps, one of the most studied molecules using this approach [87]. Importantly, in the context of polypharmacology, approved drugs or compounds in clinical trials can also be subjects of target fishing. In fact, prediction of molecular targets has become an active area of research in drug repurposing [86]. Target fishing of approved drugs can have one or more goals depending on the specific study:

- a) To identify the molecular targets of a drug for which the mechanism of action is uncertain.
- b) For those drugs with known action mechanism to identify additional molecular targets that produces a beneficial clinical effect, in other words to explore in a systematic manner polypharmacology
- c) Uncover off-targets in a systematic manner. This can lead to the prediction of secondary effects.

Examples of the application of target fishing using natural products as query compounds have been reviewed by Medina-Franco [87]. Several recent examples toward target fishing for drug repurposing have also been published [88–90].

1.5.5 Data Mining of Side Effects and Interactions for Drug Repurposing

Drug repurposing through data mining has two principal premises: (i) there is vast information (e.g., clinical, phenotypical, and experimental) regarding the drugs that are intended to be repurposed, and (ii) the obtained information is sufficient to fit a statistical model to predict whether a compound would be active against another target or disease. Text similarity is a particularly developed tool in these settings.

Through text similarity searching, the scientific literature can be mined, in order to link, often indirectly, drugs and diseases by association of terms [91]. A clear example is data mining of adverse effects; this approach assumes that drugs with similar adverse (also called off-target) effects may be active against similar diseases. Within this pipeline, Campillos *et al.* developed a model for drug repurposing with efficiency rates higher than 50% [92]. Notably, comprehensive online databases have been developed to address the problem of disperse information about drugs; these resources are a result of scientific literature mining and contain complete references about different compounds [93, 94].

1.5.6 Systems Pharmacology

Systems pharmacology has arisen as an emerging trend strongly connected to polypharmacology. The main similarity between these two lies in their fundamentals: both try to overcome the simplicity of the old-fashioned “one drug, one target” paradigm. Polypharmacology has often the connotation of “one drug, more than one target,” implying both the possibility of drug repurposing and the feasibility of multitarget treatments with a sole drug, as we have explored throughout this chapter. However, systems pharmacology is a wider concept than polypharmacology, described by the phrase “one treatment, one network.” Therefore, the focus of systems pharmacology implies the rational design of therapies accounting for the overall cellular and physiological complexity, aiming for biological networks rather than isolated targets [95]. The two main strategies emerging from systems pharmacology are (i) those based on simulations in interaction networks validated in the scientific literature [96, 97] and (ii) approaches exploiting high-throughput data such as expression or genetic microarrays [98–100]. Both approaches aim to find a differential function of pathways in pathologic processes compared to healthy states and drugs that can reverse the pathogenic features. Therefore, the objective is to develop treatments that avoid the studied pathological phenotypes [97, 100].

1.5.7 Polypharmacology Fingerprints

As part of the computational strategies to explore and eventually predict polypharmacology, Pérez-Nueno *et al.* have developed a computational polypharmacology fingerprint based on the Gaussian ensemble screening approach developed before by the same authors [101]. The newly developed fingerprint was designed to encode information related to promiscuity. In that work, the fingerprint was built using about 800 established drug targets from a public database of known drugs. In a benchmark study, the proposed fingerprint was able to predict up to 90% of the experimentally known polypharmacology associations (with no missing data). Finally, in the work the authors demonstrated that the proposed fingerprints represent a new approach to suggest molecular targets for preclinical compounds and clinical drug candidates. As the authors described in the excellent paper, the polypharmacology fingerprint represent an important addition to other *in silico* tools based on different types of descriptors that are intended to relate quantitatively biomolecular targets (e.g., protein receptors) to each other (either by computing similarity between the ligands or between the targets) [101].

1.6 Summary Conclusions

To better understand and potentially predict polypharmacology, it is necessary to explore the intersection between the chemical and biological spaces. One approach to explore such intersection is through the emerging research field of chemogenomics. To date, there are chemogenomics data sets available to

conduct drug repurposing, several in the public domain. A major challenge while working with these chemogenomic resources is that the data may be incomplete. Also, the need to conduct curation of the chemical and biological information has been recently emphasized. A broad range of novel computational strategies are being developed and implemented to mine, understand, and predict polypharmacology. For instance, PCM modeling and multitarget activity landscapes enable the simultaneous analysis of chemical and biological relationships. Using structure- or ligand-based approaches, target fishing aims to identify potential targets for a given ligand. Data mining of side effects and systems pharmacology are further examples of novel approaches employed in polypharmacology, which is a promising avenue in emerging and complex drug discovery strategy such as the development of epi-drugs.

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Part II

Selectivity of Marketed Drugs

2

Kinase Inhibitors

Peng Wu^{1,2,3,4,5}, Michael Givskov^{2,6}, and Thomas E. Nielsen^{2,6}

¹Technical University of Denmark, Department of Chemistry, Kemitorvet, 2800 Kgs. Lyngby, Denmark

²University of Copenhagen, Costerton Biofilm Center, Blegdamsvej 3B, 2200 Copenhagen, Denmark

³Harvard University, Department of Medicine, 4 Blackfan Circle, Boston, MA 02115, USA

⁴Harvard Medical School, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA

⁵Broad Institute of MIT and Harvard, Center for the Science of Therapeutics, 415 Main Street, Cambridge, MA 02142, USA

⁶Nanyang Technological University, Singapore Centre on Environmental Life Sciences Engineering, 60 Nanyang Drive, 637551 Singapore, Singapore

2.1 Overview

Kinases are phosphotransferases that are essential components of cellular signaling cascades, playing central roles in regulating proliferation, survival, apoptosis, metabolism, transcription, differentiation, and various other types of biological activities [1–7]. Being one of the largest protein families encoded in the human genome, the kinases include more than 500 members with protein kinases and lipid kinases constituting the two most significant groups [8]. The strategy of kinase inhibition using small molecules was firstly proved successful in clinical settings with the approval of fasudil in Japan in 1995 and imatinib by US Food and Drug Administration (FDA) in 2001 [9, 10]. Since then, kinases have been gradually established as a rich source of drug targets as demonstrated by the fact that more than 30 small molecule kinase inhibitors (SMKIs) have been approved by the FDA in the past 15 years (Figures 2.1 and 2.2a–c) [11], with hundreds of compounds currently in preclinical and clinical development [12]. In addition, three macrolide kinase inhibitors have also been approved by the FDA as anticancer agents and/or immunosuppressants (Figure 2.2d) [13]. SMKIs have been invaluable therapeutics in oncology [14–17]. The past decade has witnessed the implication of kinase inhibitors in a number of non-oncology indications, such as inflammatory diseases, central nervous system disorders, immune diseases, metabolic disorders, and indications in analgesia, ophthalmology, and osteoporosis [12]. It is noteworthy to mention that phosphorylation by kinases has also been widely identified in prokaryotic organisms [18, 19], such as the two-component regulatory system of GacA/GacS that regulates many aspects of bacterial metabolism and physiology including virulence and biofilm formation [20]. A significant number of small molecules

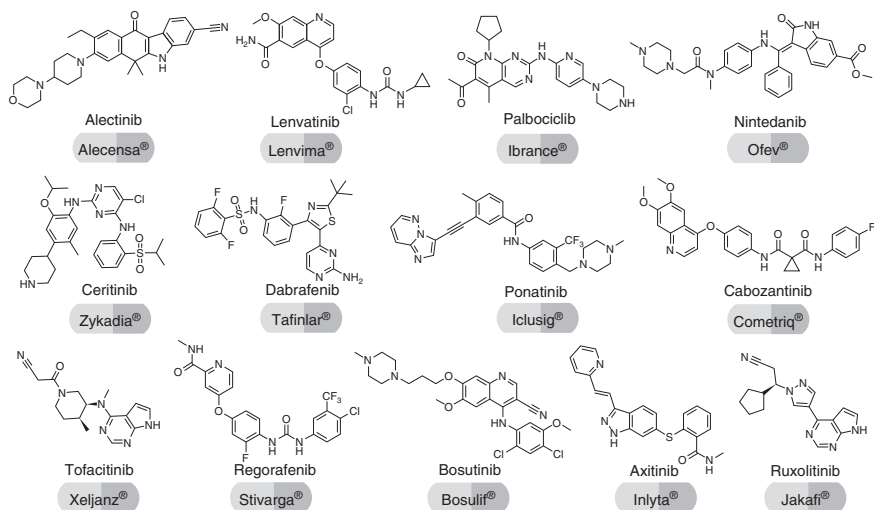


Figure 2.1 FDA-approved non-covalent type I and type II small molecule protein kinase inhibitors. Inhibitors are listed based on reverse chronological order of their first FDA approval date, that is, alectinib, which was recently approved in December 2015, is in the top left position, and imatinib, which was the first FDA-approved SMKI, is in the bottom right position. Commercial names are provided in capsules beneath generic names.

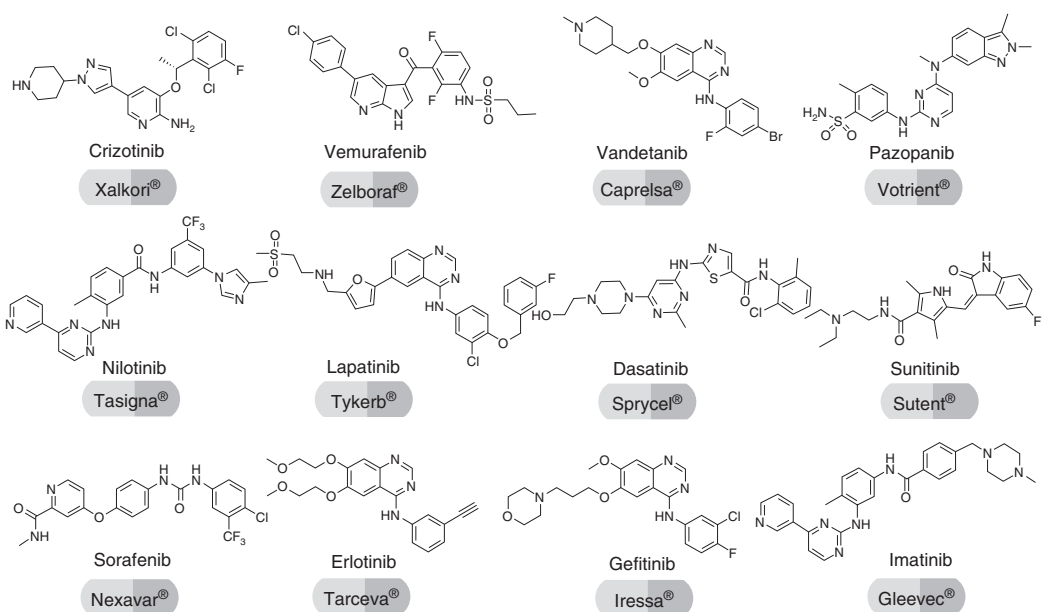


Figure 2.1 (Continued)

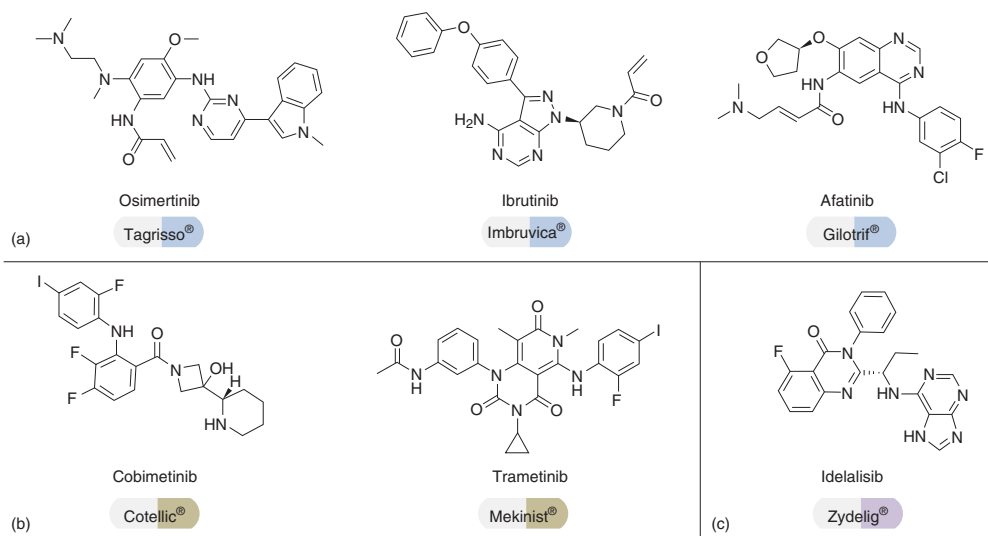


Figure 2.2 Other types of FDA-approved kinase inhibitors. Commercial names are provided in capsules beneath generic names. (a) Light blue capsules indicate approved covalent SMKIs, (b) brown capsules indicate approved type III SMKIs, (c) thistle capsule indicates the only approved lipid kinase inhibitor, and (d) pale turquoise capsules indicate approved macrocyclic kinase inhibitors.

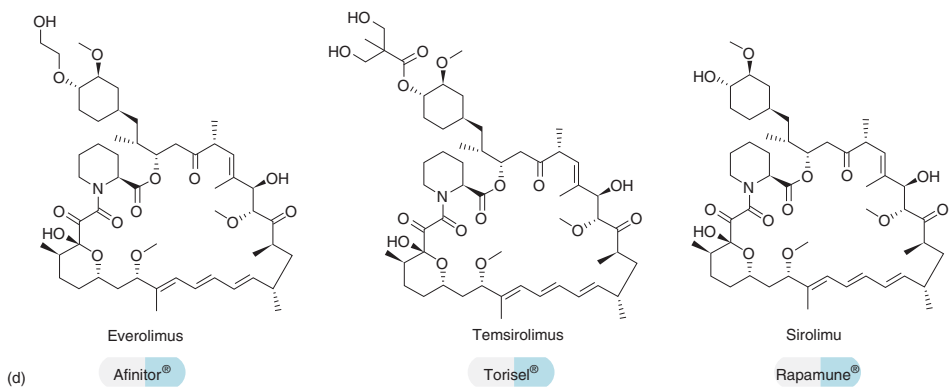


Figure 2.2 (Continued)

have been reported as inhibitors of bacterial or other prokaryotic kinases [21], while the strategy of utilizing prokaryotic kinase inhibitors as antimicrobial agents has not led to any approved drug so far.

In spite of the unparalleled success achieved in applying eukaryotic SMKIs as effective therapeutics, the field of kinase inhibition faces significant challenges [11], such as the daunting task of achieving favorable selectivity among different kinase families [22–24]. Due to the fact that a large number of SMKIs are reversible inhibitors that interact with the kinase ATP-binding pockets [25], which show a high degree of structural and sequential similarity, most reported SMKIs are multitarget inhibitors with varied levels of selectivity among different groups of kinases [26]. A lack of selectivity may lead to side effects and off-target toxicity in clinical settings, and the withdrawal of approved drugs from markets due to hidden side effects and toxicities was not uncommon in the past decade [23]. On another note, selective inhibitors are invaluable tool compounds for target validation and to understand the biology of kinases and related signaling pathways [27]. Thus, it is of significant importance to assess the selectivity and polypharmacology of SMKIs.

2.2 Kinase Profiling

Traditionally, kinase inhibitor discovery was carried out in a linear process that a single target or a few carefully selected targets were used for the screening of potential hits, followed by optimization to yield lead compounds with improved potency. Further medicinal chemistry efforts to modulate physicochemical properties and evaluate toxicities and other pharmaceutical properties were involved to the eventual identification of a candidate compound for potential clinical trials [28]. It was a time-consuming, expensive, and technically challenging process to evaluate the kinase selectivity of a hit or lead compound against a wide range of kinase targets, so typically only a few closely related kinase subset were used to evaluate selectivity in the lead optimization process [29]. Thus, it was not a surprise that some of the previously suggested selective SMKIs were later evaluated as multitarget inhibitors when more comprehensive screening assays became available [30], including the notable example of imatinib, which was originally developed as a selective inhibitor of platelet-derived growth factor receptor (PDGFR), but the compound also inhibits BCR-Abl and c-KIT [15].

Driven by a strong desire in the kinase research community to assess comprehensive selectivity of SMKIs and assisted by the development of stable recombinant proteins, panels of kinase assays constituted of hundreds of protein kinases and lipid kinases, both wild type and mutant type, have become available in the past few years [29, 31–33]. Comprehensive kinase selectivity profiling not only plays a crucial role in revealing knowledge of off-target activities and potential toxicities but also makes it possible to apply existing compounds for novel unanticipated uses [34, 35]. On the other hand, it is noteworthy to mention some limitations of the currently used profiling technologies, such as the *in vitro* screening environment may not represent kinases in physiological states and realistic disease systems. In addition, the big if not huge difference in the technologies used to

collect the selectivity data makes it difficult to make an easy comparison among available results [36]. Also, it has to be kept in mind that a simple definition of “selective” or “unselective” inhibitor in a specific literature could be misleading since assays might be carried out at concentrations that differ significantly from each other [31]. In a word, it is of extreme importance to assess the relative reference condition or control compound in analyzing profiling data of SMKIs.

Although it remains an expensive process to establish comprehensive kinase inhibitory profiles of compounds during the hit-to-lead optimization process, it is gradually becoming a standard procedure to generate inhibition data against a large kinase set to qualify the candidate selection after optimization. With the gradual improvements in screening technologies and reduced cost, large-scale

Service provider	Kinase panel	Assay mechanism
Reaction biology (USA)	 369 wild-type kinases 194 mutant kinases 20 atypical kinases 19 lipid kinases	Radioisotope binding assay
DiscoverX (USA)	 protein kinases 7 atypical kinases 3 kinases identified in pathogens 22 lipid kinase	KINOMEScan® ATP-independent thermodynamic interaction affinities
ProQinase (Germany)	 320 wild-type kinases 83 mutant kinases 17 lipid kinases	Protein kinase: radioactive filter binding Lipid kinase: ADP-Glo™ kinase assay
Carna biosciences (Japan)	 342 wild-type kinases 75 mutant kinases 5 lipid kinases	IMAP™ Mobility shift assay
Thermo fisher scientific (USA)	 394 protein kinases 11 lipid kinases	SelectScreen™ Z-LYTE™: fluorescence resonance energy transfer-based activity assay Adapta®: fluorescence-based immunoassay LanthaScreen®: kinase binding assay.
Eurofins (UK)	 protein kinases lipid kinases	KinaseProfiler™ Radioactive filter binding assay
MRC protein phosphorylation unit (UK)	 140 protein kinases 15 lipid kinases	Radioactive filter binding assay

Figure 2.3 Examples of commercially available kinase panels and profiling services. The order is based on the size of kinase panels of each supplier, data collected from the corresponding website as of January 2016. The authors have no conflicts of interests with any of the previously listed suppliers.

kinase profiling of an entire library of a large set of database is also becoming increasingly common [29].

The easy accessibility to both testing services and collections of kinase inhibitors, such as the Published Kinase Inhibitor Set (PKIS) that includes a set of 367 ATP-competitive SMKIs of 31 chemotypes [37, 38], greatly stimulates comprehensive and focused profiling of kinase inhibitors in the past decade [34, 35, 39, 40]. Some of the early profiling work using panel ranging from 30 to 400 kinases has been summarized previously [41]. To the best of our knowledge, the largest kinase panel available so far is provided by Reaction Biology [42]. A summary of the major providers for kinase profiling service, including DiscoverX [43], ProQinase [44], Carna Biosciences [45], Thermo Fisher [46], Eurofins [47], and the Kinase Profiling Centre at the University of Dundee [48], is provided in Figure 2.3. Most of these panels are constituted of a large number of wild-type and mutant kinases belonging to the eight main subfamilies of protein kinases [8], together with a few atypical protein kinases and a small number of lipid kinases. The most commonly used method in these commercially available panels is the radioactive filter binding assay using radioisotope-labeled ATP with ^{33}P -ATP being the most widely used. Other used methods include fluorescence intensity assay, fluorescence polarization assay, fluorescence resonance energy transfer assay, mobility shift assay, and enzyme-linked immunosorbent assay [32]. Among nonradioactive methods, the ATP-independent thermodynamic interaction affinity assay employed by DiscoverX has proven useful in detecting non-ATP-competitive allosteric inhibitors.

2.3 Definition and Quantification of Selectivity Levels

Kinases were grouped into clusters of subfamilies based on their sequence similarity [8, 49], with the notion that structurally related compounds often inhibit kinases in closely related clusters. Varied definitions regarding the selectivity levels of SMKIs have been proposed. The two most general classification systems used are “selective” versus “unselective” and “single-target” versus “multitarget.” We have used a simplified method to define the selectivity of SMKIs based on the number of targeting kinases [50], which is an easy way to draw the definition line but obviously not an ideal approach to reflect the complex selectivity profiles of SMKIs. Herein, for the discussion of the approved SMKIs in the following sections, we propose a dodgy but more realistic system that defines “selective SMKIs” as inhibitors that bind to small clusters of kinases or limited number of closely related kinases, “multi-inhibitors” as molecules that bind to multiple small clusters of kinases, “promiscuous inhibitors” as one that bind a large number of kinase clusters, and “pan-inhibitors” as inhibitors that interact with all or most isoforms of a kinase. A significant amount of profiling work involving a subset of kinases ranging from above 100 to more than 600 has been reported in the past 5 years [34, 35, 39]. The presence of these data and the appearance of several graphic displaying approaches make the analysis of selectivity among SMKIs an easier task. The most widely used graphic approach to present selectivity is using a kinome tree dotting with circles of varied diameter, representing different levels of potency (Figures 2.4 and 2.5) [51].

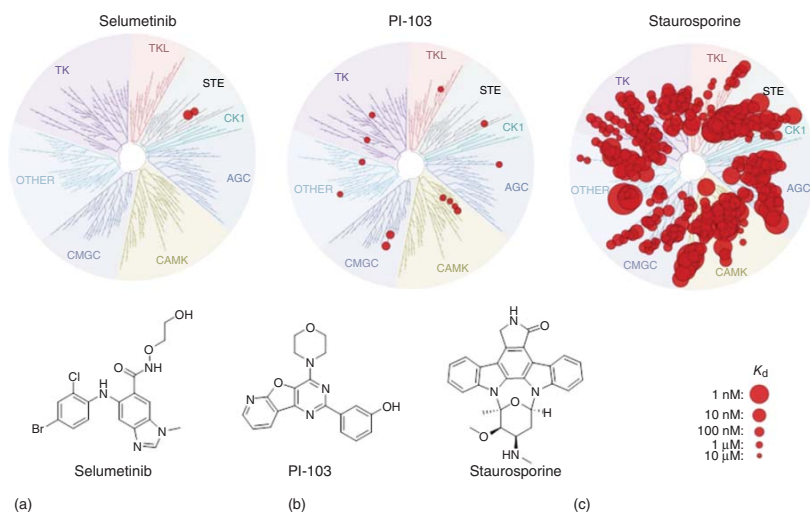


Figure 2.4 Representation of selectivity by dotted kinome tree illustration exemplified by three types of SMKIs: (a) selumetinib is a highly selective inhibitor of MEK; (b) PI-103 is a dual PI3K/mTOR inhibitor that also interacts with multiple other kinases scattered in seven subfamilies of protein kinases; and (c) staurosporine is a widely studied promiscuous inhibitor with high binding potency against numerous kinases. High binding affinity is indicated by red circles with large diameters, and low binding affinity is indicated by red circles with small diameters. Affinities weaker than 1 μM (K_d) are not indicated. The eight main subfamilies of protein kinases are shown as differently colored branches (clockwise order from TK): TK, tyrosine kinase; TKL, tyrosine kinase-like; STE, homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases; CK1, Casein kinase 1; AGC, containing PKA, PKG, PKC families; CAMK, calcium/calmodulin-dependent protein kinase; CMGC, containing CDK, MAPK, GSK3, CLK families, and other protein kinases. (With permission from Ref. [34], © Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.)

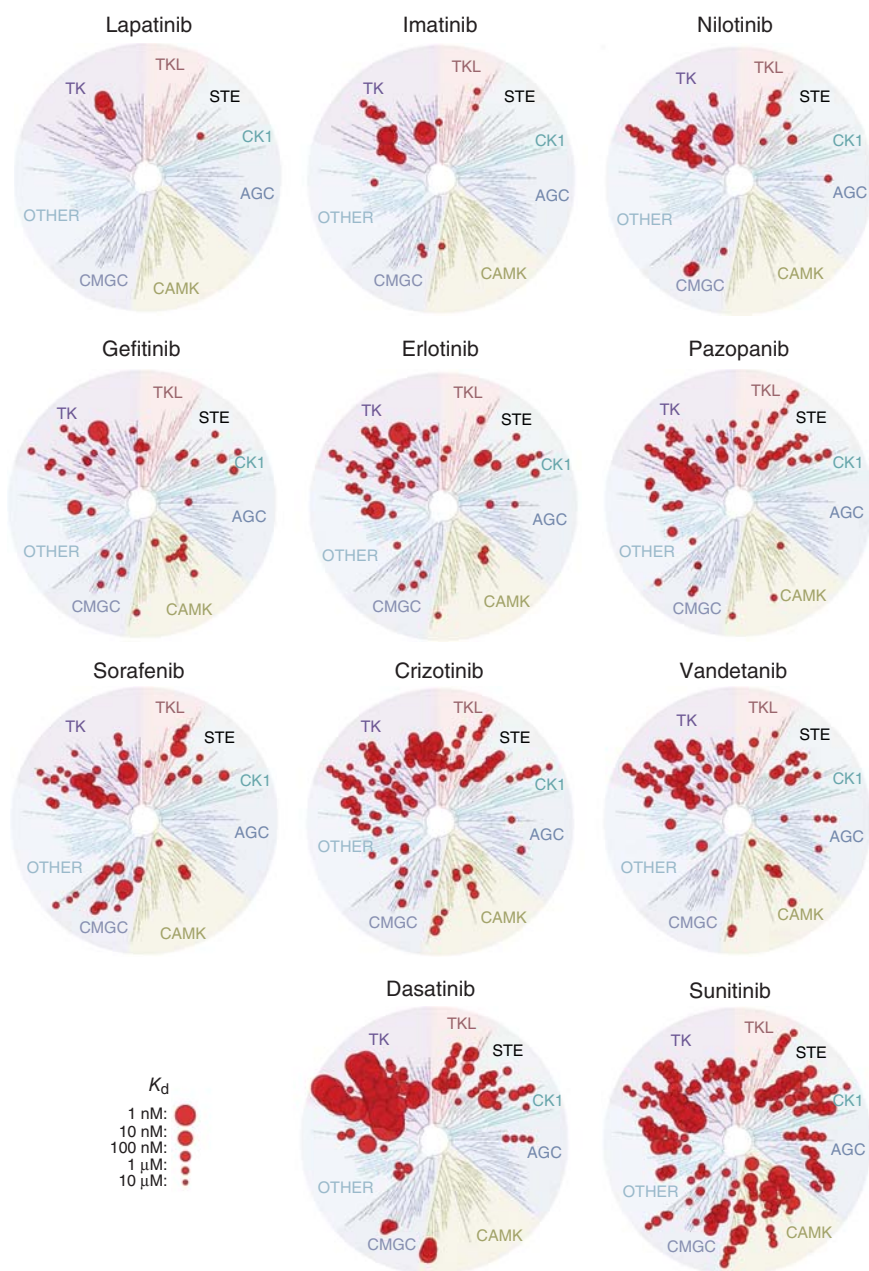


Figure 2.5 Circular kinome tree illustration of selectivity for approved SMKIs, based on K_d profiling results of 442 protein kinases. High binding affinity is indicated by large diameters, and low binding affinity is indicated by red circles with small diameters. Affinities weaker than 1 μ M (K_d) are not indicated. Selective SMKIs are positioned in the top left corner, and promiscuous SMKIs are positioned in the bottom right corner, that is, lapatinib is a representative selective inhibitor, while sunitinib is a representative promiscuous inhibitor. (With permission from Ref. [34], © Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.)

Comparison of the dotted kinome trees reflects the different selectivity of SMKIs in a straightforward manner, which retains key information about the number of targeting kinases, the structural similarity among targeting kinases, and potency against each kinase. The selectivity kinome trees of the types of SMKIs with varied selectivity are presented in Figure 2.4. Selumetinib is a highly selective type III allosteric mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MAPK/ERK also known as MEK) inhibitor, which is currently under several clinical trials for the treatment of advanced non-small cell lung cancer (NSCLC) and soft tissue sarcomas [52]. Its dotted kinome tree illustration of selectivity is simple and clear, and only dots in the serine/threonine kinase (STK) subfamily indicate its high selectivity against MEK1 and MEK2 (Figure 2.4a). PI-103 is one of the most extensively studied tool compounds, originally heralded as a dual phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor, with pan-inhibitory activities against the different isoforms of PI3Ks, while its selectivity kinome tree indicated its multitarget feature to interact with kinases scattered in almost all the eight subfamilies (Figure 2.4b) [53, 54]. In contrast, the pioneer molecule staurosporine is a widely known promiscuous inhibitor that has shown excellent inhibitory potency against a long list of kinases [55, 56]. As expected, the selectivity kinome tree of staurosporine is filled with numerous dots of varied diameters (Figure 2.4c). Other reported graphic approaches include thermal stability shift assay and heat map illustration [57] and selectivity wheel with radar plots [58, 59]. To further analyze the selectivity data in a quantitative manner, methods giving statistical and mathematical data have been proposed. Promiscuity score [57], selectivity score [39], and the Gini score [60] were among the first reported quantitative methods to evaluate selectivity, followed by the appearance of partition coefficient and selectivity entropy [61]. Both graphic and quantitative methods differ with each other in terms of presentation patterns and calculation principles, but all methods are derived based on two key points: “number of targeting kinases” and “inhibitory potency against each targeting kinase.”

2.4 Selectivity of Approved Kinase Inhibitors

Based on binding modes, FDA-approved small-molecule kinase inhibitors can be classified into three major groups: type I inhibitors bind to ATP-binding pockets of the active form of kinases with the conserved Asp–Phe–Gly (DFG) motif of the activation loop adopting an “in” conformation, type II inhibitors bind to the inactive form of kinases with the DFG motif extending outward from ATP-binding pockets, and type III inhibitors are molecules that bind exclusively to allosteric pockets that do not overlap with the ATP-binding pocket. The detailed binding modes and structural features for most of the approved SMKIs have been recently summarized in a systematic way [11], providing a molecular basis for the explanation of the varied selectivity of SMKIs. Although extensive efforts have led to the development of highly selective inhibitors, for the reasons mentioned in first section of this chapter, there is no established correlation between selectivity of SMKIs and therapeutic value [23]. In some cases, a multitarget

inhibitor may be more effective in the treatment of certain diseases due to the cross-reactive nature of this type of inhibitors and the fact that off-target activities can contribute positively through synergistic effects. For example, most of the FDA-approved type I and type II SMKIs are multitarget inhibitors that have been proved to be valuable therapeutic anticancer agents (Figure 2.6), which is

SMKIs	Main target(s)	Therapeutic indication(s)
Alectinib	ALK	NSCLC
Lenvatinib	VEGFR2, VEGFR3	Thyroid cancer
Palbociclib	CDK4, CDK6	Breast cancer
Nintedanib	VEGFR, EGFR, PDGFR	Idiopathic pulmonary fibrosis
Ceritinib	ALK	NSCLC
Dabrafenib	B-RAF	Metastatic melanoma
Ponatinib	BCR-Abl, KIT, RET, FGFR, SRC, VEGFR, PDGFR	CML, acute lymphoblastic leukemia
Cabozantinib	VEGFR, MET, RET, FLT1/3/4, AXL, TIE2	Thyroid cancer
Tofacitinib	JAK1-3	Rheumatoid arthritis
Regorafenib	VEGFR, PDGFR, FGFR, RAF, KIT, RET	Metastatic colorectal cancer, GIST
Bosutinib	BCR-Abl, SRC, LYN, HCK, CDK, MAPKK	CML
Axitinib	VEGFR, KIT, PDGFR	RCC
Ruxolitinib	JAK1, JAK2	Myelofibrosis
Crizotinib	ALK, MET	NSCLC
Vemurafenib	B-RAF	Thyroid tumor, metastatic melanoma
Vandetanib	VEGFR, EGFR	Multiple endocrine cancers
Pazopanib	VEGFR, PDGFR, KIT, FGFR	Advanced RCC, soft tissue sarcoma
Nilotinib	BCR-Abl, PDGFR, KIT	Various leukemias, such as CML
Lapatinib	EGFR, ErbB2	Advanced metastatic breast cancer
Dasatinib	BCR-Abl, SRC, LCK, FYN, KIT, PDGFR	CML, lymphoblastic leukemia
Sunitinib	VEGFR, PDGFR, KIT, FLT3, RET	RCC, GIST
Sorafenib	VEGFR, FGFR, PDGFR, RAF, FLT3, KIT, RET	Advanced RCC, hepatocellular carcinoma
Erlotinib	EGFR, SLK, ILK, ARG	NSCLC, pancreatic cancer
Gefitinib	EGFR, GAK	NSCLC
Imatinib	BCR-Abl, PDGFR, KIT, RET	CML and other leukemias, GIST, etc.

Figure 2.6 FDA-approved inhibitors and their targeting kinases and therapeutic indications (Part I). The targets for which the approved SMKIs have shown inhibitory activities include but are not limited to the ones listed here. CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma.

SMKIs	Selectivity/classification	Main target(s)	Therapeutic indication(s)
Osimertinib	Covalent EGFR inhibitor	EGFR	Metastatic EGFR T790M mutation-positive NSCLC
Ibrutinib	Covalent BTK inhibitor	BTK	CLL, mantle cell lymphoma
Afatinib	Covalent ErbB inhibitor	EGFR, ErbB2, ErbB4	Metastatic EGFR L858R mutation-positive NSCLC
Cobimetinib	Selective allosteric type III MEK inhibitor	MEK1, MEK2	Unresectable or metastatic melanoma with a BRAF V600E or V600K mutation
Trametinib	Selective allosteric type III MEK inhibitor	MEK1, MEK2	Metastatic melanoma with a BRAF V600E or V600K metastatic melanoma
Idelalisib	Isoform-selective lipid kinase (PI3K) inhibitor	PI3K δ	Relapsed CLL, follicular B-cell non-Hodgkin lymphoma, small lymphocytic lymphoma
Everolimus	Isoform-selective macrocyclic mTOR inhibitor	mTORC1	Advanced RCC, breast cancer, other types of tumors, immunosuppressant
Temsirolimus	Macrocyclic mTOR inhibitor	mTOR	Advanced RCC
Sirolimus	Macrocyclic mTOR inhibitor	mTOR	Immunosuppressant

Figure 2.7 FDA-approved inhibitors and their targeting kinases and therapeutic indications (Part II). The targets for which the approved SMKIs have shown inhibitory activities include but are not limited to the ones listed here. CLL, chronic lymphocytic leukemia; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma.

understandable considering the complex signal cascades leading to tumor genesis and progression. In comparison, success of SMKIs in therapeutic fields outside oncology is limited due to the lack of efficacy of previously tested SMKIs [41].

Currently available FDA-approved kinase inhibitors include 3 macrolides and 31 SMKIs, most of which are unselective inhibitors in the category of non-covalent type I and type II inhibitors, two are representative type III allosteric MEK inhibitors, one is a lipid kinase inhibitor, and three are covalent inhibitors (Figures 2.2 and 2.7). Representative examples of type I–III SMKIs are illustrated in Figure 2.8 together with their co-crystal structures with main targeting kinases.

2.4.1 Non-covalent Type I and Type II SMKIs

The kinase inhibitors' approval started with the type II inhibitor imatinib and type I inhibitor gefitinib, and all approved SMKIs continued to fall into this category until the approval of the first allosteric inhibitor, trametinib, in 2013. To date, a total of 25 non-covalent type I and II SMKIs are FDA approved (Figures 2.1 and 2.6), and it is a common phenomenon that SMKIs in this group often inhibit kinases outside of the originally targeted subfamily. A scrutiny of available selectivity data of these compounds revealed the following notable trends. Firstly, due to the fact that inhibitors in this category target the highly conserved ATP-binding pockets of kinases, most type I and II inhibitors are multitarget inhibitors with new off-target activities being identified after more elaborate profiling tests. Secondly, selectivity of type I and II inhibitors differs significantly depending on the main targeting kinases. For example, B-Raf inhibitors including dabrafenib and vemurafenib [62–64] and Janus kinase (JAK)

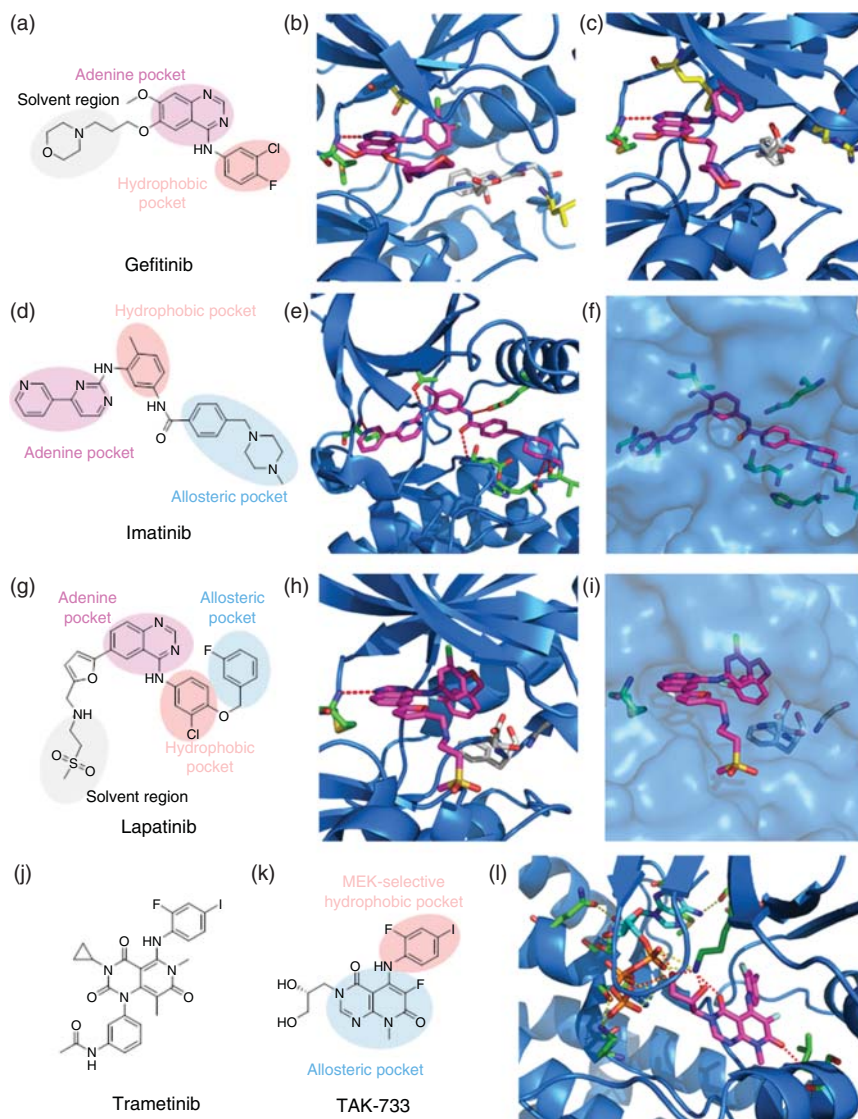


Figure 2.8 Representative examples of different types of approved SMKIs. Type I inhibitor: (a) gefitinib and its depicted binding mode with EGFR, (b) gefitinib co-crystal structure with EGFR (PDB ID: 2ITY), and (c) gefitinib co-crystal structure with mutant L858R + T790M EGFR (PDB ID: 4I22). Type II inhibitors: (d) imatinib and its depicted binding mode with BCR-Abl, (e, f) imatinib co-crystal structure with the kinase domain of Abl (PDB ID: 1IEP), (g) lapatinib and its depicted binding mode with EGFR, and (h, i) lapatinib co-crystal structure with EGFR (PDB ID: 1XKK). Type III inhibitor: (j) chemical structure of trametinib, (k) trametinib analog TAK-733 and its depicted binding mode with MEK, and (l) TAK-733 co-crystal structure with MEK1 (PDB ID: 3PP1). SMKIs are shown in magenta backbones, ATP is shown in cyan backbone, hydrogen bonds are indicated by red broken lines, residues interacting with SMKIs and ATP through hydrogen bonds are shown in green backbones, residues of the Asp-Phe-Gly motif of the activation loop are shown in white backbones, and residues 858 and 790 in the gefitinib EGFR co-crystal structure are shown in yellow backbones. (Wu *et al.* 2015 [11]. Reproduced with permission of Elsevier.)

inhibitors including tofacitinib and ruxolitinib are inhibitors with identified potency against mainly a defined subset of kinases or only the subfamily containing targets they intend to interact originally [65, 66], while Abl inhibitors such as dasatinib [67], bosutinib [68, 69], and ponatinib [70] and vascular endothelial growth factor (VEGFR) inhibitors such as sorafenib, sunitinib, and regorafenib are usually multitarget or promiscuous inhibitors since there are a large number of structurally related kinases with Abl and VEGFR [71]. Thirdly, even for kinases that have structurally related subfamilies, it is still possible to achieve certain degree of high selectivity by utilizing some unique structural features in the vicinity of binding pocket, which is the case for the endothelial growth factor (EGFR) inhibitor imatinib (Figure 2.8d–f) and Abl inhibitor lapatinib (Figure 2.8g–i) [2, 72–74], which are type II inhibitors utilizing not only the adenine pocket of the ATP-binding pocket but also an allosteric pocket formed due to the conformation change of the DFG motif [75]. However, these results are not sufficient enough to conclude that type II inhibitors are not more selective than type I inhibitors, as have been shown by a recent binding mode analysis [76].

Extensive kinase profiling and the resulting selectivity kinome tree have been reported for the early approved SMKIs in this group (Figure 2.5) [34, 35, 39]. It is easy to identify selective and promiscuous inhibitors by comparison of the selectivity kinome trees, that is, lapatinib is one of the most selective inhibitors in this group showing only main inhibitory dots representing EGFR and ErbB2 in the TK subfamily, and sunitinib, which inhibits more than 50% of the tested kinase panel, has a fully occupied dotted tree like that of staurosporine, although less intense. It is noteworthy to mention that in spite of all the reported graphic and quantification methods relating to selectivity, the distinction between selective, multitarget, and promiscuous inhibitors remains a highly debatable topic. For example, gefitinib was originally studied as a selective type I EGFR inhibitor (Figure 2.8a–c), although later studies have identified that gefitinib exhibits a broad selectivity profile covering all the eight main subfamilies of protein kinases, high potency against EGFR (binding constant $K_d < 1$ nM), and moderate to weak potency against many other kinases (binding constant K_d ranging from 13 nM to 10 μ M). On this basis, it is acceptable to state that gefitinib is a selective EGFR inhibitor [39], especially in comparison with inhibitors like dasatinib and sunitinib. The approval of gefitinib has inspired a large amount of subsequent optimization work based on structural modification, and dedicated research efforts have led to the approval of several SMKIs of the same chemotype but with varied or improved selectivity profiles, including erlotinib, lapatinib, vandetanib, bosutinib, lenvatinib, and afatinib [11].

2.4.2 Allosteric SMKIs

Trametinib and cobimetinib are the only two allosteric inhibitors approved so far, both of which are highly selective and potent MEK1 and MEK2 inhibitors [77, 78]. The co-crystal structure of the trametinib analog TAK-733 with MEK1 showed the distinct binding mode with that of type I and type II inhibitors (Figure 2.8j–l) [79]. The approval of these inhibitors indicated the uprising

of resorting to the strategy of targeting allosteric pockets outside the highly conserved ATP pocket as a promising alternative to overcome limitations of type I and type II SMKIs [80–82]. The biggest challenge in the development of allosteric inhibitor at this stage is the lack of efficient evaluating assay that could specifically identify allosteric inhibitions, as reflected by the fact that some of the reported allosteric pockets are only identified serendipitously by crystallographic analysis [82]. It is still too early to conclude whether allosteric inhibitors will lead to superior therapeutic effects in comparison with other types of SMKIs, but this newly raised strategy has paved a different road to access highly selective kinase inhibitors.

2.4.3 Lipid Kinase Inhibitor

Idelalisib is the only approved SMKI that target the lipid kinase PI3K, in spite of considerable efforts from both academia and pharmaceutical industry in trying to address this target in the past 20–30 years [53, 83, 84]. It utilizes an allosteric pocket of the δ isoform of PI3Ks, resulting in high isoform selectivity [85]. Like allosteric MEK inhibitors, the small data set of approved molecules make it difficult to summarize a general therapeutic prospective, but based on available pre-clinical and clinical data, PI3K inhibitors may have limited single-agent activity, and combination strategies are needed to assist their clinical applications [86, 87].

2.4.4 Covalent Inhibitors

Osimertinib, ibrutinib, and afatinib are inhibitors that incorporate a chemically active Michael acceptor electrophile that forms a covalent bond with accessible nucleophilic cysteine residues of targeting kinases [88]. In comparison with reversible inhibitors, the irreversible binding feature of most covalent inhibitors makes it easier to enrich and measure target and off-target selectivity in living systems [89]. A proteome-wide selectivity evaluation involving ibrutinib and afatinib in cancer cells and *in vivo* was reported recently with the aim of establishing a general strategy for the characterization of potential targets of covalent kinase inhibitors. The results showed that covalent kinase inhibitors intend to interact with not only other kinases with functional cysteine residues but also receptors, enzymes, and other uncharacterized proteins that bear accessible cysteine residues [90]. Considering the large number of kinases and other proteins with functional cysteine residues [91], the strategy of covalent inhibition itself will not lead to high selectivity. However, the combination strategy of a non-covalent inhibitor with reasonable selectivity and a reactive electrophilic group that targets specific cysteine residues has led to the development of highly selective inhibitors [92]. In a word, additional structural features of targeting kinases need to be utilized for the design of selective covalent inhibitors.

2.5 Conclusion and Perspective

Selectivity is one of the most controversial topics in kinase research. Most approved SMKIs are multitarget or unselective inhibitors that bind with the

highly conserved ATP pocket of kinases. Covalent SMKIs based on the structures of type I and type II inhibitors with the addition of an electrophile have shown potential to interact with a range of kinases and proteins with a functional cysteine residue. Selectivity is more likely achieved against kinases with only few closely related homologs or unique structural features in the binding site. High selectivity has been achieved by using the emerging strategy of allosteric inhibition utilizing unique allosteric pockets that do not overlap with the ATP-binding pocket. Guided by structure-based analysis, a few approaches are being utilized with the aim of developing inhibitors with improved selectivity. First of all, the structural insights gained from X-ray crystallography have greatly promoted the development of highly selective SMKIs [93]. Secondly, rational chemical modifications based on structure–activity relationship of reported unselective SMKIs have successfully been made to eliminate inhibition against unwanted kinases. Besides, new scaffolds representing unexplored chemical spaces are being synthesized to expand the currently available molecular space that aimed to target novel protein families and yield compounds with different selectivity. Currently available data is not sufficient to establish correlation between selectivity of SMKIs and their therapeutic value, given the crucial functions of kinases in cellular signaling. It is reasonable to conclude that multitarget SMKIs may be more suitable for the treatment of cancers, while highly selective SMKIs may have the potential to overcome side effects and off-target toxicity and find use in broader clinical applications of kinase inhibitors outside the oncology area.

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3

Repositioning of Drug – New Indications for Marketed Drugs

Ren Kong¹ and Stephen T. Wong²

¹Institute of Bioinformatics and Medical Engineering, School of Electrical and Information Engineering, Jiangsu University of Technology, Changzhou, P. R. China

²Weill Cornell Medicine, Houston Methodist Hospital and Research Institute, Department of Systems Medicine and Bioengineering, 6670 Bertner Avenue, Houston, TX 77030, USA

3.1 Introduction

The R&D productivity of drug development has decreased over the years in comparison with the average number of dollars spend on each new drug approval or each Investigational New Drug (IND) application received by the US Food and Drug Administration (US FDA) [1]. Finding new indications for existing drugs, having been through several stages of clinical development, becomes an attractive and cost-effective means of drug development by avoiding the big failure rate in toxicity or pharmacokinetic profiling. The process of identifying new use outside the original medical indications for old drugs is known as drug repositioning, redirecting, repurposing, or reprofiling [2–4]. Due to its efficiency, drug repositioning attracts attention not only in industry but also in academia. When searching for the terms “drug repositioning,” “drug redirecting,” “drug repurposing,” and “drug reprofiling” in the NCBI PubMed database, no hits were found in and prior to 2002. However, the publications increased dramatically in recent years (Figure 3.1). From January 2014 to September 2014, there are 150 publications with these phrases in text, showing great efforts and enthusiasm in the field of drug repositioning.

In the early decades, serendipitous discovery was the major way to find new indications. The most famous and successful example is the development of sildenafil (Viagra) in 1980s–1990s, which was originally intended for cardiovascular disease but finally approved for male erectile dysfunction. Quite a few of old drugs have been approved with new indications (Table 3.1). However, the obvious candidates for repositioning are limited. Traditional drug discovery technologies such as phenotype screening/high-throughput screening can be applied to screening the old drug collections to identify possible candidates [7–10]. Although great success has been made, the traditional methods are limited by low knowledge of elucidated mechanisms and hard-to-satisfy unmet medical needs. Creative approaches integrating computational methods and experimental validation have been explored in recent years for both drug repositioning and

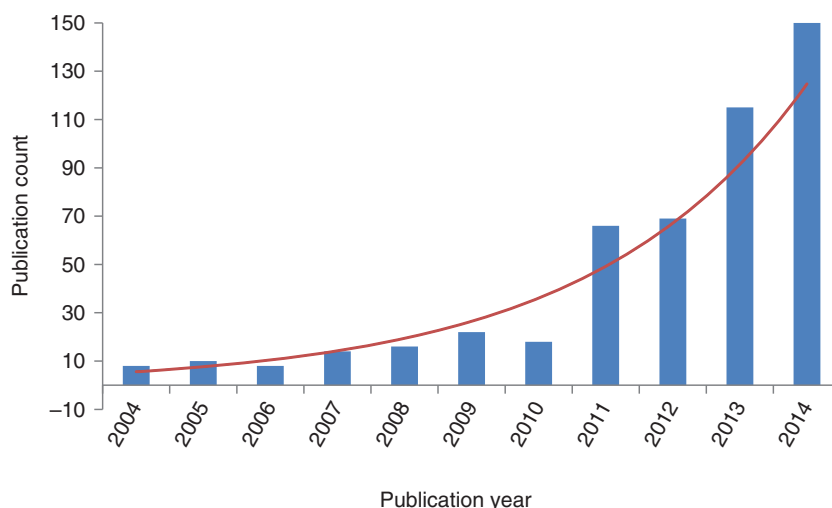


Figure 3.1 Increase of publications in NCBI PubMed with the phrase “drug repositioning,” “drug redirecting,” “drug repurposing,” or “drug reprofiling” in the text.

Table 3.1 List of repositioned drug with approved new indications [5, 6].

Drug	Original indication	New indication	Year
<i>Repositioned antidepressant drugs</i>			
Bupropion	Depression	Smoking cessation	1997
Cymbalta	Depression	Diabetic peripheral neuropathy	2004
Dapoxetine	Analgesia and depression	Premature ejaculation	2009
Fluoxetine	Depression	Premenstrual dysphoric disorder	2000
Milnacipran	Depression	Fibromyalgia syndrome	2009
Sibutramine	Depression	Obesity	1997
<i>Repositioned neurological drugs</i>			
Atomoxetine	Parkinson's disease	Attention deficit hyperactivity disorder (ADHD)	2002
Chlorpromazine	Antiemetic; antihistamine	Nonsedating tranquilizer	1954
Galantamine	Polio, paralysis, and anesthesia	Alzheimer's disease (mild to moderate dementia)	2001
Lidocaine	Local anesthetic	Arrhythmia	2010
Ropinirole	Hypertension	Parkinson's disease and idiopathic restless leg syndrome	1997 and 2005
Tofisopam	Anxiety-related conditions	Irritable bowel syndrome	2014 (Phase II)

(Continued)

Table 3.1 (Continued)

Drug	Original indication	New indication	Year
<i>Other repositioned drugs</i>			
Allopurinol	Tumor lysis syndrome	Gout	1967
Amphetamine	Stimulant	Hyperkinesis in children (ADHD)	1943
Celecoxib	Osteoarthritis and adult rheumatoid arthritis	Colon and breast cancer	2014 (Phase II and III)
Eflornithine	Anti-infective	Reduction of unwanted facial hair in women	2000
Finasteride	Benign prostatic hyperplasia	Alopecia	1997
Lumigan	Glaucoma	Hypotrichosis simplex	2009
Methotrexate	Cancer	Rheumatoid arthritis	1999
Mifepristone (RU486)	Pregnancy termination	Cushing's syndrome	2012
Minoxidil	Hypertension	Alopecia	1988
Paclitaxel	Cancer	Restenosis	2004
Phentolamine	Hypertension	Reversal of soft tissue anesthesia	2008
Raloxifene	Osteoporosis	Breast cancer risk reduction	2007
Sildenafil	Angina	Male erectile dysfunction	2005
Tadalafil	Inflammation and cardiovascular disease	Male erectile dysfunction	2011
Thalidomide	Morning sickness	Multiple myeloma	2003
Topiramate	Epilepsy	Migraine	2004
Zidovudine	Cancer	HIV/AIDS	1985

Year listed here is the approval year for new indications except phase descriptions in parentheses.

traditional drug development methods [11]. This kind of strategy aims to make full use of information of known drugs, targets, biomarkers, pathways, genomes, and diseases [11] by employing methods and tools from cheminformatics, bioinformatics, network biology, and systems biology to integrate available information and elucidate the unknown mechanisms. In this chapter, we will introduce the methods and examples in the field of drug repositioning.

3.2 New Uses from Adverse Effects

3.2.1 Dapoxetine for Premature Ejaculation

Lilly developed dapoxetine originally for analgesia and antidepressant. It is a selective serotonin reuptake inhibitor (SSRI) with rapid onset and short half-life, which did not allow for once-daily dosing, a huge drawback for the competitive market of antidepressant drugs. The drug development was discontinued for a while. However, it was noticed that a common side effect of SSRIs is delayed ejaculation. More than 20% of men in the United States have a problem of

premature ejaculation [12]. Considering the possible new application, the pharmacokinetic characteristics of dapoxetine, such as rapid onset and short half-life, become advantageous. Clinical trials were launched in 2001 and the compound was approved to treat premature ejaculation by the US FDA in 2009 (trade name Priligy). As the first oral medication for premature ejaculation, about \$4 billion market volume was estimated.

3.2.2 Sildenafil for Erectile Dysfunction

Sildenafil is a phosphodiesterase-5 (PDE5) inhibitor originally developed by Pfizer to relax coronary arteries and allow greater coronary blood flow, therefore alleviating angina. However, the clinical experiment on healthy individuals showed no cardiovascular effect of this drug. In contrast, the researchers received several reports from the volunteers in the clinical trial to describe that they had an adverse effect as unusually strong and persistent erections. It was found later that PDE5 is also a key enzyme in the biochemical pathway mediating erections [13]. To validate the proof of concept, a quick small trial was set up on impotent men. Sildenafil has suitable pharmacokinetic and pharmacodynamic properties (rapid absorption, relatively short half-life, no significant effect on heart rate and blood pressure) for an oral agent to be taken, as required, prior to sexual activity. It was shown that the drug could induce penile erections, typically within 30–60 min [14, 15]. A large-scale study was performed on 3700 subjects worldwide in the 1990s. Sildenafil was effective in 63% of men tested with lowest dose level and 82% of men with highest dose. Due to its high efficiency, it was approved by the FDA for use in treating “erectile dysfunction/impotence” in 1998 with trade name of “Viagra.” In the first year when it was marketed, Pfizer obtained about 1 billion dollars in sales. To date, over 20 million men have taken sildenafil in the United States alone [16]. The success story of Viagra indicates that looking into the basic mechanism of side effect can lead to identification of new indications with great economic achievements.

3.3 New Uses Based on Known Mechanism of Action

3.3.1 Duloxetine for Stress Urinary Incontinence (SUI)

In the 1980s, Eli Lilly discovered a compound named duloxetine for depression. The mechanism of action (MOA) of duloxetine is to block the reuptake of both serotonin and noradrenaline in the synaptic cleft. Lilly intended to use this compound as an improved version of its highly successful drug, fluoxetine (Prozac). However, despite the influence on mood, serotonin and noradrenaline are also known to have significant activity in the spinal cord to affect urethral sphincter motor neurons, resulting in urethral resistance and protection from incontinence. The experiments on animal model showed that duloxetine significantly suppresses bladder activity through 5HT receptor mechanisms and enhances external urethral sphincter activity through 5HT₂ and alpha-1-adrenergic mechanisms under conditions of bladder irritation [17]. Thus duloxetine was proposed for treatment of stress urinary incontinence (SUI), a condition characterized by leaking of urine associated with sharp increases in intra-abdominal pressure,

such as laughing, coughing, and sneezing, which is commonly seen in women who have given birth to several children. There is no other drug treatment available for SUI, but the surgery and incontinence pads are options. Clinical trials showed significant effect of duloxetine on SUI [18]. Duloxetine was approved both as antidepressant drug with trade name “Cymbalta” and as SUI control drug with trade name of “Duloxetine SUI” in Europe.

3.3.2 Thalidomide for Erythema Nodosum Leprosum (ENL) and Multiple Myeloma

Thalidomide is well known to the public due to its bad reputation from its original indication in the 1950s – treatment for morning sickness of pregnant women. It was marketed in England and Germany as a sedative. At that time, no regulatory approval was required and the drug was assumed “safe” for treatment on humans. About 15,000 infants whose mother had taken this drug were born with severe skeletal birth defects. Lessons were learned from the tragedy, leading to the introduction of a law in Germany, known as “Arzneimittelgesetz,” requiring proof of safety of drugs. The reuse of thalidomide was quite accidental [19]. Erythema nodosum leprosum (ENL) is an agonizing inflammatory condition of leprosy characterized by large, persistent, painful boils and inflammation. The disease progress even leads to blindness. Patients suffer from such severe pain that they become unable to sleep. In 1964, Dr Jacob Sheskin in the University Hospital of Marseilles prescribed thalidomide to an ENL patient who had not slept for weeks due to severe pain. Thalidomide was the only drug in the hospital’s infirmary, and he believed that the drug might help the patient sleep. After a few weeks, the patient was cured miraculously not only of the lost sleep but also of the sores and pain. Thus the only drug for treatment of ENL appeared. The clinical trial sponsored by the World Health Organization on 4552 patients showed about 99% of the patients obtained a complete remission in less than 2 weeks [19]. In 1998, it was approved for treatment of ENL with trade name “Thalomid.” The subsequent MOA research showed that thalidomide is a potent inhibitor of tumor necrosis factor α , having antiangiogenic properties [20, 21]. The activity makes it a candidate for anticancer treatment and also explains its dramatic effect in limb development in human fetus [21]. Ninety-two percent of thalidomide is prescribed for off-label usage in cancer treatment. In 2006, the FDA approved it for treatment of multiple myeloma, a bone marrow cancer.

3.4 New Uses from Genome, Network, and Signal Pathway Analysis

3.4.1 Identification of Sunitinib and Dasatinib for Breast Cancer Brain Metastasis

One of the characteristics of cancer pathology is its heterogeneity and complexity [22]. Because of the genetic heterogeneity within individual cancers, no magic drug exists to cure of all cancer types or subtypes. Zhao *et al.* introduced a rapid and highly efficient drug repositioning strategy composed of six computational modules and two experimental modules for breast cancer metastasis

(Figure 3.2) [23]. The computational modules included differential analysis, enrichment analysis, cancer signaling bridge (CSB) analysis, network mechanism analysis, survival analysis, and repositioning analysis; the two experimental modules included target validation and drug efficacy measurement.

Microarray data from cohorts of breast tumor from patients with brain, lung, and bone relapse were collected for computational analysis. Partek (<http://www.partek.com>) and Ingenuity Pathway Analysis (<http://www.ingenuity.com>) were used to perform the differential and enrichment analysis to identify the most differentially expressed genes (gene signatures, $P < 0.001$, Student's t -test) between patients with tissue-specific metastasis and those patients without metastasis and to uncover the related enriched signaling pathways based on the gene signatures. A new concept, CSB, was used to connect the signal pathways with “cancer proteins,” the proteins closely involved in cancer disorder [24]. Basically, CSBs are specific network motifs that are those recurrent and statistically significant subgraphs or patterns in a protein–protein interaction (PPI) network [24, 25]. Only those motifs with at least one protein in a signaling pathway and at least one cancer protein outside the signaling pathway are defined as CSBs. The incorporation of information about protein–protein physical interactions in CSB enables the discovery of unknown specific MOA.

For example, a specific CSB contains four proteins, BRCA1, HSPA8, GRB2, and NPM1, with four PPIs – BRCA1↔HSPA8, BRCA1↔NPM1, GRB2↔HSPA8, and GRB2↔NPM1. The coding gene of NPM1 is mutated in acute promyelocytic leukemia with unknown signaling mechanism. By using CSB, the NPM1 could be connected to EGF pathway upon GRB2 protein or E2F transcription factor pathway upon BRCA1 protein. Network mechanism analysis was conducted based on the outputs from differential and enrichment analysis and the signaling network extended by CSB. Based on the network modeling, the pre-signaling network was obtained to involve the maximal number of signal pathways and include the most differentially expressed genes in the gene signature. Then survival analysis module was applied to correlate the putative signaling pathways with the available clinical information of patients, such as metastasis-free survival time. Hierarchical clustering on the paths and Kaplan–Meier survival analysis were conducted iteratively to filter out the high confidence protein pathways (HCPP), which are pivotal to patient survival and metastasis occurrence [26]. Drugs targeted to the proteins or genes in the high confidence pathway have high potential to interfere with specific gene profiling in the cancer cells. Fifteen repositioned drug candidates were identified by using the computational modules. Among them, 10 drugs satisfy the “rules of five” for CNS drugs, indicating their potential to penetrate the blood–brain barrier (BBB). By literature research, it is found that vorinostat [27], pazopanib [28], and XL184 [29] have shown efficacies on inhibiting brain metastases in recent independent preclinical or clinical studies. Dexamethasone is, currently, often used in the clinic to improve brain metastasis symptoms, and thalidomide is used in combination with temozolomide or radiation therapy for treatment of brain metastasis [30, 31]. Two of the drugs, sunitinib (approved for treating advanced renal cell carcinoma and gastrointestinal stromal tumors) and dasatinib (approved for treating chronic myelogenous leukemia), with no reports connecting them to brain metastasis of breast cancer, were selected via their

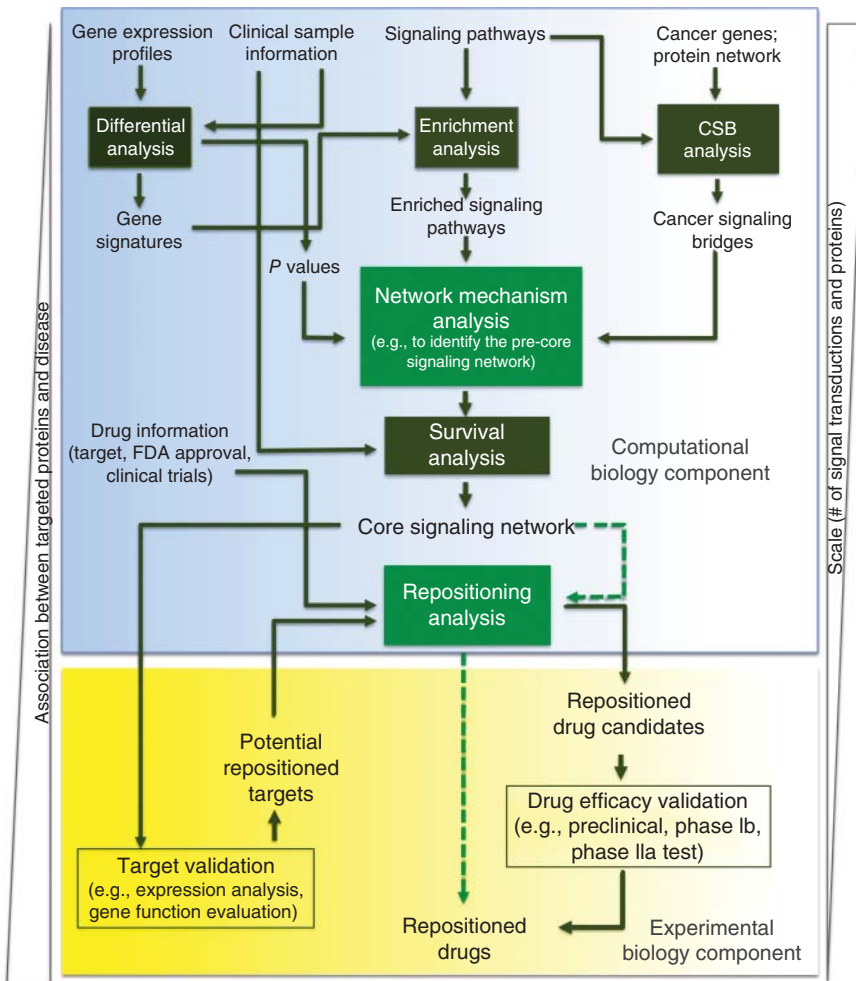


Figure 3.2 An overview of the technical pipeline for cancer drug repositioning. (Reprint from Ref. [23].) The pipeline comprises six modules for the computation biology analyses (differential analysis, enrichment analysis, CSB analysis, survival analysis, signaling network analysis, and repositioning analysis) and two modules for experimental biology analyses (target validation and drug efficacy validation). Signaling network analysis, which is the core of the computational component, is to refine the general signaling networks to the core signaling network that is specific to the cancer of interest. Differential analysis and enrichment analysis modules provide the differential genes and enriched signaling pathways for the mathematical model, whereas CSB analysis supplies the essential cancer signaling network for the mathematical model. Survival analysis enables further narrowing of the signaling networks down to the core signaling network based on the metastasis-free survival times of patients. Repositioning analysis identifies the repositioned drug candidates from the available drug information, integrating with the two experimental biology modules, target validation, and drug efficacy validation.

targets in the signaling network (RET and KDR for sunitinib and FYN for dasatinib) and validated *in vitro* and *in vivo*. The results showed that both drugs could significantly decrease the brain colonies occurrence. The drugs are in phase II clinical trial for breast cancer brain metastasis.

3.5 New Uses Based on New Target Identification (Off-Target Effects)

3.5.1 Antidepressant Drug, Amoxapine, for Alleviating Cancer Drug Toxicity of Irinotecan

Irinotecan (CPT-11) is a type I DNA topoisomerase inhibitor that is commonly used as a chemotherapeutic agent for the treatment of malignances, such as cancers of the brain, colon, and lung, and refractory forms of leukemia and lymphoma [32]. Late-occurring diarrhea is its major adverse effect, with a high incidence rate of 88% of patients. About 20–30% of patients suffer from severe diarrhea (CTCAE grades 3–4) and have to stop the administration [33]. This side effect increases patient suffering and, more importantly, prevents dose intensification and efficacy in a significant fraction of patients undergoing irinotecan treatment [34]. No effective therapy exists for this unmet medical need. Bacterial β -glucuronidase (GUS) enzyme in the intestines plays a pivotal role in CPT-11-induced diarrhea via reactivating the nontoxic CPT-11 metabolite, SN-38G, back to toxic SN-38 [35–41]. The Prestwick Chemical Library® (www.prestwickchemical.com), which includes 1280 approved drugs, was screened for the ability to inhibit the enzymatic activity of purified bacterial GUS. The antidepressant drug amoxapine (AMOX) was identified as a potent *in vitro* GUSi and determined to be one of the most promising potential drugs against bacterial GUS [42]. The molecular modeling work showed the atomic details of the binding modes of AMOX and its metabolites *in vivo*.

AMOX and its major metabolites, 7-hydroxyamoxapine and 8-hydroxyamoxapine, bound in the active site of GUS as the native ligand (inhibitor 2) in crystal structure and share common pharmacophore features: two aromatic features near bacterial loop residue F365 and one hydrogen bond donor feature toward E413, but with different chemical patterns (Figure 3.3) [43, 44]. As a result of such binding with GUS, *in vitro* assays showed not only AMOX but also its metabolites are potent GUS inhibitors, likely contributing to the *in vivo* antidiarrheal efficacy of AMOX. Significant suppression of diarrhea was obtained by combining the administration of AMOX and CPT-11 in xenograft model mice, as well as improved efficacy in reducing tumor growth. As an old drug with known pharmaceutical properties and toxicity profile, the original antidepressant drug AMOX could be applied to human subjects in a relatively short period to prevent CPT-11-induced diarrhea with minimal side effects and even strengthen the efficacy of this chemotherapy.

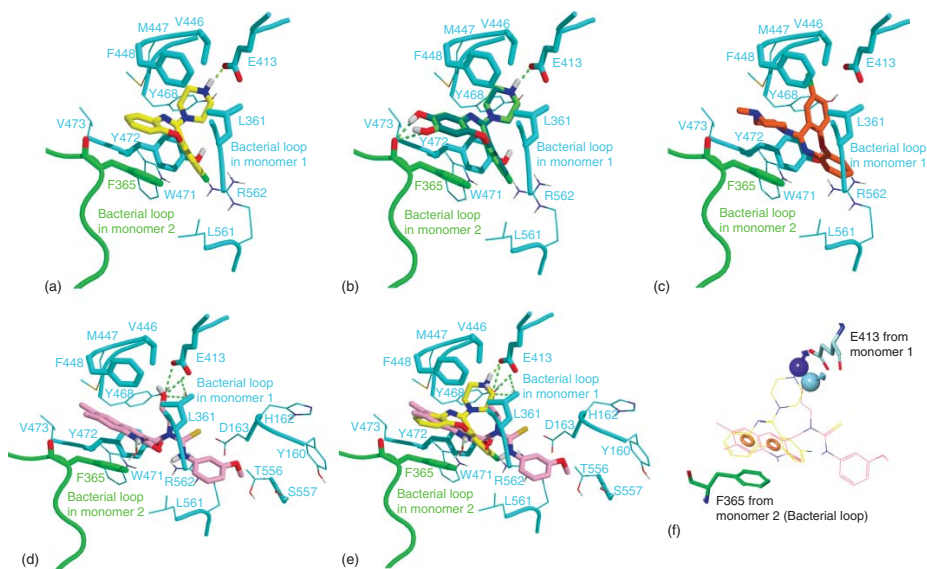


Figure 3.3 Binding modes of (a) amoxapine (yellow), (b) 7-hydroxyamoxapine (lime) and 8-hydroxyamoxapine (dark green), and (c) loxapine (orange), (d) inhibitor 2 (pink) from 3LPF, and (e) amoxapine and inhibitor 2 in the same active site. (f) Pharmacophore features for amoxapine and inhibitor 2 (Reprint from Ref. [43].) The compounds and close contacting residues are shown in sticks. Residues in 5 Å around the ligands depicted: the active site are shown in lines. The primary monomer is colored in cyan and bacterial loop from the adjacent monomer is colored in green. The hydrogen bonds are drawn in lime dash lines. In the mode of loxapine, the critical hydrogen bond with E413 is missing. The pharmacophore based on amoxapine and inhibitor 2 is composed of two aromatic ring features (shown in orange spheres) and hydrogen bond donors pointed to E413 (purple and cyan spheres).

3.6 Computational and Systematic Drug Repositioning

Phenotypic and target-based screenings are indisputable cornerstones for not only drug discovery but also the drug repositioning process. However, the number of phenotypic or target-based assays for potential indications exceeds the screening capacity of most companies. Computational drug repositioning, which has boosted in recent years, provides a systematic and rational strategy for effective drug repurposing [11, 45]. Even though most of the published examples driven by computational methods are supported just by preclinical validation, clinical examples should show up in the near future. The computational methods make use of the available large data sets, such as genomic and transcriptomic data, clinical reports, medical literature, and public data resource for protein and compounds, to disclose the potential drug-new indication connections for rational drug repositioning. Representative methods include transcriptomics guided repositioning, clinically observed side effects repositioning, disease-related genetics guided repositioning, network and pathway analysis guided repositioning, and off-target effects indicated repositioning. For a specific case, several methods could also be integrated based on available information to achieve the repositioning purpose.

3.6.1 Methods Based on Knowledge of Side Effects

Clinical observations for side effects of drugs are traditional and reliable ways of finding new indications. For example, the most successful case, sildenafil (Viagra), has achieved billions of sales each year. Indeed, the promiscuous binding behavior of drug and its possible metabolites *in vivo* not only includes the side effects but also opens novel therapeutic possibilities. Despite the serendipitous discovery, computational methods were developed to systematically explore the side effects of drugs and drug candidates in clinical trial for opportunities of new indications [46, 47]. Yang *et al.* constructed a database of disease–side effect associations using the information of drug labels from SIDER (<http://sideeffects.embl.de/>) and drug–disease relationships from PharmGKB (<https://www.pharmgkb.org/>). Both side effects and indications are treated as measurable behavior or physiological changes in response to particular drugs. If the drugs treating the disease X share common side effect Y, then there might be some underlying MOA linking disease X and side effect Y. Side effect Y could be assumed to be a phenotype “biomarker” for disease X. Other drugs with the same side effect Y might have potential to treat disease X and could serve as repositioned drugs for this disease, which is the newly predicted drug–disease relationship. The authors also built a quantitative structure–activity relationship (QSAR) based on chemical structures to predict the side effects for compounds with no information available. When effects in the database are queried, the diseases associated with the queried effect are provided for possible new uses. Of all the newly hypothesized drug–disease relationships, 27% of them had already been published in clinical trials. For example, transplant rejection drugs have a common side effect of increased cytomegalovirus infection. Other drugs with the same side effect could also have the potency for transplant rejection. Methotrexate, an antineoplastic

drug having cytomegalovirus infection as a side effect, is also used as off-label drug for preventing transplant rejection [46, 48].

3.6.2 Methods Based on Transcriptomics Data (Transcriptional Profile)

In 2006, Broad Institute scientists published a paper in *Science* announcing the “Connectivity Map” (C-Map) data source for systematic discovery of functional connections of diseases, genetic perturbations, and drugs [49]. C-Map listed the gene expression signatures of human cell lines in condition with or without drug treatment, as well as with or without genetic reagent treatment. It is the first time to systematically measure the gene expression profiling changes for human cell lines in response to various drugs or genetic reagents, a technique known as transcriptomics. Currently, using the reduced number of genomic measurements along with streamlined methodologies, the build 02 version of C-Map contains more than 7000 expression profiles for 1309 drugs and compounds. The data set enables great potential to connect human diseases with genes and drugs, which may not have been disclosed before. An intuitive application is that the strong negative correlation between disease signatures and drug transcriptomic signatures could strongly suggest the potential therapeutic use of a drug for a disease.

An algorithm has been developed to quantitatively score the correlation, while tools are provided to match user-provided disease state gene signatures or phenotype model gene signatures for the whole data set to identify possible repositioning drug candidates (<http://www.broadinstitute.org/cmap/>). Researchers interested in a particular disease need to upload the disease signatures, which are a set of significantly up- or downregulated genes, onto the online server. Correlation scores for each compound in the data set with the specific disease signature are given, and those compounds with strong negative scores are believed to be potential drugs for this disease. The results could be validated experimentally in the same disease or phenotype model.

Two limitations exist for C-Map methods, however. One is the availability of disease signatures, and the other is the capacity of compounds with transcriptomics in the released database. The Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) provide publicly available gene expression repositories, which are contributed by thousands of individual investigators, making it possible to access numerous disease signatures. On the other hand, the cost of platform is reduced by using only necessary genes for distinguishing patterns rather than the whole genome, enabling the possibility of high-throughput screening for millions of compounds. The promising results of using C-Map have been shown in several recent publications. By using the disease signature derived from a rat model of osteoarthritic pain, phenoxylbenzamine was identified and further validated in the *in vivo* model [50]. Ursolic acid is identified for muscle weight loss [51] and the anticonvulsant drug topiramate for inflammatory bowel disease [52], which has been validated using *in vivo* model. Sirota *et al.* generated 100 disease signatures based on the GEO data set and probed in C-Map. Many novel disease–drug pairs were uncovered [53]. One of the predictions, cimetidine for lung carcinoma was validated *in vitro* and *in vivo* [53].

Another application is classification of the compounds in the data set based on transcriptional profiling. A network was built to connect the compounds in C-Map based on the gene expression similarity (MANTRA, <http://mantra.tigem.it>) [54]. Some of the tightly connected drugs were identified to share modes of action, strongly indicating that the remaining drugs in the same group may have similar therapeutic uses. Fasudil (a rho-kinase inhibitor) was found to be closely connected with deoxy-D-glucose. As deoxy-D-glucose is for autophagy, fasudil was suggested to have the same function in terms of the network. A subsequent *in vitro* assay confirmed the new use of fasudil. Many algorithms have been developed to utilize the C-Map data. Notably, Jin *et al.* implement the CSBs and the Bayesian factor regression model to build a new network based on C-Map for drug repositioning [24]. Even the candidates identified by using C-Map are currently in preclinical stages, it should be not far before those promising candidates enter into clinical trials.

3.6.3 Methods Based on Genome-Wide Association Study (GWAS)

Genome-wide association study (GWAS), also known as whole-genome association study (WGA study), compares the DNA of whole genome from two groups of individuals: people with disease and similar people without disease (control). The results from GWAS provide one of the strongest evidences to connect specific genetic variants, single nucleotide polymorphisms (SNP), to specific human disease (<http://www.gwascentral.org/>). An online database was developed to collect the SNP–trait associations from published GWAS [55]. The larger data set led to the increased disclosure of gene–disease relationship, which could be used for drug repositioning. A drug could be repurposed if its known target is newly discovered to be genetically associated with a disease that is not among the known drug indications. NTE5, a gene encoding an enzyme involved in adenosine metabolism, has been linked to a dominant and adult-onset form of arterial calcification [56]. An antithrombotic drug, dipyridamole, could be potentially used as treatment for this disease as it inhibits reuptake of adenosine.

3.6.4 Methods Based on Network and Pathways Analysis

By using data from genomics, proteomics, or transcriptomics, with known signaling pathways or metabolic pathways and PPI network, the network or pathway-based methods implement computational algorithms to identify disease-specific pathways or subnetworks, which provide pivotal biomarkers or key targets for drug repositioning [23, 57, 58]. The computational method is helpful in narrowing down the large protein number involved in a general signal pathway down to a few proteins in the specific disease-oriented signal network and uncovering unknown MOA. Drug repositioning could be achieved either directly by using known drugs associated with targets in the uncovered disease-specific pathway or indirectly by disease similarity based on the subnetwork. Zhao and Jin's work addressed distinct signaling pathways of metastatic subtypes of breast cancer, followed by repositioning sunitinib and dasatinib for breast cancer brain metastasis [23]. Li and Suthram's work showed the algorithms to compare disease similarity based on subnetwork modules or canonical signal pathways, enabling repositioning drugs from one disease to another [59, 60].

3.6.5 Methods Based on Off-Target Effects

Different from discovering new usage of drugs via uncovering new role of targets or signal pathways in disease, off-target effects show new targets for drugs in addition to the original target it was tested for. Although drugs are designed or tested for specific targets, most drugs have promiscuous target profiling [61]. The *in vivo* behavior of drugs is often nonspecific, and the possibility of a drug binding to biomolecules other than its original targets can lead to adverse effects. On the other hand, the off-target effects provide potential drug repositioning opportunities when the addressed targets play a pivotal role in other diseases. Computational methods for systematically excavating known off-target effects were developed in recent years and include large-scale molecular docking, protein binding site similarity, ligand structure similarity, and knowledge-based complex network inference.

In silico molecular docking, which computationally evaluates the binding modes and binding affinities of compounds and proteins, was applied in the proteome scale to disclose potential targets exhaustively [62–65]. Dakshanamurthy *et al.* performed extensive computations on 3671 FDA-approved drugs across 2335 human protein crystal structures [63]. Over 58% of known drug–target associations were accurately predicted to rank in the top 40 of the 3671 drugs list. New drug–target associations were identified and confirmed experimentally *in vitro*. Mebendazole, an antiparasitic with recently discovered and unexpected anticancer properties, was predicted to structurally bind with VEGFR2 and further validated by kinase assay and angiogenesis assay. The anti-inflammatory agent celecoxib was predicted to bind with cadherin-11, an adhesion molecule important in rheumatoid arthritis and poor prognosis malignancies for which no targeted therapies exist, and the activity was confirmed by surface plasmon resonance assay. Luo and Yang applied docking experiments on 162 drugs with known indications and adverse effects and 845 proteins with structural information to predict chemical–protein interaction (CPI) [64, 65]. The results indicated not only the possibility of drug repositioning but also adverse effects. An online server, DRAR-CPI, is available to identify drug repositioning potential and adverse drug reactions via chemical–protein interactome [65]. A protein binding site similarity method, sequence order-independent profile–profile alignment (SOIPPA), which detects common binding sites among proteins unrelated in sequence and function, was applied in repositioning old drugs for multidrug-resistant tuberculosis [66, 67]. The nicotinamide adenine dinucleotide (NAD)-binding Rossmann fold and the *S*-adenosyl methionine (SAM)-binding fold proteins share similar binding pockets with common physiochemical properties [66]. Entacapone and tolcapone are drugs for catechol-*o*-methyltransferase (COMT), a member of SAM-binding family protein, for Parkinson's disease.

The primary target of tuberculosis is enoyl-acyl carrier protein reductase (InhA), an enzyme belonging to NAD-binding family. Drugs originally for Parkinson's disease were therefore recommended for treatment of tuberculosis and validated in *in vitro* assay. As not all the target protein structures have been resolved, potential targets were also predicted based on their ligand structural and physiochemical properties [61, 68, 69]. Remarkably, 30 of the predicted new drug–target associations were validated experimentally [69]. Cheng *et al.*

used the known drug–target, target–disease, and disease–gene association from publicly available data resources including DrugBank and KEGG to construct a complex network of associations [70]. The network-based inference (NBI) was used to recommend previously unconnected drugs with targets with a predictive score. High scoring drugs were considered more likely to interact with a specific target. Predicted drug–target associations were tested in the in-house available *in vitro* experiments for estrogen receptors and breast cancer cell line showing the potency of proliferation for old drugs, simvastatin and ketoconazole [70].

Notably, the evolutionary similarity of protein in human genome and parasites genome indicates the possible off-target effects in both sides, and the most efficacious drug repositioning may be achieved by using anti-infective drugs for corresponding targets in human, or the reverse [2, 67, 71].

3.7 Perspective

The most recognized success story of sildenafil (Viagra) indicates that a drug repositioning strategy provides not only potential great economic benefit for the pharmaceutical companies but also significant health benefits for the appropriate patients. A repositioned drug with proven bioavailability and known safety profiles would avoid the high failure rate caused by absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties, reduce the development cost, and shorten time spent on R&D process [5, 6, 72]. Serendipitous discovery from clinical observations has led to a lot of successful cases. Target-based or phenotype screening on existing drug collections, the traditional tools in drug discovery pipeline, also are powerful ways for repositioning. Obstacles exist for the traditional methods. First is the availability of drugs. Although many vendors provide various commercially available drug collections, none of them cover all the drugs that have been approved or investigated worldwide. The National Institutes of Health's National Center for Advancing Translational Sciences collection has a collection of 3800 approved or investigated compound entities (NCGC Pharmaceutical Collection) [73]. The electronic resource about the compounds is freely available; however, the physical collections of small molecules for screening can only be obtained through collaboration [74]. Second, even though screening is limited to existing drugs, numerous assay types make it exceed the screening power of any companies. If we use orphan diseases as example, defined by the FDA Orphan Drug Act as <200,000 US prevalence, there are approximately 6000 diseases, most of them having no medical solutions. Thus, computational methods are becoming mainstream for systematic drug repositioning by utilizing the tremendous increased publicly available data, including chemical–target interactions, PPIs, target–disease associations, signal pathway and networks, generic transcriptome of drug treatment, disease-related genome signatures, clinical observations, and electronic health records (Table 3.2). Several successful stories have been validated *in vitro* and *in vivo*, and some of them are in phase II clinical trials [23]. Further collaboration between clinical pharmacologists and translational bioinformaticians could certainly accelerate the drug repositioning process.

Table 3.2 Databases used for drug repositioning studies.

Main function	Databases	Website	References	Descriptions
Chemical–target associations	PubChem	https://pubchem.ncbi.nlm.nih.gov/		Compounds, physicochemical properties, bioassays, bioactivities, targets
	ChEMBL	https://www.ebi.ac.uk/chembl/		Compounds, physicochemical properties, bioassays, bioactivities, targets
	ChEBI	http://www.ebi.ac.uk/chebi/init.do		Chemical entities of biological interest
	BindingDB	http://www.bindingdb.org/bind/index.jsp	[75, 76]	Compounds, physicochemical properties, bioassays, bioactivities, targets
	Psychoactive Drug Screening Program K_i (PDSP K_i)	http://pdsp.med.unc.edu/pdsp.php		K_i value for compounds and target interactions
	MATADOR (manually annotated)	http://matador.embl.de/	[77]	Manually curation for direct and indirect protein–chemical interactions
Drug/target/disease/ metabolism/side effect information	SuperTarget	http://bioinf-apache.charite.de/supertarget_v2/	[77]	Drugs, targets, pathways, activities
	STITCH	http://stitch.embl.de/	[78]	Chemical–protein interactions
	GPCR-Ligand Database (GLIDA)	http://pharminfo.pharm.kyoto-u.ac.jp/services/glida/	[79]	GPCR protein and ligand interactions
	ChemSpider	http://www.chemspider.com/		Compounds, physicochemical properties, bioactivities, targets
	DrugBank	http://www.drugbank.ca/	[80–83]	Drugs, physicochemical properties, bioactivities, targets
	Therapeutic Target Database (TTD)	http://bid.d.nus.edu.sg/group/cjttd/	[84–87]	Drugs, physicochemical properties, bioactivities, targets, diseases
				(Continued)

Table 3.2 (Continued)

Main function	Databases	Website	References	Descriptions
Chemical collections	Pharmacogenetics Knowledge Base (PharmGKB)	https://www.pharmgkb.org/	[88]	Genetic variation on drug response
	DrugMap Central (DMC)	http://r2d2drug.org/index.html	[89]	Drugs, physicochemical properties, bioactivities, targets, diseases, side effects
	ChemFrog	http://www.chemfrog.com/		Compounds and vendor information
	ZINC	http://zinc.docking.org/		Commercially-available compounds for virtual screening and vendor information
Target information	iScienceSearch	http://www.isciencesearch.com/		Integrated compound information from a wide variety of databases
	Chemicalize (ChemAxon)	http://www.chemicalize.org/		Compounds, physicochemical properties, literatures
	RCSB Protein Data Bank (PDB)	http://www.rcsb.org/pdb/home/home.do		Biomolecules structure information
	OPM	http://opm.phar.umich.edu/		Membrane protein and its orientation in membrane
	Proteopedia	http://proteopedia.org/wiki/index.php/Main_Page		Education usage for 3D structure model
	TOPSAN	http://www.topsan.org/		Protein structure and function
Adverse effects and clinical trial information	UniProt	http://www.uniprot.org/		Protein sequence, function, and annotations
	SIDER	http://sideeffects.embl.de/	[90]	Drug and side effects associations
	FAERS	http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm082193.htm		Side effect reporting system in US FDA

FDA label information	Adverse Reaction Database	http://www.hc-sc.gc.ca/dhp-mps/medeff/databasdon/index-eng.php	Side effect reporting system in Canada
	Iowa Drug Information Service (IDIS)	http://itsnt14.its.uiowa.edu/	Drug treatment information from journals and public databases
	ClinicalTrials.gov	https://clinicaltrials.gov/	Clinical trial reporting system
	FDALabel	http://labels.fda.gov/	Drug label information (US FDA)
	DailyMed	http://dailymed.nlm.nih.gov/dailymed/index.cfm	Drug label information (US FDA)
Pathway information	Structured Product Labeling (SPL)	http://www.fda.gov/forindustry/datastandards/structuredproductlabeling/default.htm	Drug label information
	NCI Pathway Interaction Database (NCI-PID)	http://pid.nci.nih.gov/	[91] Biomolecular interactions and cellular processes assembled into authoritative human signaling pathways
	Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.genome.jp/kegg/	[92] Drugs, proteins, pathways
Protein interaction information	BioCarta	http://www.biocarta.com/genes/index.asp	Signal pathways and kits provider
	Reactome	http://www.reactome.org/	Signal pathways and bioinformatics tools
	Pathway Commons	http://www.pathwaycommons.org/about/	[93] Signal pathways and bioinformatics tools
	Human Protein Reference Database (HPRD)	http://www.hprd.org/	[94] Manual curation for human protein information
	Biological General Repository for Interaction	http://thebiogrid.org/	[95] Curation of protein interactions
	STRING	http://string-db.org/	[96] Known and predicted protein interactions
	Mammalian protein–protein interaction database (MIPS)	http://mips.helmholtz-muenchen.de/proj/ppi/	[97] Manual curation of protein interactions from individual experiments

(Continued)

Table 3.2 (Continued)

Main function	Databases	Website	References	Descriptions
Genetic data or information	IntAct	http://www.ebi.ac.uk/intact/	[98]	Molecule interactions from literature curation and user submission
	Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu/dip/Main.cgi	[99]	Curation of protein interactions
	NCBI-GEO	http://www.ncbi.nlm.nih.gov/geo/		Public functional genomics data repository
	Sequence Read Archive (SRA)	http://www.ncbi.nlm.nih.gov/sra		Biological sequence data
	Stanford Microarray Database	http://smd.princeton.edu/		Microarray database
	ArrayExpress	http://www.ebi.ac.uk/arrayexpress/		Public functional genomics data repository
	Princeton University MicroArray database (PUMAdb)	https://puma.princeton.edu/		Microarray database
	CellMiner	http://discover.nci.nih.gov/cellminer/home.do		Molecular and pharmacological data sets for the NCI-60 cell lines
	Oncomine	https://www.oncomine.org/resource/login.html		Cancer microarray database and integrated data mining platform
	Cancer Cell Line Encyclopedia (CCLE)	http://www.broadinstitute.org/software/cprg/?q=node/11	[100]	DNA copy number, mRNA expression, mutation data and more, for 1000 cancer cell lines
	dbSNP	http://www.ncbi.nlm.nih.gov/projects/SNP/		Short genetic variations
	Online Mendelian Inheritance in Man (OMIM)	http://omim.org/	[101]	Online Catalog of Human Genes and Genetic Disorders
	Connectivity Map (C-Map)	https://www.broadinstitute.org/cmap/	[49]	Catalog of gene-expression data collected from human cells treated with chemical compounds and genetic reagents

Source: Adapted from Ref. [11].

This quote from the noted chemist, Christopher Lipinski, is particularly apt: “although a bit of an exaggeration, there is a lot of truth in the saying that we do not need to find new drugs; rather we need to find the patients who can benefit from existing drugs” [102].

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4

Discovery Technologies for Drug Repurposing

Naiem T. Issa¹, Stephen W. Byers^{1,2}, and Sivanesan Dakshanamurthy^{1,2}

¹Georgetown University Medical Center, Lombardi Comprehensive Cancer Center, Department of Oncology, Washington, DC 20057, USA

²Georgetown University, Department of Biochemistry & Molecular Biology, 3900, Reservoir Road, Washington, DC 20057, USA

4.1 Introduction

Drug repurposing, or repositioning, is a biopharmaceutical discovery paradigm that focuses on establishing new therapeutic indications for already approved drugs. High failure rates associated with the exorbitant cost of translating the conception of a therapeutic through preclinical/clinical testing and ultimately to the market drive this effort [1]. Repurposing streamlines the movement of approved drugs with known safety and toxicity into the phase II clinical trial level, thereby reducing the cost and time to market while maximizing success [1–6]. Such economic feasibility is important not only for associating blockbuster drugs with prevalent diseases but also for resurrecting orphaned drugs [7, 8] and lowering barriers for treating rare diseases [9–11]. Presented here is a discussion of phenotypic and target-based screening methods followed by *in silico* computational approaches that are driving contemporary drug repurposing efforts.

4.2 Biological Drug Screening Methods

4.2.1 Phenotypic Screening

When targets for a disease are unknown, phenotypic screening can be utilized. Phenotypic screening, classically referred to as “forward pharmacology” [12], provides the advantage of recapitulating disease states and assessing the physiological outcomes of pharmacological interventions. Such models maintain signaling pathways and protein expression levels, allowing for appropriate assessments of drug effect. Typically, phenotypic screening is used to discover lead compounds that are further refined for absorption, distribution, metabolism, excretion, and toxicity (ADMET) optimization [13]. In the case of FDA-approved drugs with known ADMET properties, phenotypic screening allows for quick identification of new indications and streamlined entry into late-phase clinical

trials. The advantages and disadvantages of phenotypic screening models in drug discovery and repurposing are discussed next.

4.2.1.1 Animal-Based Screening

Small nonmammalian animal models are used for preclinical testing of drugs. Intact whole organisms maintain cellular microenvironments and tissue-specific biochemical attributes that occur in disease states. These models are thought to increase both the chance and efficiency of validating bioactive compound “hits” in mammals.

Low-cost and genetically tractable small animal disease models include worms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), frogs (*Xenopus laevis*), and zebrafish (*Danio rerio*) [14, 15]. Prevalent diseases that have been modeled by these systems include Alzheimer’s disease [16, 17], malignant melanoma [18], and Duchenne muscular dystrophy [19]. In addition to their versatility, these models can be formatted in 96-well plates for high-throughput screening (HTS) assays [20–24]. Multiple drugs can be screened against a single disease phenotype or, conversely, a single drug can be screened against multiple disease phenotypes quickly. Efforts in automating data analysis for HTS [25–28] also enhance workflow and bridge the gap between lead identification and development of the market product.

Drawbacks to small animal phenotypic screenings include establishing an effective dose as drugs are provided via media [29] and organism-unique pharmacokinetic parameters. However, the latter is less of a concern in drug repurposing than in *de novo* drug discovery as many biological and pharmacokinetic effects in humans have already been characterized. Additionally, there exist definite anatomical, physiological, and molecular differences between humans and small animal models, making disease phenotypes possibly organism specific. Although these subtleties result in suboptimal attrition rates, they provide the significant advantage of probing diseases in the absence of known biochemical mechanisms and also help in the identification of drug targets and pathways.

4.2.1.2 Cell-Based Screening

Cellular phenotypic screening methods are complementary to small animal models. Their small size and simpler culture conditions render cellular systems applicable to HTS. Multiple cell lines corresponding to various aspects of a disease (i.e., MCF-7 cells for cancer [30, 31] and primary bronchial epithelial cells for cystic fibrosis [32, 33]) provide greater analytical resolution as subtle pathological differences can be exploited.

Cell viability assays are among the most common types for identifying drugs that kill cells. Biomarkers of cell viability typically include death-defining enzyme activities, such as the release of intracellular lactate dehydrogenase [34] and protease [35], mitochondrial activity (MTT assay [36]), and cellular metabolism (ATP content assay [37]). Signaling pathway assays are also utilized to probe known pathways (i.e., mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)). Assay end points are typically the expression of a reporter gene (i.e., luciferase) or fluorescence protein [38–40] and do not require knowledge of protein–protein interactions (PPIs). Similarly, disease-related

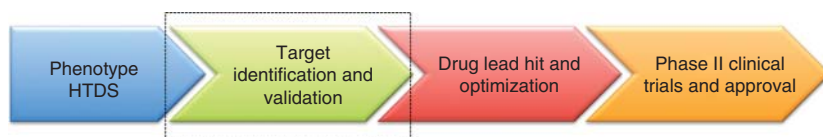
assays utilize macro-phenotypic end points, where disease-specific cellular and morphological changes serve as markers. For example, in neurodegenerative diseases like Alzheimer's and Parkinson's disease, neurite outgrowth assays are employed to assess cytoskeleton abnormalities [41]. Another example is in metabolic diseases like Niemann–Pick disease type C, where a filipin staining assay is used to measure lysosomal cholesterol accumulation [42].

Phenotypic approaches, whether organismal or cell based, attempt to recapitulate the disease state and provide high-level information about pharmacologically induced perturbances. Clinical success is often limited to the reliability and accuracy of the model being used, but mechanistic information of pharmacological effect may remain unknown. However, information regarding mechanisms of action is important for elucidating modes of efficacy and identifying causes of side effects, both of which are essential for FDA approval. Target-based screening, discussed next, is utilized for this type of investigation.

4.2.2 Target-Based Screening

Target-based screening is a hypothesis-driven approach that helps to find drug mechanisms of actions. This allows for the identification of drugs against a disease-implicated target or pathway. In the preclinical setting, a target is first identified and validated. Readers interested in target identification are directed to [43–50] for further discussion. A target-specific HTS assay is then developed for subsequent drug identification and development. A schematic of this process is shown in Figure 4.1. Results of target-based screenings are becoming

(a) Phenotypic screening approach



(b) Target-based screening approach



Figure 4.1 Workflows of phenotypic and target-based screening approaches in drug discovery and repurposing. (a) Phenotypic screening requires organismal or cell-based models that are subjected to high-throughput drug screening (HTDS). Once the requisite change in phenotype is achieved, the lead drug can be optimized or, in the case of drug repurposing, taken directly to phase II clinical trials. If desired, identification of the drug's target can be pursued (dashed box), but target knowledge is not necessary. (b) Target-based screening is reliant on the identification and validation of a target associated with a disease of interest. An assay specific for that target needs to be developed and amendable to HTDS. However, though the assay may indicate an association between the target and a drug, the desired organismal effect may not be achieved as the target may only be one of many contributors to the disease state. Thus, target-based screening approaches tend to be less successful for discovering first-in-class drugs compared with phenotypic screening approaches, but are more useful for follower drugs that are optimized for reducing off-target effects and maximizing efficacy.

increasingly centralized for public use, found in databases such as PubChem Bioassays [51], RCSB [52], DrugBank [53], Therapeutic Target Database [54], Comparative Toxicogenomics Database [55], and CARLSBAD [56], among others. Information contained in these databases includes drug–protein target associations, half maximal inhibitory concentrations (IC_{50}), half maximal effective concentrations (EC_{50}), dissociation constants (K_d), and ligand binding affinities (K_i).

A major limitation of the target-based approach is the reductionism of a disease to a single gene product or mechanistic pathway [57–59]. While a drug may exhibit strong potency against a given target through binding or cell-based assays, its effect on other targets (“off-target effects”) remains unknown. Thus, it may be advantageous to begin with phenotypic screening to identify lead drugs and then determine mechanisms of target and pathway effects. The majority of new molecular entities (NMEs) for first-in-class drugs have been derived from phenotypic screening, whereas the follower drugs more often result from target-based screening [60]. This reflects a general methodology in drug discovery – first is the need to find a drug that disrupts the disease at the organismal level (phenotypic screening) followed by the refinement of those drugs to decrease side effect and toxicity profiles while increasing efficacy for optimal therapy (target-based screening).

Target-based screening is currently a time-consuming process that requires proper target identification, assay development, and biological validation. However, computational and network pharmacology approaches that integrate big data genomics are facilitating the process of identifying disease-causing targets and drug–polytarget associations for enhanced drug repurposing success. These approaches are discussed next.

4.3 *In silico* Tools for Drug Repurposing

Although drug repurposing is a cost-effective alternative to *de novo* drug discovery, HTS methods still pose many challenges such as resource availability and model validity. *In silico* methods have been adopted to address these. These methods are diverse and utilize data spanning low-level biochemical assays to high-level semantic data retrieved from literature text mining and actual patient data within electronic medical records (EMRs). Network analysis further provides system level analytics where associations across various modalities accurately predict biological outcomes. The subsequent section provides an overview of the latest *in silico* methods and network analyses being applied for drug repurposing.

4.3.1 Docking

Docking is the computational process of fitting a ligand into a potential binding site within proteins by predicting the free energy of binding. Ligand–protein complexes exhibiting negative free energies of binding are considered to be biologically plausible. In the case of drug repurposing, approved drugs are docked

Table 4.1 Docking algorithms used in computational drug repurposing.

Algorithm	Country	References
AutoDock4	USA	[64]
eHiTS	UK	[65]
FlexX	Germany	[66]
DOCK	USA	[67]
GLIDE	USA	[68]
GOLD	UK	[69]
Lead Finder	Canada	[70]
LUDI	Germany	[71]
MS-DOCK	France	[72]
Surflex	USA	[73]

into a target not involved in the approved indication. The repurposing of the antipsychotic haloperidol to HIV-1 protease inhibitor is one of the first successful docking efforts [61]. Other examples include nilotinib for inhibiting MAPK14 [62] and the drug profile matching (DPM) algorithm [63], which predicted celecoxib, a selective COX-2 inhibitor, to interact with the dopaminergic system.

Many docking scoring functions (SFs) exist to predict free energies of binding. Examples of the most popular SFs currently used are found in Table 4.1 [64–73]. However, their underlying methods differ significantly and lead to inconsistencies in outcomes [74]. Some SFs performed better when hydrophobic intermolecular interactions dominated, while others were more accurate for hydrophilic interactions. The topic of choosing the “right” docking SF is currently being debated, highlighting the need to better understand intermolecular interactions in computational models.

Presently, docking as a sole method for predicting drug–target interactions is considered unreliable. Better understanding of how non-covalent interactions contribute to binding and how different protein systems exhibit different binding properties (i.e., buried vs solvent-exposed pockets) is critical. Further discussion on docking strategies and limitations can be found in [75–80].

4.3.2 Chemoinformatics

Chemoinformatics, computational analysis of drug chemical properties and structures, uses a ligand-centric perspective to explore the drug space in the absence of protein structure information. Ligand-centric approaches assume that structurally and chemically similar drugs have similar properties, bind similar targets, and cause similar biological effects [81–83]. Discussed next are some methods used to quantify similarities between drugs.

One measure of drug similarity is through three-dimensional shape. A fundamental principle driving biological recognition is “form fits function.” For a drug to elicit a particular effect, it must adopt a precise conformation to

“fit” into the pocket and stabilize the protein structure. In addition to shape, proper orientation of the drug’s chemical functional groups is important for optimal drug–protein interactions. The computational conformer selection (CSS) approach [84] uses a Tanimoto-combined score to measure drug similarity based on the combination of shape and chemical functional groups, leading to the repurposing of the cardiovascular drug *S*-bepiridil to MDM2, an important oncologic target. A spherical harmonic-based “consensus shape” method also enriches virtual screening performance for targets whose pockets can accommodate drugs in multiple ways [85]. Similarly, Screen3D is a novel shape-based screening algorithm with considerable success [86].

Pharmacophore screening is another chemoinformatic method. A drug’s pharmacophore is the unique three-dimensional geometric representation of chemical and steric features contributing to recognition by a target. If a drug’s bioactive conformation is known, pharmacophore models can be used to screen large drug databases efficiently for other drugs with similar features. Successful applications of pharmacophore screening include the prediction of antiviral drug activity profiles [87] and micromolar inhibition of phosphodiesterase 5 (PDE5) by rolofylline, an adenosine A1 receptor antagonist [88].

Another chemoinformatic method of establishing drug similarity is the similarity ensemble approach (SEA) [89]. SEA relates protein targets based on the chemical similarity of their ligands. This similarity is quantified by the Tanimoto coefficient based on drug structural features represented through standard two-dimensional topological Daylight fingerprints. SEA thus establishes links between unexpected protein targets based on this similarity, leading to the hypothesis and subsequent experimental confirmation that methadone, an opioid, could antagonize the muscarinic M3 receptor. Since its inception, SEA has been applied to the discovery of new targets for FDA -approved drugs [89]. According to a 2009 report, 77% of predictions were confirmed via experimental testing. SEA is also being used to understand the nature of multiple drug effects through network polypharmacology [90], discussed below.

4.3.3 Protein Binding Site

Paralleling the notion that similar drugs exhibit similar effects, it is also thought that similar protein pockets bind similar drugs. A general schematic of this logic is shown in Figure 4.2. Methods that exploit the binding site focus on structural aspects, as the orientation of binding site residues cannot be extracted from the amino acid sequence. Moreover, proteins that are characterized by different families or folds may have conserved binding site properties, which explains why some drugs associate with seemingly unrelated proteins [91]. An example of this is celecoxib binding to COX-2 and carbonic anhydrase [92].

When a crystal structure or homology model for a target is available but in the absence of a drug, the first step is to identify the putative binding pocket. The biochemistry and structural biology of druggable protein pockets are a subject of great study, and an introduction by Perot *et al.* [93] may be a good primer for the interested reader. Computational algorithms that search for pockets are divided into two main categories: geometric and energy-based methods.

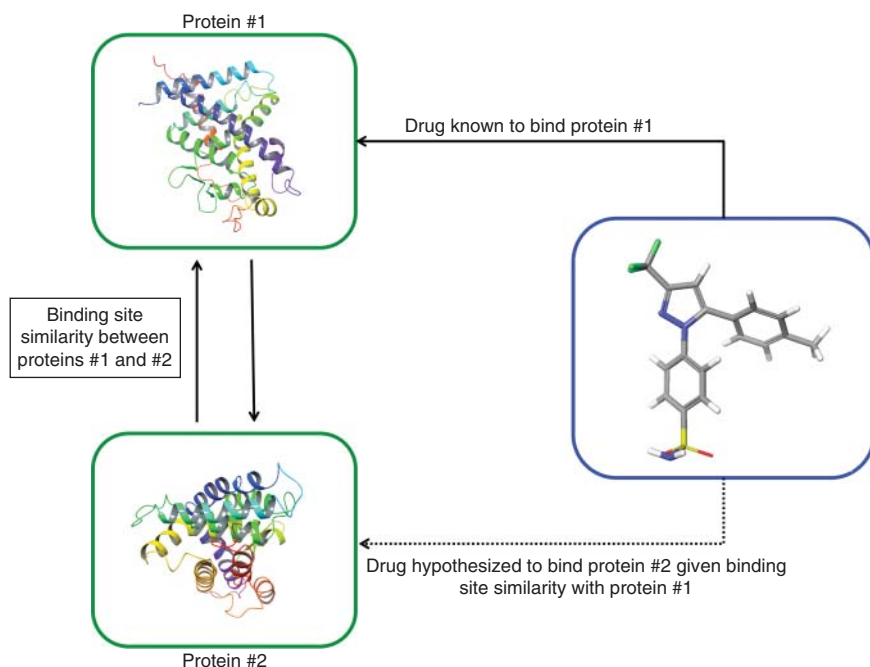


Figure 4.2 Schematic depicting drug repurposing through similarity between protein binding sites. For a given drug known to be associated with protein #1, it is hypothesized to also associate with protein #2 if there is sufficient binding site similarity between the two proteins determined by a variety of methods, such as structural alignment.

Geometric algorithms fit virtual shapes (i.e., spheres) into the solvent-accessible space to identify cavities [94]. Alternatively, energy-based methods utilize probe molecules at different points to estimate the interaction energies between them and the protein [95]. Once a putative binding site has been identified and deemed “druggable” [96], it is compared with other well-characterized binding sites to establish similarities. A detailed discussion on the different alignment algorithms is given by Haupt and Schroeder [97]. Other alignment-free methods also exist that functionally annotate binding pockets for similarity calculations [98–102].

In addition to binding site similarity, structure- (or receptor-) based pharmacophore screening is another protein-based strategy for drug repurposing. Like drug-centric pharmacophores, structure-based pharmacophores are geometrical representations of binding site residue functional elements that may contribute to drug binding [103]. In the case of crystallized apoproteins, pharmacophores encompass a greater number of features than those identified by a complexed drug. Thus, pharmacophore screens of apoproteins retrieve a greater diversity of drugs and binding modes. Purely structure-based pharmacophore algorithms include fingerprints for ligands and proteins (FLAP) [104], SNOOKER [105], and Discovery Studio [106]. Application of structure-based pharmacophore screening has also resulted in drug repurposing successes, such as identification of natural inhibitors for human rhinovirus (RhV) coat protein [107] and micromolar inhibitors of the HtrA protease, a target of *Helicobacter pylori* [108].

Target-based computational approaches have a unique utility when drug information for the target of interest is not known. While advantageous, the information provided alone is prone to many false positives in virtual screenings. An optimal rational drug repurposing scenario is when both drug-centric and protein-centric methodologies are integrated into a holistic approach, discussed next.

4.3.4 Combining Drug-Centric with Protein-Centric Approaches

When crystallized drug–target complexes are available, combining drug- and protein-centric approaches is feasible. One drug repurposing paradigm that performs this integration is “Train, Match, Fit, Streamline” (TMFS) [109], which is an amalgamation of drug docking and functional contact points with shape- and drug-derived chemical signatures. Query drugs for a repurposing study have multiple energy-minimized three-dimensional conformations created, and the shape of each conformer quantified using a spherical harmonic expansion method [110] to establish a conformer shape database. The shapes of these conformers are then compared with the shape of the reference drug in its crystallized bioactive conformation. Conformers with the greatest shape similarity to the reference drug were selected for screening. This conformer shape is also compared with that of the protein target binding site, which is also quantified via a ProtoMol method based on the reference drug position [111]. Shape comparisons are quantified using a Euclidean distance metric. Chemical-specific properties used include the drug’s solvent-accessible surface area, volume, and the number of hydrogen bond acceptors and donors, among others. A Tanimoto similarity metric is used to quantify the similarity of these physicochemical properties between the reference drug and chosen conformers. Once all terms are quantified and normalized, they are combined in a “z-score” equation that allows query drugs to be ranked from greatest to least repurposing potential for the given protein target. As proof of concept, TMFS was able to repurpose the anti-hookworm medication mebendazole to vascular endothelial growth factor 2 (VEGFR-2) kinase and celecoxib to Cadherin-11, both important targets for cancer pathogenesis. While this is a singular example, TMFS illustrates how virtual screening outcomes can be enriched for accuracy by integrating drug- and protein-specific descriptors.

4.3.5 Network Pharmacology

While drug repurposing is heavily reliant on establishing biologically plausible drug–target interactions, it is important to contextualize this information within particular cell systems, tissues, and diseases. Disease states are considered systems of dysregulated interacting components that collectively contribute to pathology. To provide a system-based framework, network pharmacology has come to the fore in drug discovery/repurposing [112, 113]. The reader is encouraged to peruse the following article [114], which provides an elegant introduction into the field of network pharmacology.

Network pharmacology attempts to link drugs to gene products, cell signaling pathways, biochemical functions, and ultimately diseases (Figure 4.3). Many computational methods exist to perform this on large-scale data and provide

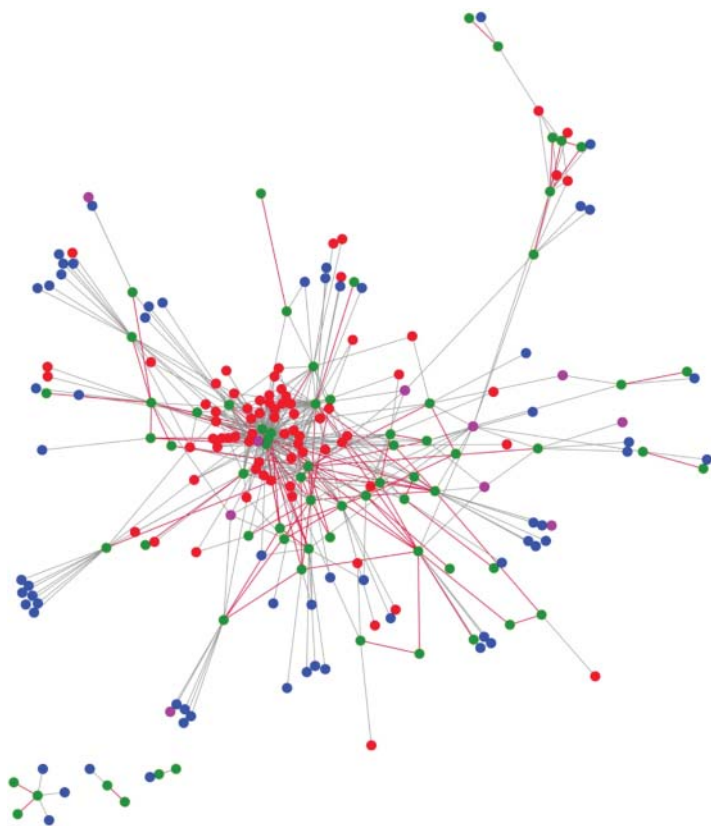


Figure 4.3 Hypothetical network polypharmacology. Hypothetical multitiered network connecting drugs (blue nodes) to protein targets (green nodes) and further to cellular pathways (red nodes) and clinical diseases (purple nodes). Protein–protein interactions are designated using red edges. Nodes found toward the center tend to have many relations, whereas those found toward the periphery exhibit fewer associations. Network analysis highlights drug promiscuity and off-target interactions, as well as protein similarity through the drug chemical space.

researcher tools to perform customized analyses. One such platform is VisANT [115], which is publically accessible for drug repurposing initiatives. Users are able to visualize biological networks and formulate drug repurposing hypotheses based on network associations. Another tool is DrugMap Central [116], where multilevel drug data is also presented visually for researcher-driven drug repurposing studies.

Fundamentally, empirical drug–protein target associations drive higher-order drug associations in network pharmacology. Such empirical information exists and has been validated in multiple databases, such as DrugBank [53], Therapeutic Target Database [54], and PubChem [51]. However, the vast drug–protein interaction space has been minimally explored and given limitations in technologies, resources, and time. In the absence of such knowledge, multiple efforts have established computational tools in an attempt to infer drug–protein and

drug–disease relations. For example, Cheng *et al.* hypothesized drug–target interactions based on the integration of known chemical, side effect, and therapeutic information to predict the association of the muscarinic acetylcholine receptor M1 with tramadol [117]. Several groups also utilized network integration of drug side effect profiles to predict the repurposing of tramadol for depression [90, 118–121].

PPIs and cellular pathways are also used to link drugs to diseases. In the former, the premise is that if two diseases shared drug-associated gene products via their PPI networks, then the drug may be repurposed for both diseases [122]. Pathways are also utilized to provide a broader context of PPIs and disease perturbations. For example, the platform CauseNet quantifies the likelihood of a drug–disease association via the implicated pathway of a drug–target association [123]. DrugRouter is another platform for inferring drug-specific pathways via protein, metabolic, and transcriptional interaction networks, leading to the hypothesis wherein thalidomide could be repurposed for Alzheimer’s disease [124].

In the absence of drug–target information, understanding whole-cell and whole-tissue drug effects becomes reliant on other “omics” methodologies (i.e., genomics, transcriptomics, proteomics, and metabolomics). Drug- and disease-induced changes at these different biological levels can be integrated for holistic analysis. The connectivity map (cMap) is a platform that utilizes gene expression data for diseased and drug-induced phenotypes [125]. A drug may be repurposed for a disease if both states exhibited inversely related gene expression signatures. Drugs with similar gene expression signatures are also considered to exhibit similar phenotypic effects, despite chemical and structural differences. cMap has been used to identify novel antiviral agents against the human influenza A virus [126]. MANTRA is also an explorable drug network created from the merging of cMap expression profiles for the same drug treatment across different cell lines. This led to the unexpected prediction that Fasudil, a potent vasodilator, could modulate autophagy [127]. Functional module connectivity map (FMCM) further refines cMap data by applying a gene selection by trend of progression procedure (GSToP) for greater accuracy [128].

Network pharmacology places drug effect predictions into physiologically relevant contexts where phenotype depends on the differential interactions of multiple proteins and pathways depending on tissue type and disease state. Thus, drug repurposing predictions are enriched with a better understanding of mechanisms of action and off-target effects.

4.3.6 Mining of Big Data

As biochemical and clinical data capture, analysis, and dissemination exponentially increase with time, “big data” analytics provide a unique approach to drug repurposing. Large-volume big data allows investigators to formulate new associations and connections across multitiered data structures, thus revealing previously unknown drug–target, drug toxicity, and drug efficacy signatures [129]. This is especially beneficial in the area of personalized medicine, where pharmacogenomics combined with other “omic” platforms guide patient-specific drug choice.

One form of data that is becoming increasingly utilized in biomedicine is free text found in literature (i.e., journal articles and prescription pamphlets) and EMRs. Free text, a disordered data structure, is incredibly robust for documenting observations. To harness the vast amount of information buried in free text, literature and text mining tools provide structure to the data and extract biologically meaningful associations [130–135]. A community effort called BioCreative [136] has also been created to hasten the advancement of biological knowledge extraction systems.

A well-established literature-based discovery method is Swanson's ABC model of co-occurrence [137]. Scientific concepts from the literature are said to be related if they co-occur with a third concept (i.e., two diseases are related if they co-occur with a protein target, side effect, or drug). An application of the ABC model is a support tool that applies natural language processing (NLP) techniques to MEDLINE citations [138]. This algorithm discovered bibliographic evidence for repurposing thalidomide for acute pancreatitis and myasthenia gravis, though experimental and clinical validation is needed. While the method of co-occurrences is helpful for formulating novel associations, the number of hypotheses is exceedingly great and the associations may not be semantically meaningful for biological relevance. To enrich co-occurrence findings with biological meaning, multiple studies have looked to linguistics, stochastic mathematics, graph theory, and network analysis. For example, a network-based approach anchored in the "distributional hypothesis of linguistic theory" is used to measure the strengths of co-occurrence relations [139]. This mathematical model predicted that imatinib, originally approved for chronic myelogenous leukemia, could be repurposed for Creutzfeldt–Jakob disease and also provides a sound physiological rationale. Another co-occurrence approach for MEDLINE records applies a random network model for measuring the statistical significance of shared relationships, finding chlorpromazine to reduce the progression of cardiac hypertrophy in a rodent model [140].

Literature-based discovery and hypothesis generation has garnered great attention in various aspects of biomedical sciences. In particular, SemMedDB [141], a repository for semantic predictions extracted from the entirety of PubMed citations, has been established for noncommercial use by the National Library of Medicine and has been the springboard for many studies and discoveries. These include endeavors for clinical decision-making and usefulness [142, 143], discovery of physiological mechanisms for clinical diseases [144], and revealing novel drug–drug interactions [145] in addition to drug discovery and repurposing [146, 147].

4.4 Conclusion

Drug repurposing is a cost-effective and clinically viable solution for the currently ailing pharmaceutical pipeline. Computational methods that facilitate identification of new targets and diseases for known drugs considerably enhance the drug development process. With advances in technology and computational prowess, biological information is also obtained and analyzed with greater dimensionality

and resolution. This explosion in knowledge coupled with computational tools to process this “big data” represents a vast space from which to form clinically and patient-oriented drug repurposing strategies with enriched success. Combining DNA next-generation sequencing with other “omics” technologies allows for individualized “big data” records to drive medical decision-making and personalized drug development. Computational tools that holistically integrate biochemical and high-level end point clinical data are poised for optimal success in identifying disease-related targets/mechanisms and elucidating therapeutic strategies while minimizing toxicity.

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Part III

Unselective Drugs in Drug Discovery

5

Personalized Medicine*Christian Noe and Volker Baumann*

*University of Vienna, Institute for Medicinal Chemistry, Department for Life Sciences, Althanstr. 14,
1090 Vienna, Austria*

5.1 Roots of Personalized Medicine

The advent of molecular biology in the last quarter of the twentieth century meant a fundamental change for medical and pharmaceutical sciences. The genome level was added to human physiology and revealed the close relation between genes and disease. The upcoming concept that each disease is connected to one specific gene mutation was fascinating [1]. Consequently, gene therapy – somatic and germ line – became the great goal and was envisaged not just to complement pharmacotherapy but to replace it completely within short time, not later than the turn of the century [2, 3]. Biotech industry popped up and – providing the financial prerequisite – opened the chance for implementation to a plethora of game-changing therapeutic concepts. Many mind-popping concepts failed, some of which – above all gene therapy [4, 5] and immunotherapy [6–16] – are still great and justified expectations nowadays. The failures of early biotech with its exaggerated hopes were to a large degree due to the ignorance of biotech managers on the strict rules and long timelines of drug research. New regulatory rules had to be set up of the new type of medicines, the biologicals [17]. Not surprisingly for experts, but surprisingly for biotech researchers, also they had to follow the regulatory system, which has been the stronghold of quality-based progress in modern drug therapy [18].

It is evident that any gene-based therapy, including therapeutic cancer vaccines and other agents addressing the immune system, has to be adjusted to the requirements of an individual patient and would not fit into the traditional business concept of pharmaceutical industry to sell medicines for an optimum number of patients [19]. Reducing a disease to one or a few mutated genes and adjusting the medicine to the requirements of a specific patient carrying these mutations means basically an individualizing approach. This intent to increase precision was the origin of what is now called “personalized medicine” [20]. Equally, the term “precision medicine” is in use to describe this new approach [21].

5.2 The Return of the Active Pharmaceutical Ingredients (APIs)

At the turn of the millennium, results from sales of biotech medicines still did not match the promises. Exaggerated hopes of biotech for paradigmatic changes in therapy had already narrowed down to realistic but very fruitful levels. Nevertheless, there was still an expectation in the biotech community that large but highly target-specific biomolecules might one day fully replace the traditional drugs, for which the name “small molecules” had been coined. The biologicals, a new class of medicines, had been established. Therapeutic humanized proteins, such as EPO, insulin, and many others, filled the market of medicines, all of which manufactured effectively by methods of recombinant biotechnology. Antibodies have become the most straightforward and successful therapeutic option in biomedicine [22, 23]. Today they are among the leading goals of drug discovery, designed as specific, bi-specific [24, 25], or even tri-specific antibodies [26–31]. Frequently they complement standard drug therapy, for example, in the case of trastuzumab (Herceptin) [32, 33], which in the meantime is part of the standard therapy against specific forms of breast cancer [34, 35].

From the point of history, an almost cynical situation arose, when a life-threatening disease appeared at the same time as the biotech boom came up. AIDS became the first great target and challenge of the biotech age. The problem was difficult above all, because, due to the instability of its genome, the AIDS virus was certainly a particularly unsuited target to be addressed by those immunotherapeutic approaches, which had been chosen to combat it [36]. There had been already a number of disappointing therapeutic approaches when Erik de Clercq found out in screening experiments that conventional active pharmaceutical ingredients (APIs) and drug candidates could block effectively the replication of the AIDS virus [37]. Combinations of “small molecules” attacking in parallel different molecular targets at the virus are the standard therapy nowadays [38]. This was probably the most relevant individual event to readjust thinking in pharmaceutical research and development (R&D) resulting in its present setting. Nevertheless, an ongoing shift of research funds continued in Europe away from established pharma research units and centers, which were reduced or closed down [39, 40].

De Clercq’s finding was certainly an important momentum to bring the “small molecules” back to attention. It contributed certainly also to the revival of a renewed drug discovery process building on the fundamentals of “classical” pharmaceutical R&D, but complementing it with new elements [18, 41]. Most important was the incredible increase in knowledge on physiological function and diseases, which was generated by the booming molecular biology and cell biology research. Recombinant test systems on cellular, tissue, and animal levels, highly specific assays, and biomarkers [42] were now complemented by the introduction of combinatorial techniques to synthesize test molecules to increase precision and speed of testing and hit finding tremendously. High-throughput screening (HTS) based on ligand binding assays [43, 44] became a new fashion [45, 46]. An ambitious new streamline of research was

established: finding of a potential target – validation of the target – finding a hit on the target – defining a site of attack at the target – selecting a lead compound to be optimized – selecting the candidate for clinical development – carrying out preclinical development – entering in the phases of clinical development – and finally reaching market authorization. Like the genome-based therapies, also this phenotypic approach aiming at high drug specificity was highly reductionist, this time on the drug design side. Broad classification of subtypes of receptors turned subtype specificity of a drug into a standard goal. Design specificity could be increased in an impressive way applying cloned receptor [47–49] for binding and functional studies and implementing *in silico* design [50]. Redefinition of classical diseases based on their genomic background has turned them into syndromes and created novel families of drugs [1, 51]. These are summarized by the World Health Organization (WHO) as “international classification of disease” [52]. Medical and molecular research drive innovation within this framework for healthcare. Nowadays drug research has grown into a new normality, in which – on the fundamentals of biomedical research – biotech pharma and traditional pharma are evolving in parallel, rather divided by their respective manufacturing technologies than by the conceptual understanding of the options to treat a disease in general.

In contrast to gene therapy with individual patients, options to market a drug, which is designed exactly to the requirements of an individual patient, are obviously limited in any case due to commercial reasons both with biologicals and APIs. The turnover for any product is determined by the number of selling transactions. This holds also true for the blockbuster status for a drug with a turnover of more than 1 billion USD, the dream of pharma managers [53]. Multiplying the price with the number of sold units is the decisive criterion in generating a turnover. The reduction of the number of potential customers by designing a medicine to the needs of one specific person can be only compensated by increasing the price. Public or individual financial affordability puts clear limits to this option. This situation has led to the new acronym of “nichebuster,” defining a medicine for a very small number of patients suffering from absolutely life-threatening genetic diseases [54, 55]. Extremely high prices – certainly also resulting from the generally high development cost for a new medicine – are paid for these drugs, making them profitable products. From an industrial point of view, a business concept based on the design of drugs for a few specific persons only cannot be valid. Nichebusters will have to remain a niche in the pharma sector, reserved for very rare life-threatening diseases [56–58]. Nevertheless, orphan regulations have been set up to allow, under cost-saving conditions, development of drugs for rare diseases, the number of which increased swiftly due to new molecular medicine-based classifications [59].

5.3 Systems Pharmacology

The millennium year 2000 marked a milestone also for science. The human genome project was completed in this year [60, 61]. The sequence of the genome is the ultimate reduction of a human being and was a truly important research

goal. However, being in the focus of public attention, the completion of this project fostered a lot of simple questions – comparably to other great projects such as the landing of man on the moon. There was no beneficial immediate consequence to be extracted from the knowledge of the gene sequence. Gene therapy was suffering from unpredictability. In drug therapy, the high precision did not yet work. Nucleic acid sequences had become important tools in research addressing gene sequences perfectly [62–64], but they were far from regular use in drug therapy [65], because they did not properly reach their target in the cell of a human body [66–71]. Although being announced for years to be the “next big thing to come,” and in spite of many efforts to optimize them by chemical modifications, nucleic acid drugs even nowadays do not yet meet all the requirements of bioavailability to make use of their huge potential as one of the most specific classes of APIs. Not even the reshaped pharmaceutical R&D strategy came up to the ambitious expectation [72, 73]. Frequently, the early steps went perfectly well providing an excellent drug candidate attacking at a highly specific site of a drug target. But there was the request that the target had to be new and the therapeutic effect had also to be of unprecedented quality. Unfortunately, it appeared more often than expected that an excellent drug candidate addressing a completely new target with high efficacy was associated with completely new side effects, thus bringing the project to an end [19, 74]. Drug safety became a critical issue [75–77]. At the latest then it became clear that in a living body, even the most specific drug will reach and address many other enzymes and receptors and will not reach its molecular target without an appropriate pharmacokinetic profile [18]. Failure in blocking specific receptors and pathways made clear that blocking any specific site in a physiological system may easily result in deviation of the “traffic” via alternative pathways, exactly as it happens with the car traffic in the streets of a large town. Even highest target specificity was obviously not enough to achieve the goal. Clever selection to block several pathways at the same time became the solution, leading to the rise of drug selectivity-based research concepts. Researchers were reminded on those comprehensive metabolic pathway maps, which had been set up more than 30 years earlier and could be found at the wall in almost every laboratory. Molecular biology had contributed in the meantime to a huge increase in complexity of these metabolic systems [78, 79]. Effective research involving big data numbers was now accessible by the use of large computing capacity. The opportunity to apply computer techniques beyond visualization of detailed molecular structures and interactions and also to manage the huge amount of data generated in the drug research process became essential in a new setting of biomedical research [80, 81]. Based on the results of reductionist molecular biology research, a new type of systems biology grew up, refocusing the attention to a comprehensive overall understanding of biological networks, their function, and – in biomedical research – their dysfunction. Systems pharmacology has evolved as the new hope [82–90].

5.4 The Patient in the Focus of Research

With the declining boom of the stock market-driven biotech industry and depleted pipelines of drug candidates with blockbuster status [91], toward the end of the last century, an implementation crisis in drug research was generally felt in “big pharma” and fostered new broad initiatives to combine forces between academic, industrial, and regulatory research to remove bottlenecks and speed up the timelines of drug development. Most prominent were the “Critical Path” Initiative (<https://c-path.org/>) of the FDA [92] and the “Innovative Medicines Initiative” (IMI, <http://www.imi.europa.eu/>), a public–private partnership (PPP) between the European Commission and the European Federation of Pharmaceutical Industry Associations (EFPIA) [93]. “Noncompetitive” research was funded with projects aiming at an improvement of the general research infrastructure and optimizing and speeding up the whole sequence of the pharmaceutical R&D process. In view of this optimization process, it became soon clear that there had been a strong bias in efforts focusing on early phases of discovery – such as target finding – which were many years away from the market and the patients, sometimes raising hopes of concerned patients with promises to be fulfilled realistically at the best a decade later. A new request was imposed that drug research projects should not only start up from basic findings, such as the function of a gene, but also should consider equally that patients need help here and now: “The medical need of patients has to be the driving force in drug research!” This message added a second meaning to the term “personalized medicine.” Now, it meant more than just genome-driven therapy or perfect phenotype-based design of drug candidates. The word itself indicated the general paradigm change in industrial pharmaceutical research shifting attention from the early stages of discovery to market and patient near activities. In view of the R&D process, it meant adaptation of the design of clinical studies to arrive at a higher precision by application of biomarkers, surrogate markers [94], or other novel research tools [49, 95–97], lowering at the same time cost driving factors, such as number of patients to be recruited. It also meant to reduce fantastic therapeutic options to an economically realistic level by considering the effectiveness of a drug [98–104], and it meant to observe systematically the long-term benefit of medicines by pharmacovigilance [74, 105–107]. Above all it meant that “the patient has to be in the focus of drug research!” Based on this statement, an observation at the bed of the patient is of equal relevance as a potential starting point for a research project, as a new finding in basic biomedical research.

5.5 Personalized Therapy

The term “medicine” has a double meaning. On the one hand, “medicine” means a formulated drug, while on the other hand, it stands for medicine as a discipline,

above all to connection with therapy. The term “personalized” is derived from the Latin word “persona,” which was originally used for the mask of an actor, by which his role was defined and through which he was speaking to the audience. Consequently, the use of the attribute “personalized” should be restricted to a human person and should – in the best case – include the personality. Since a drug cannot have a personality, it might be helpful to increase the precision of definitions by speaking rather about “individualized medicines” for highly profiled (individualized) properties to be particularly suited for a specific group of persons or even a single person. To avoid misunderstandings it seems advisable to use the term “individualized medicine” for a highly profiled medicine, while in view of patient-related aspects, the term “personalized therapy” might describe a therapy adjusted to the individual requirement of a specific person better than the term “personalized medicine.”

It is remarkable that most of all therapy in human history has been “personalized therapy,” because strictly speaking each therapeutic talk between the doctor and the patient is an act of personalized therapy. A medieval pharmacist preparing a specific medicine for a patient took also part in personalized therapy. In any case, each surgical intervention is an element of personalized therapy. However, this is not what nowadays is described as “personalized medicine.” The term was brought up as the big new goal of biotechnology. It roots in industrial research and was originally not within the scope of medical doctors in their practice and surgeons in hospitals. Thus, it is not surprising that patient-related aspects in “personalized medicine” have not yet been systematically and sufficiently considered in pharma R&D. Nevertheless, progress in individualizing drugs will automatically result in a convergence with personalized therapy. This will not only lead to new settings and research areas in drug research itself but will also require new business models of biomedical industry. Nowadays, drugs are manufactured in industrial plants and reach the patient via established distribution lines. Patients are treated – at least in nontrivial cases – in hospitals. The upcoming gene and immunotherapeutic avenues [4–16, 19] will require a new type of hospital, where patient treatment will be carried out within a sophisticated setting, in which on the one hand new diagnostic tools, above all biomarkers [108, 109], will specify the need of the patient [110] and on the other hand novel technologies will be applied to adjust the medication exactly to those needs under strictly controlled conditions of the “good practices.” This will provide the perfect frame for personalized therapies. In the following chapters, several – by no means all – aspects of important development lines both for “individualized medicines” and “personalized therapies” will be discussed.

5.6 Gene Therapy

The success of a drug depends above all on two factors: pharmacodynamics and pharmacokinetics. While the optimization of the pharmacodynamic effect of a drug at its target has found many approaches for implementation, pharmacokinetics, which is a much younger discipline of drug research, has been a traditional

source of failures in clinical studies. Although it is clear that a drug can exert its beneficial effect at the target only, if it can reach the target in the body, this requirement tends to be underestimated in its complexity, as well as those undesired effects, which a highly receptor-specific drug can have, if it exerts activity at an unforeseen site in the “universe” of a human body [111, 112], where an absolute specificity does not exist. The drawbacks that have hampered progress in gene therapy were not due to flaws of the principle, but were above all caused by pharmacokinetics. Transfer of genes or gene segments is far from trivial, particularly, if it does not happen in the controlled environment of a cell culture. Not surprisingly, viral infection [113–117] was identified to be a suited mechanism for gene transfer [118–122]. Equally not surprisingly, infections could not be fully controlled and did not only occur with the target cells [123]. Fatal incidences happened causing massive setbacks of gene therapy [124–127].

The frustration of gene therapists of not being able to transfer a therapeutic effect that had been proven in a cell culture to the organism of the patient fostered automatically concepts to carry out the therapeutic intervention outside the body and to replant the treated cells. Human cell culture provides a perfect setting to design a gene therapeutic medication. *Ex vivo* therapy allows to follow the result of the therapeutic intervention and to replant only those cells, which have been successfully transfected without sign of potential side effects. Techniques of cell transplant, above all stem cell transplant with leukemia patients, and even organ transplant, from the heart to the hands, had been successfully implemented before gene therapy came up and provide a solid repertoire to prevent cell rejections.

“Gene surgery” or “molecular surgery” is the final goal of gene therapy [4]. Introducing an additional gene into a cell via a virus is much less elegant than “curing” the gene itself. Cutting out a defect gene sequence and replacing it by the correct sequence would certainly be a method beyond serious ethical concerns, if carried out to prevent serious diseases and being safe by precise intervention at the target site. There is a long tradition of more than 25 years aiming at selective cleavage of gene sequences making use of stabilized hammerhead ribozymes [128–130] or man-made engineered zinc finger nucleases [131–133]. Sophisticated strategies are slowly overcoming the complexity of the task to design an applicable therapy. Regardless of the drawbacks of the first gene therapy approaches, *ex vivo* approaches with modification or selective editing of patient cells are growing. Thus, promising results were obtained in clinical studies [69], with deletion of CCR5 gene in T cells to confer HIV resistance, opening an approach additional to antiretroviral drug therapy (ADT) [134–137]. Just recently, the door for gene surgery has opened wide by the discovery and application of the stunning technique of CRISPR/Cas [138–146]. This technique allows a clean site-specific excision of a pathogenic gene sequence, followed by its replacement by the corrected segment. There is certainly still some way to go to arrive at a validated gene surgery method. With CRISPR the limiting factor at present is the small size of the insert used for gene editing and false hits based on off-targeting amplified genes causing genomic effects rather than target gene-specific proliferation inhibition [147–149]. In view of the progress in preimplantation diagnostics, reproductive medicine preimplantation gene

correction may be expected to be among the next things to come. In view of the precise interventions at the molecular level, it can be predicted that gene therapy, particularly nucleic acid therapy, will remain a big task and challenge both for biologists and medicinal chemists.

5.7 Regenerative Medicine

Cell culture is one of the key techniques applied in molecular biology. The tremendous progress in this field has been shifting biomedical research and pharmacological testing significantly away from classical molecular pharmacology and traditional animal pharmacology. Humanized test systems from cell to animal [150] in the best case, making use of cells of a specific patient nowadays provide an excellent basis for pharmacological studies, without extrapolation from nonliving to living or animal to human conditions. It has become the key tool in drug discovery as well as the product source in regenerative medicine.

In its broadest definition, regenerative medicine will range from the use of vitamins for body recreation at the one side to the transplant of whole organs at the other side. In the focus of regenerative medicine is above all tissue engineering [151–153]. There is a swift progress in technology of cell culture, including three-dimensional [154–159] approaches and advanced organoid cell culture [160]. Cells may be somatic cells, progenitor cells, (embryonic) stem cells, or germ cells. They may be genetically manipulated or not. Gene therapy makes up just a minor part of this rapidly growing field. A common feature of tissue engineering is the *ex vivo* intervention [69, 161, 162]. Also common is the close interaction of biologists, pharmacologists, technologists, and medical doctors in R&D and the requirement for settings to implement regenerative medicine in practice.

Like gene therapy, cell therapy – particularly immunotherapy to fight cancer – has been one of the great hallmarks [7] of the biotech age [6, 8–12, 14, 16, 19, 116, 163–168]. Cell therapies are not new in principle. Since the times of the discovery of the blood groups, it is known that the cell–cell recognition takes place with high specificity. Blood transfusion based on blood groups has been in use for more than a century. As with gene therapy, high specificity of recognition is a key feature of cell therapy, but in this case the immediate target is not located in the cell nucleus but at the surface of the cell. Most immunotherapeutic projects aim at boosting the human immune system to recognize and destroy a cancer cell. Like gene therapy also this approach has been connected to serious drawbacks but will in the long run be an important sector of personalized therapy [20, 134].

5.8 Individualized Medicines

At the latest, starting from the days of Paracelsus, who wanted to extract the “arcanum” – the “secret” – from the medical plants, it has been a major goal of drug

research to increase the efficacy of medicines. The wish for better drug molecules has remained unchanged until today; however the source driving these optimizations has changed over time. Toward the end of the nineteenth century, pharmaceutical industry grew out from the art of chemical synthesis; many classes of established drugs root in natural products, which were isolated and structurally optimized [169–171]. Natural product modification together with serendipitous observations from organic synthesis and pharmacological tests were the main driving force of progress until the middle of the twentieth century [172]. From the side of the patient, upcoming knowledge on physiology, metabolites, and hormones added a second discovery line of new drugs in the early twentieth century [171]. Particularly, the rise of biochemistry around the middle of the twentieth century shifted the focus of research from the active compound to the target [18]. From now on drugs were above all classified along their target and their function as agonist, antagonist, inverse agonist, or modulator. In the meanwhile, molecular biology and cell biology have enhanced options of testing and have added further qualities to pharmacology by receptor cloning and assay development.

In parallel to the rise of pharmacology, medicinal chemistry became increasingly effective in the second half of the twentieth century and led to many new classes of drugs for many different indications. In a competitive way, rules and design strategies were set up to optimize drug quality, resulting in “generations” of new drug families with steadily improved qualities. The concept of bioisosterism [173–178] – for example, that of benzene and thiophene – opened not just a way toward me-too drugs, but revealed that minor changes in structure or substitution of a compound were fundamentally decisive for the activity of a drug and for its specificity [173, 178]. A strictly defined interaction between drug and receptor was suspected to cause this specificity. Thus, increase of specificity of drug–receptor interaction by structural modulation of drug candidates became a priority in drug discovery. This was the starting point of what is nowadays called ligand-based drug design. Although at that time nothing was known about receptor structures, more and more pharmacologically relevant receptors were identified and classified. Subtyping of receptors (first by experimental pharmacology, later by gene analysis) followed and sharpened specificity even more [179, page 55]. Visualization of the structures of drugs, in the early phases making use of structural drug models (e.g., Dreiding models [180]), provoked the topic of “chiral drugs,” adding a further quality request to drug design, which is to avoid carrying, along with the API 50% of its weight as distomer, a useless burden to the body [181–183].

Nowadays, details of receptor binding sites can be visualized by means of *in silico* methods. Even more impressive, high performance methods of crystallography, NMR and cryo-EM provide detailed structures of receptors of increasing complexity, frequently even co-crystallizing a binding ligand demonstrating the situation at the binding site [184–190]. The pharmacological study of the four isomers of glycopyrrolate led to unexpected results, which were found to be due to differences in the kinetics of drug–target interaction of the isomers at the subtypes of the muscarinic receptors [191–193]. Tiotropium [185, 194, 195] became the first blockbuster of a new class of drugs, defined by a new quality

of pharmacokinetics, the receptor kinetics [186, 187]. Resulting from this, pharmacokinetics nowadays does not only mean just the distribution of a drug in the body, but includes the dynamic process at the receptor, by which each individual drug molecule approaches the receptor (on-kinetics), binds to it (drug residence), and finally leaves it (off-kinetics) [196, 197]. Since not only receptor action but also most metabolic processes in the body are governed rather by organic reaction kinetics than by reaction thermodynamics, it may be expected that a new physical chemistry-based strategy in drug discovery might evolve in the near future. Although strategies and methods to optimize drug specificity have reached impressive levels today, there is still room for further improvement, as can be seen by the variety of approaches, which are the core content of this book.

This chapter will not deepen further the topic of increasing drug specificity, but will just mention imatinib (Glivec, Gleevec), one of the first great achievements of “post-biotech pharma” [184, 198]. This tyrosine kinase inhibitor (TKI) is targeting BCR–ABL-positive leukemia by selectively inhibiting the kinase activity of the disease-causing fusion gene. It addresses directly only the disease-causing target and is therefore a truly individualized drug. Approved for chronic myelogenous leukemia (CML) [198], imatinib established a new class of drugs, the kinase inhibitors, which are important players in protein activation. With ATP being their natural ligand, kinase inhibitors had been considered for a long time to be “undruggable.” Designed in a way to inhibit specifically the function of mutated oncogenes only, they stand for a new quality in drug research [188, 199].

While target and cell specificity are well-identified goals of pharma research, the aspect of tissue and organ specificity has not yet reached the same level of quality. Of course, the importance of pharmacokinetics is known and plays its role in drug design, but traditionally this task has been left over to the pharmaceutical technologist, who had to find a formulation or drug carrier to get the drug to its target. Due to the usually late consideration of pharmacokinetics in drug discovery, there is still much open room to “ab ovo” design of APIs to act (almost) specifically only in the target tissue or organ. It may be assumed that pharmacokinetics will become a driver also in API discovery in the near future and will not remain almost exclusively an area of competence of formulation specialists.

5.9 Stratified Medicines

Biomedical research breaks down diseases from symptoms and established diagnosis to the cellular level [56]. A major result of biomedical research was the finding that many of the classical diseases are based on the dysfunction of different genes. For example, the abnormal excitation of cells, which leads to epilepsy, may be due to overactivity of excitatory ion channels as well as underactivity of “silencing” ion channels [200]. Equally inefficient decarboxylation of the excitatory amino acid glutamate may be a cause [201]. Based on this, epilepsy today is to be classified rather as a syndrome comprising a set of diseases of different origin. Mutations of genes coding for ion channels were identified in

patients suffering from epilepsy conferring a genetic aspect to the disease cause. Consequently, each of these epilepsies has a different optimal treatment. Obviously, all those clinical studies that had been carried out for nowadays generic antiepileptic drugs could not consider these newer results [202, 203]. Their results are heavily biased, because the number of nonresponders was probably depending rather on the diversity of the diseases of the recruited patients than on the efficacy of the tested drug. Molecular medicine-driven reclassification of diseases together with genome-driven stratification of medicines is progressing fast [204]. Drug candidates nowadays are highly specific for their molecular target. Of course, the resulting increase in the “number of diseases” has an impact on patient recruitment, for example, with some subforms of epilepsy being candidates for orphan registration. At the same time, lower numbers of patients bearing an exactly specified disease make it increasingly difficult to reach the blockbuster status with a drug [56].

In fact, an amazing situation is given, when highly specific drug candidates, which have been designed based on extensive pharmacological testing, reach the clinical phase of development to be tested on a high number of mostly male patients to exclude the risks that might be associated in testing a woman in reproductive age. However, male and female patients frequently do not react equally on a medicine. A “standard test human” does not exist.

The more detailed knowledge on diseases together with the increase in available patient data allows novel gene- and biomarker-based optimization of patient recruitment for clinical studies. Human diversity beyond the disease-causing genetic factors is vast, for example, gender and age. This opens the relevant development task to design stratified medicines suited for specific groups of population [205]. This can be achieved in rather straightforward manner by means of drug repurposing. Due to this, population-based stratification is an important issue and can be easily addressed. Nevertheless, the number of studies addressing either children or elderly persons is rather limited. Cost of clinical studies is high, even in the case of repurposing. Off-label use may take place easily. Payors of healthcare are not prone to allow increased prices for basically existing medicines, which were optimized to meet the needs of a subpart of the population. In view of the questionable effectiveness of a project of population-based stratification, a repurposing approach, which is important in view of patient needs, remains unsolved.

5.10 Drug Selectivity

Specificity of a drug means the property to select one single target out of a group of several targets, whereas selectivity means to select several targets out of a group [206–208]. This has been the most serious argument of traditional medicine (pharmacognosy), where the proven effect of a known active compound is complemented by beneficial activities of other components of the mixture [209, 210]. Similarly, the “dirty drug” concept claims that the interaction of a drug with more than one target may be desirable [211–214].

Although such concepts seem to be scientifically weak, they will retain their role in pharmacotherapy until they are replaced by more efficient development strategies.

Nowadays, bioanalytical methods have reached an impressive level, both with evaluating the metabolome and the proteome of a cell. The impact of a drug on the body down to the cellular level can be shown with high precision. Proteomics- and metabolomics-guided drug discovery will allow to determine the activity profile of a drug beyond the known target [111, 112]. Thus, differential proteomics revealed that unexpected off-target effects on proteins appeared, after applying oblimersen [215], a thioate-type nucleic acid drug, to cancer cells to silence the apoptosis regulator bcl-2, while such effects were absent when the same target was addressed via siRNA [216–226]. Addressing several targets at the same time to block effectively a patho-biochemical pathway is certainly a desirable property of a selective API. Optimized target selectivity will also be the strategic research goal of a novel cell function-based combination therapy, which is a more straightforward approach in comparison with the attempt to design multitarget activity into a single API [204].

5.11 Smart Innovation

Pharma industry is traditionally composed of two groups, namely, the researching pharma industry and the generic industry [227]. While the discovery of new drugs with high market potential and significant superiority over existing medication has become a risky task increasingly difficult to reach for the researching industry, the generic industry has a comparatively easy life, because also the best blockbuster will be generic after a decade [228]. The mentality there is purely commercial. To register a generic drug means at the same time to copy an outdated “model” without the slightest improvement, thus freezing technological development [72]. The concept of generics is anti-innovative, forcing at the same time research-based industry into increasingly demanding projects. Rethinking of the system of generic regulation seems to be adequate, following to some degree the regulatory frame of biologicals, where the development of biosimilars [228–230] needs careful investigation with additional clinical studies. A result of these regulations is “biobetters” [231, 232], biologicals with slightly improved properties. An initiative seems advisable to stop anti-innovative generic drug registration and to replace it by modified regulations for clinical development and registration of medicines from patent-free APIs, which favor minor improvements.

The progress in biomedical science is increasing knowledge on diseases very fast and opens an easier identification of suited targets for therapy. The concept of drug repurposing [233–235] roots as well in this option and in the fact that for many older drugs details of their pharmacological action are not known at the level of present understanding [210]. More than 3000 registered drugs [210] and tens of thousands of drug candidates or withdrawn drugs, respectively, are immediately available. Their further optimization might provide improved medicines

acting at known targets. In addition, pharmacological network-based strategies will support the design of new combinations of both established and new drugs based on molecular medicine-based testing. Imaging techniques can be used to shed a clear light on the distribution of a drug in the body. Optimizing of pharmacokinetics of established APIs to reach the desired target organ or tissue is a further field for a variety of smart complementary approaches for innovation in drug research.

5.12 Electronic Health

“Big data” has become a catchword and stands for the huge and increasing amount of potential information that is collected by means of computers [81, 236]. This applies also for the health sector [237]. Nowadays drug research is virtually impossible without the use of electronic data. In fact, drug research has become one of the drivers in the application of robotics in combination with artificial intelligence [49, 238–243]. Equally, efficient pharmacovigilance is to be built on information technology (IT) [244].

While IT-supported collection and management of knowledge is highly welcomed in handling of information related to medicines, the situation is much more delicate in view of handling data derived from patients [245]. The topic of electronic health records has become a heavily discussed political issue including aspects of ethics and potential abuse of data [246]. In any case, anamnestic and diagnostic data on a patient are generated in a steadily increasing amount, ranging from basic data of laboratory medicine to the sequence of the whole genome. Highly specific biomarkers are designed to support diagnosis and therapy [49]. Comparative analysis of patient data on regional or even global level becomes almost indispensable in view of rare diseases and will become an important trigger for drug research projects [247]. If properly used, electronic health will be of highest benefit for the health system in general and will become a fundament of personalized medicine. Respecting rights and dignity of patients will be a prerequisite to achieve this.

5.13 Doctor and Patient

For centuries, general practitioners have been the backbone of the whole health system. Traditionally they have been writing prescriptions for pharmacists to mix individual medicines for their patients. They treat both chronic and acute illnesses and provide preventive care and health education to patients. Their holistic approach goes beyond the mere treatment of a disease. They take into consideration also social and psychological factors. By this add-on their therapy becomes a truly personal therapy. Particularly in rural areas they are even indispensable elements of social life. Due to several circumstances their role in primary healthcare is questioned nowadays in many countries by upcoming primary healthcare centers [248–250]. The concept of such centers was originally

brought up to respond to the need of patients in regions with insufficient coverage of medical doctors. Apart from availability and cost factors, a main argument for their promotion is the importance of assessment of a steadily increasing number of diagnostic parameters. This argument does not hold against general practitioners, if personalized medicine is understood in its meaning of treating a person as perfectly as possible. The first appropriate step in treating a patient individually is not to assess his diagnostic parameters, but to discriminate whether the person is hypochondriac, whether psychological factors play a dominant role, or whether social factors might be involved [251]. Based on this analysis, a triage is to follow to decide whether it is a rather trivial disease to be treated directly, whether it is a disease that requires the intervention of a specialist, or whether the patient represents a special case. This is the most efficient and cost-saving approach. Of course, in times of molecular medicines with renewed understanding on diseases, with new classes or drugs and new therapies coming up, and with diagnostic tools ranging from gene analysis to IT-based health assessment, it is essential for a general practitioner to be able to handle also these tools appropriately. Thus, for the benefit of patients and the social system, it might be advisable to redesign and expand the professional qualification of general practitioners to evolve into “physicians for personalized medicine.”

5.14 The Competent Patient

Within a book on drug research, it might seem inappropriate to deal with the competence of patients. However, patients buy and use medicines. They are the final customers of pharmaceutical industry, and the customer should be satisfied [227]. Pharmaceutical industry is a highly ethical field of economics and is reaching a very high level of technology. Nevertheless, surveys on the image of industry reveal that pharma industry has almost the worst image of all [252–255]. This is a paradox. The negative opinion of many people on pharma industry is mainly due to a fundamental inability of fact-based judgment on medicines. This situation has even a negative impact on public health, because the mistrust is a door opener for useless so-called alternative medicines. Generally, there is a very low level of knowledge on diseases among the population. The knowledge on medicines is almost absent. School education usually does not take this into account [256]. Disease prevention by proper nutrition and lifestyle should not be only a political message, but is to be based on individual knowledge on physiology and disease. Therefore, the topic of “health literacy” is an essential element of personalized medicine [253]. Both school education and continued education on disease prevention and treatment are to be promoted. The result will be not only an increased health awareness and an improved level of discussions on diseases in social media but also a more precise and selective use of medicines, above all in the increasing segment of over-the-counter (OTC) medicines [257–259].

5.15 Conclusion

It is by no means the aim of this chapter to propose to abolish the term “personalized medicine.” However, in view of drug research, it is important to promote the awareness among the involved scientific community that a drug research project is not a one-way street from gene to patient, but can also start out from the need of patients. Adjusting the quality of a drug or a therapy exactly to the requirements of one specific person means at the same time merging the concepts of individualized drugs and personalized therapy. To achieve this goal, it is not only necessary to open minds but also to consider that new business models for pharma industry are to be found that make the admirable achievements of drug research affordable to the whole population.

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6

Drug Discovery Strategies for the Generation of Multitarget Ligands against Neglected Tropical Diseases

Annachiara Gandini, Federica Prati, Elisa Uliassi, and Maria L. Bolognesi

*Alma Mater Studiorum — University of Bologna, Department of Pharmacy and Biotechnology,
via Belmeloro 6, 40126 Bologna, Italy*

6.1 Introduction

Thanks to the pioneering work of first alchemists and then medicinal chemists, mankind has benefited enormously from the discovery of new drugs that can prolong life, improve the quality of life, or even both [1]. Today, dramatic increase in average life expectancy ranks as one of most notable societal achievements. Undoubtedly, innovative medicines have led to improved treatments of certain forms of breast cancer, hypertension, and AIDS.

As drug designers and discoverers, however, our work is far from over. There are many other diseases that remain incurable and still threaten humanity: cancer, neurodegenerative and infectious diseases, and other deadly conditions affect millions of people in developed and developing countries. In addition, a health-care system struggling with rising costs and gaps in quality calls for the discovery of new and improved medicines in terms of both efficacy and sustainability [2].

In the years, many changes are taking place in the way we discover new cures for currently incurable diseases. One of the concepts that has gradually evolved over time to encompass the new scientific advances is the one dealing with drug selectivity.

Basics of drug discovery teach us that the efficacy and safety profile of a drug is determined by its interactions with the molecular target(s) it encounters in its journey in the human body. Thus, the therapeutic action is the result of the interaction with the so-called on-targets, whereas the interaction of a drug with undesired proteins (off-targets) causes toxicity and adverse effects.

Accordingly, twentieth-century drug research aimed to discover drugs that could hit a single target thought to be fully responsible for the etiology of a given disease. Particularly in the case of G-protein-coupled receptors (GPCRs), there was a growing impetus to identify small molecules able to discriminate among the different receptor subtypes that were being pharmacologically characterized and validated as molecular targets. Similarly, target-based approaches had centered on exquisitely selective drugs able to recognize a certain enzyme against members of a homologous enzyme family [3].

This so-called “one-molecule–one-target” paradigm led to the discovery of many successful drugs and will likely lead to many more in the future.

However, the concept of selectivity applied to drugs over the years has acquired a broader meaning. From the frequent failures, we have learned that very often modulation of a single target can be insufficient to produce the desired therapeutic effect [4]. This is particularly true in the case of complex multifactorial neuropsychiatric conditions, or cancer, caused by a multitude of genetic and environmental factors acting in concert [5]. Along the same lines, the single-target strategy searching for new anti-infective drugs directed to single proteins essential for microorganism survival has appeared inherently inadequate [6]. The weakness in this approach is that a single amino acid mutation in the target protein is often enough to confer drug resistance [7]. At the same time, researchers have retrospectively found that many effective marketed drugs target multiple proteins simultaneously. The average target number for the drugs approved by the Food and Drug Administration (FDA) from 2000 to 2009 has been calculated to be 2.5 [8].

As a consequence, we and others have shifted our focus from the development of drugs selective for single molecular targets (“magic bullets”) to the development of selectively nonselective drugs (“magic shotguns”) able to simultaneously modulate multiple targets [9].

However, designing drugs with such a predefined multitarget profile is an intricate and exceedingly difficult task for medicinal chemists. In this chapter, by choosing the field of neglected tropical diseases (NTDs) as the background therapeutic area, we will describe our recent efforts to develop multitarget drugs for solving this vexing medical problem.

6.2 Drug Discovery for NTDs: The Past, the Present, and the Future

Initially, the term NTDs referred to diseases where there was little private investment in the development of new drugs and thus required public investment. However, in 2010 the *First World Health Organization (WHO) Report on Neglected Tropical Diseases* brought NTDs to the forefront. Under this umbrella, WHO identified 17 infectious diseases that affect over a billion of the poorest population of the world and pose a significant economic burden to developing economies [10]. NTDs are not only a plague for low- and middle-income countries [11]; leishmaniasis and Chagas’ disease have appeared in Europe [12] and the United States [13], although disproportionately afflicting the poor.

The WHO released a follow-up report in 2012, closely followed by the 2012 *London Declaration to control, eradicate, or eliminate by 2020 ten NTDs*.

Among the various NTDs, we have focused on three diseases most significant in terms of morbidity and mortality: human African trypanosomiasis (HAT), Chagas’ disease, and leishmaniasis. They are caused by parasitic protozoa of the family *Trypanosomatidae* (*Leishmania* and *Trypanosoma*) [14]. Sixty-nine million people in Africa are estimated at risk of HAT (or sleeping sickness),

a disease caused by two parasites of the genus *Trypanosoma*. *Trypanosoma brucei rhodesiense* is responsible for the chronic human trypanosomiasis, while *Trypanosoma brucei gambiense* for the acute form. Both cause debilitating illness and mental suffering and are fatal if left untreated. HAT has a tendency to occur in epidemics and in some areas during some periods of time has killed more people than AIDS [15].

In Central and South America, in 2010 5.7 million people were infected with Chagas' disease, a deadly disease caused by *Trypanosoma cruzi*. The chronic stage of the disease can last for years as parasites invade the internal organs (myocardium and intestine), causing irreversible damage. In many countries, it is still a severe cause of illness and premature death [16].

Leishmaniasis is caused by over 20 species of related intracellular parasites from the genus *Leishmania*. With clinical presentations that vary from asymptomatic infection to cutaneous, mucocutaneous, or visceral disease, it has widespread incidence, ranging from Mediterranean to tropical countries in four continents. More than 12 million people worldwide are infected with leishmaniasis [17]. The different forms can cause internal organ damage, skin ulcers, and disfiguration of the nose and mouth. Due to an increase in epidemics in countries such as India and Sudan and in coinfections with HIV, there is alarm at the sharp rise in visceral leishmaniasis (VL), the particularly serious and, without treatment, always fatal form of the disease [18].

The commonality of the three diseases is that they are in principle amenable to control by mass drug treatments [19]. However, treatment remains highly unsatisfactory. This is exemplified by three drugs currently in use (Figure 6.1). Introduced back in the 1940s, melarsoprol (**1**) is still the first-line treatment for HAT. As an organic arsenic derivative, it is highly toxic (5–10% mortality following treatment, mainly due to reactive encephalopathy) [20]. Nifurtimox (**2**) is considered by most experts as the mainstay treatment for Chagas' disease. It is cheap, easy to use, and less toxic than **1**, although it is badly tolerated [20]. Miltefosine (**3**), originally developed as an anticancer agent, has the merit of allowing oral administration. However, cure rates for the cutaneous form differ among *Leishmania* species and also between the same species from different endemic areas. More importantly, **3** is teratogenic and therefore strictly contraindicated in pregnancy, so effective contraception should be assured. The appearance of resistance is a further common drawback of all these molecules [20].

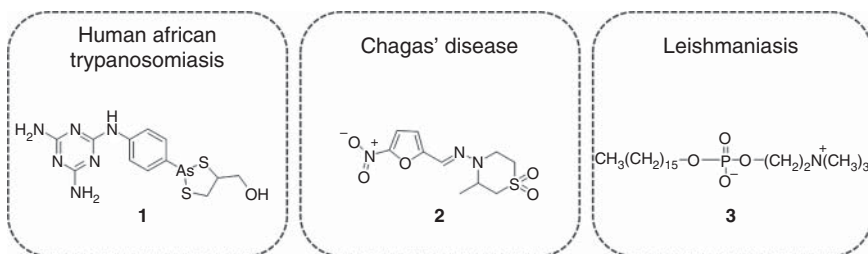


Figure 6.1 Chemical structures of anti-trypanosomatid drugs melarsoprol (**1**), nifurtimox (**2**), and miltefosine (**3**).

Against this backdrop, we envisaged a multitarget drug design strategy as a way to produce novel, effective, less toxic, and more cost-effective drugs [6, 20, 21]. A multitarget-directed ligand (MTDL) is a single chemical entity that simultaneously recognizes different targets inside the parasite. Thus, such a ligand (i) could be highly effective for treating the diseases, (ii) could reduce the development of resistance by the multiple targets, and (iii) could simplify the therapeutic regimen with positive consequences for patient adherence and caregiver compliance.

On these rational bases, we and other several groups [22] have been engaged in the design of multitarget drugs by exploiting, in most cases, a molecular hybridization approach.

It is worth mentioning that a numerous class of hybrid anti-trypanosomatid molecules is represented by the so-called targeted drugs, which combine a framework with antiparasitic activity with a fragment recognized by a parasite transporter [23].

In the following, we will report on the different approaches exploited by us to obtain innovative hybrid small molecules that, moving away from the conventional idea of “selectivity,” are able to address several antiparasitic targets and show an expanded activity profile.

6.3 Search for New Anti-Trypanosomatid MTDL Hits: A Phenotypic Approach

Modern drug discovery for NTDs is carried out using both target-based and phenotypic approaches. As very few molecular targets have been validated and considering that for many currently registered drugs the mechanism of action is likely to involve interaction with a number of different targets [24], a screening approach to determine whether compounds can stimulate any phenotypic changes in the parasite has been slightly preferred. Indeed, the screening of small molecules for phenotypic effects induced in “unbiased” complex living systems, which mimic aspects of disease biology or host disease relevant pathways, has gained momentum [24].

In the phenotype-based approach, hit molecules are obtained first, followed by target deconvolution to identify the molecular targets underlying the observed phenotypic effects [25]. This can be rather complicated, emerging as a drawback step of the approach.

In particular, the phenotypic screening has the advantage of identifying compounds directly active against the parasite, meaning that issues, such as low cell uptake and important cell efflux, have already been ruled out. Another positive feature is that usually parallel screens against mammalian cells are performed. This is fundamental to evaluate compound's selectivity, thus allowing the rapid filtering of the cytotoxic ones. In fact, achieving therapeutic selectivity has been one of the major obstacles in drug discovery. In particular, the main goal is to achieve a potent activity against the parasites, with no or minimal activity toward the host cells. Accordingly, the Special Programme for Research and Training in Tropical Diseases at the WHO (WHO/TDR) established that an anti-trypanosomatid hit

must possess a selectivity index (SI) >100, which is defined as the ratio of the compound's ED values on mammalian and parasites cells.

All in all, activity in phenotypic screening might be translated to human disease more efficiently than that in target-based screens.

When looking for new hit molecules to be tested in a phenotypic approach in NTDs, another important issue is the cost-effectiveness. Hence, as for the library synthesis, a strategy that can yield molecules with high hit rates and a concomitant reduced library size is highly desirable. In this context, the concept of natural product-derived compound libraries is particularly attractive [26]. This approach recognizes natural product frameworks as appropriate starting point, as they have been evolutionary selected and biologically pre-validated [27]. Additionally, natural products, due to their mode of generation, are naturally able to bind to multiple targets. This is because they have a complex chemical structure and their synthesis involves a range of enzymes, each of which has distinct architectures and molecule-binding cavities [28]. Thus, natural products interact with all of them during their biosynthesis. Finally, natural products are attractive also because they serve plants and animals as potent defense chemicals with an innate multitarget mechanism of action [29].

In light of this, a phenotypic workflow for discovering multitarget drugs may consist in the design and synthesis of natural-like compounds, in testing them in a whole-cell-based assays, and, finally, in angling potential targets by means of target deconvolution strategies [30].

In 2008, we reported a focused library built around the 1,4-naphthoquinone and 1,4-anthraquinone natural scaffolds, showing promising anti-trypanosomatid activities in a phenotypic screening [26]. Naphthoquinones and other related quinone-based compounds (such as plumbagin, aloe emodin, lapachol, and diospyrin) are one of the major natural product classes with significant activity against *Leishmania* and *Trypanosoma* (Figure 6.2) [31].

For instance, lapachol was reported to exhibit marked anti-trypanosomal and leishmanicidal activity while displaying no serious toxic effects in humans [32]. In view of the well-known biological properties of this class of compounds, it is highly possible that naphthoquinones exert their anti-trypanosomatid profile by means of a multitarget mechanism. Furthermore, it can be hypothesized that in addition to a possible target-related mechanism, the general free radical generation mechanism of quinones, probably also at the basis of their general cytotoxicity, may contribute to the multitarget profile of these molecules [33]. Indeed, it has been reported that parasitic protozoa are particularly sensitive to oxidative stress [34]. Based on the 1,4-naphthoquinone and 1,4-anthraquinone moieties, we synthesized 16 compounds that incorporated, at position 2, different aromatic groups, able to mimic a structural element of triclosan, which was previously demonstrated to kill both procyclic and bloodstream forms of *Trypanosoma brucei* (Figure 6.2) [35]. All the compounds were then tested against *T. brucei rhodesiense* (trypomastigote), *T. cruzi* (intracellular amastigote), and *Leishmania donovani* (axenic amastigote) and also their toxicity against mammalian L6 cells (rat skeletal myoblasts) was tested.

The lead of this series, 2-phenoxy-1,4-naphthoquinone (B6; **4** in Figure 6.3), showed an ED₅₀ of 80 nM against *T. brucei rhodesiense*. It also showed an SI of 74,

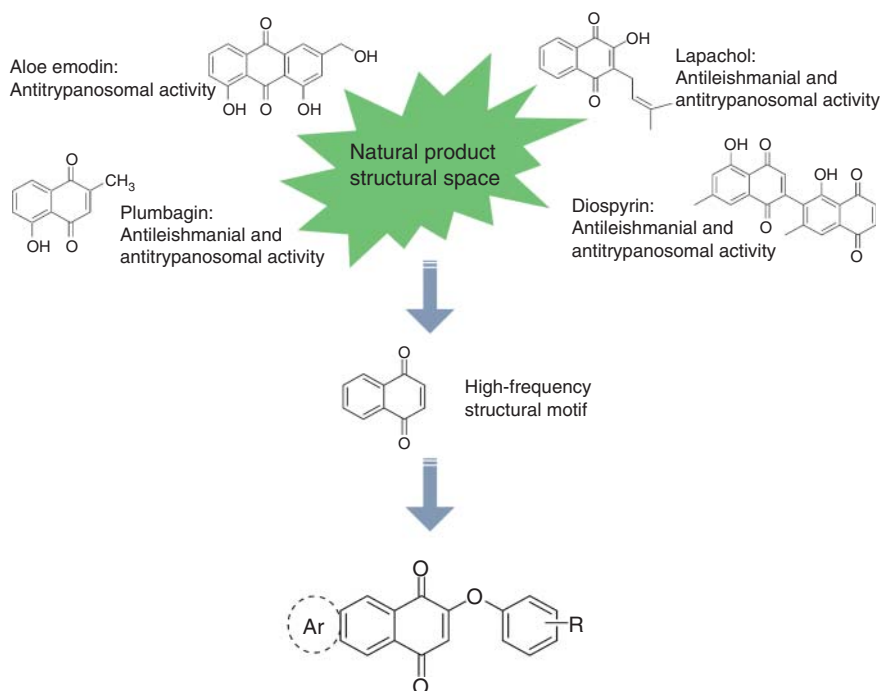


Figure 6.2 Design strategy toward the naphthoquinone- and anthraquinone-derived library.

which is very close to the specifications required for an anti-trypanosomatid hit. However, the target(s) responsible for the biological profile of this class of compounds has remained undisclosed.

Thus, by means of a chemical proteomics approach, we aimed at identifying the putative molecular target(s) of **4**. In particular, using its immobilized derivative (2-(4-aminophenoxy)naphthalene-1,4-dione), we isolated its targets from protein parasite extracts.

Two potential targets of **4** were identified, namely, glycosomal glycerol kinase (*TbGK*) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (*TbGAPDH*). In biochemical experiments, **4** inhibited both enzymes with IC_{50} values in the micromolar range (Figure 6.3). However, mathematical modeling shows that *TbGK* inhibition contributed to **4** trypanocidal activity only under low oxygen conditions. In fact, this enzyme under most physiological conditions does not play an essential role in trypanosome's metabolism and is thus considered as a suboptimal drug target. On the other hand, *TbGAPDH* is a vital parasitic enzyme and a well-validated molecular target for drug discovery [36]. Furthermore, evidence from several biochemical and computational experiments suggested for **4** a *TbGAPDH* covalent inhibition, probably through a cysteine trapping mechanism [30].

However, in order to fully account for the phenotypic nanomolar activity of **4**, we looked for other mechanisms of action. Indeed, as highlighted before, it is well known that quinones display a multitarget profile and can interact with the

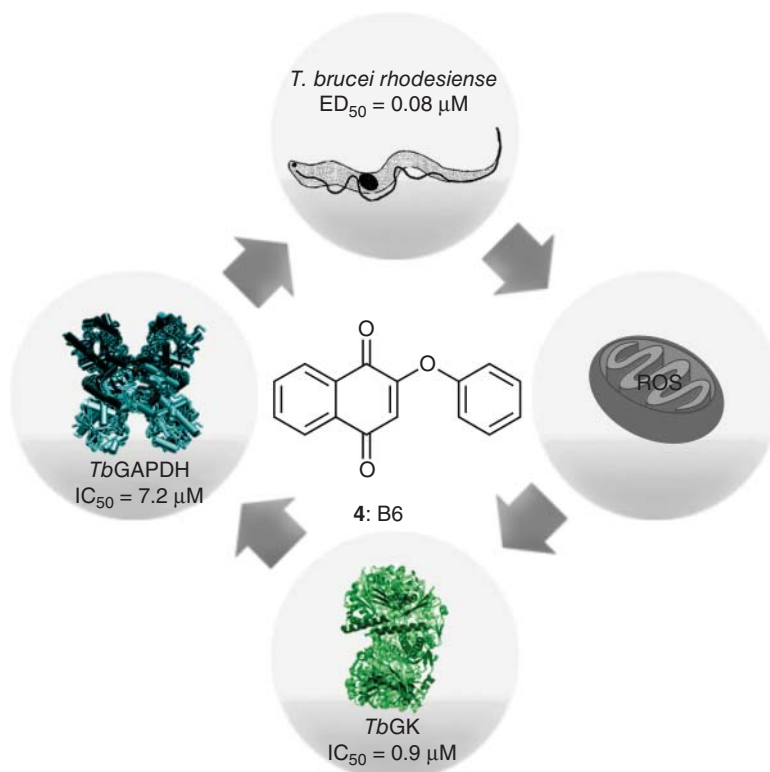


Figure 6.3 Chemical structure and multitarget profile of **4**.

mitochondrial respiratory chain [37]. Thus, we performed further experiments using trypanosomal isolated mitochondria and permeabilized trypanosomes. The obtained results supported the idea that **4** interacts with additional targets located in the *T. brucei*'s mitochondrion by generating reactive oxygen species (ROS).

In conclusion, we were able to demonstrate that the developed quinones can represent a promising class of natural-like multitarget compounds against *T. brucei*, with a concomitant glycosomal and mitochondrial mechanism of action. However, as already pointed out, the selectivity for parasite versus human enzymes must be considered as a key issue in the development of hit candidates against NTDs. Thus, the discovery of **4** was exploited as a starting point for the development of more selective compounds through a target-based approach.

6.4 Search for New Anti-Trypanosomatid MTDL Hits: A Target-Based Approach

As already highlighted, target-based approaches to NTDs are a widely used route for drug discovery programs aimed at identifying new lead compounds for such

diseases [38]. This approach has especially reinforced by the publication of the “TriTryp” (*T. brucei*, *T. cruzi*, and *L. major*) genome sequences that has revealed a high number of new kinetoplastid drug targets [39].

In this post-genomic era, multitarget-based design strategies have gained always wider acceptance. However, despite the high promise (see preceding text), these still represent a challenging task for medicinal chemists. This is because they must deal with two peculiar issues that are (i) the balance of affinities toward the multitarget proteins and (ii) the selectivity toward the corresponding human counterpart. Indeed, it is essential to establish a similar level of modulation for each target (i.e., affinity balance) in order to obtain the optimal efficacy and safety. This is based on the assumption that the same degree of *in vitro* activity against each target will presumably lead to similar levels of target modulation *in vivo* [40]. At the same time, the selectivity toward the human orthologs is another critical concern in designing multitarget compounds for NTDs. As already emphasized, for a therapeutic application, selective drugs specifically directed to the pathogen's targets without affecting the host's ones are crucially needed [41].

With this in mind, we will discuss our recent efforts toward the rational design of dual inhibitors of trypanosomatid enzymes [42].

As in any target-based approach, the first step is represented by the careful selection of the two targets (target A and target B) that ideally should be (i) pharmacologically validated and (ii) located on complementary parasite cell pathways. As for the first point, a validated antiparasitic target has to fulfill specific criteria that have been comprehensively reviewed by Gilbert [43]. Regarding target selection in relation to the desired synergistic effect, it should be emphasized that synergistic potentiation can be achieved only if the selected targets are located on functionally complementary pathways. Conversely, additive effects can be observed if targets are located on the same path. However, both cases (additive and synergistic) require lower drug doses, and therefore a better safety profile can be in principle achieved [44].

Then, the molecular frameworks (and the underlying pharmacophores) of single-target molecules responsible for binding to targets A and B must be identified and amalgamated in one hybrid compound through a framework combination approach. As depicted in Figure 6.4, the resulting hybrid molecules can be viewed as *linked*, *fused*, and *merged* derivatives depending upon the overlapping degree between the starting frameworks [6, 45]. In *linked* hybrids, the molecular frameworks are combined by a chemical linker that can be cleavable by metabolic enzymes releasing *in vivo* the two individual frameworks, or metabolically stable, leading to an indivisible molecule. When the two frameworks are connected without a linker, the hybrids are referred to *fused* compounds, whereas *merged* hybrids denote an overlap of a common chemical moiety present in both starting frameworks. Intuitively, increasing the degree of overlap of the two frameworks leads to the decrease of both molecular weight and structural complexity of the resulting hybrids.

On this basis, we focused on the search for dual *T. brucei* and *T. cruzi* GAPDH/trypanothione reductase (TR) inhibitors directed toward two enzymes that have been identified as validated targets for anti-trypanosomatid drug discovery [38, 46].

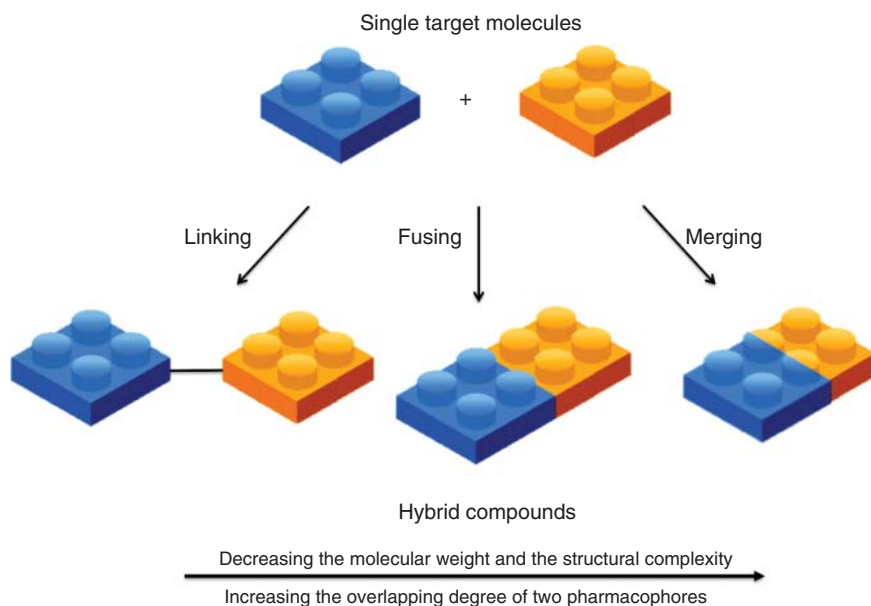


Figure 6.4 Framework combination approach toward hybrid compounds for NTDs.

In the case of GAPDH, its validation derives from the consideration that glycolysis represents the sole source of energy supply for the bloodstream form of *Trypanosoma* parasites [47]. Moreover, the unusual compartmentalization of this pathway inside the specific organelles named glycosomes [48] makes the development of selective GAPDH inhibitors (which should exhibit minimal or no affinity for the human counterpart) particularly attractive [49].

GAPDH is one of the key enzymes involved in glycolysis, which plays an essential role in the control of the glycolytic flux. Indeed, it catalyzes the oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. Structurally, GAPDH is a homotetramer of approximately 156 kDa constituted by two different domains: the catalytic site and the cofactor domain. The design of selective active site inhibitors is difficult because the active site of an enzyme is often well-conserved in the course of evolution. This is certainly true in the case of glycolytic enzymes [50]. In spite of that, the availability of crystallographic structures of *T. brucei* [51], *T. cruzi* [52], and also human GAPDH (hGAPDH) [53] allows to gain information about possible subtle structural differences to be used for the design of selective inhibitors. The two enzymes of *Trypanosomatidae* share 80–90% of identity and 45–48% with the human enzyme. Table 6.1 shows the main homologies and differences between the human and parasitic enzymes arising from alignment studies of primary sequences, as well as from computational studies aimed at determining the main physicochemical properties of the binding sites. Due to the high degree of homology of *T. brucei* with the human counterpart, the catalytic domains of the two orthologs look very similar with the catalytic residues (Cys and His) and the two anionic sites (named Ps and Pi) responsible for the phosphate recognition conserved in both enzymes.

Table 6.1 Homologies and differences between *Tb*GAPDH and *h*GAPDH.

Enzyme	<i>Tb</i> GAPDH	<i>h</i> GAPDH
Endogen ligand	Glyceraldehyde-3-phosphate	Glyceraldehyde-3-phosphate
Homologies and differences in the residues of the binding domains	NAD ⁺ site Gln91, Leu113, Pro190, Phe36, Ile37	NAD ⁺ site Asn95, Val116, Pro193, Phe37, Ile38
Overall structural homologies and differences	Catalytic site Cys166, His194, Arg249, Asp210	Catalytic site Cys165, His193, Arg247, Asp213
	Additional loop (residues 66–76) at the NAD ⁺ site	No additional loops
	Anionic sites (Ps and Pi)	Anionic sites (Ps and Pi)
	Additional lipophilic cleft (Pro190, Phe36, Ile37) at the interface between the catalytic and the NAD ⁺ sites	No additional lipophilic clefts

The cofactor domains, described by a Rossmann fold motif in both enzymes, differ only for the presence of an additional loop (residues 66–76) at the *Tb*GAPDH NAD⁺ binding site [51]. Furthermore, an additional lipophilic cleft present in *Tb*GAPDH but not in *h*GAPDH has been identified. This is described by a small pool of hydrophobic amino acids (Pro190, Phe36, Ile37) located at the interface between the catalytic and the NAD⁺ sites. This small cleft may be exploited in the rational design of selective inhibitor of trypanosomatidic GAPDH.

In the case of TR, validation builds on the fact that trypanosomatid protozoa possess a unique thiol metabolism, with metabolic peculiarity with respect to mammalian host [54].

An unusual low molecular weight thiol, namely, *N*¹,*N*⁸-bisglutathionyl-spermidine (or trypanothione), discovered as the major redox reactive metabolite in trypanosomatids [55], makes the enzymes involved in its metabolism as particularly appealing [56]. In this pathway, TR is responsible for reducing trypanothione disulfide (TS₂) to dihydrotrypanothione [T(SH)₂] and for maintaining a reducing intracellular milieu [57]. Albeit unique, TR resembles the human glutathione reductase (GR) for their similar physiological function and also in structure and mechanism [58]. Indeed, both enzymes are involved in scavenging free radicals and oxygen-reactive species [46a]. Being both NADPH-dependent flavin-containing disulfide oxidoreductases, TR and GR share about 30% sequence identity [59]. As depicted in Table 6.2, the key residues involved in catalysis (Cys–Cys–His) are conserved, as well as the FAD and NADPH binding sites. However, the critical observation that the endogen ligand of each enzyme shows significant differences in dimension, charge and lipophilicity, indicates that it should be possible to selectively inhibit the parasite TR and not the human GR. The specificity of the substrate is largely determined by five amino acids at the catalytic site (see Table 6.2 for details), which make the

Table 6.2 Homologies and differences between TR and GR.

Enzyme	TR	GR
Chemical features of endogen ligands	Trypanothione Net charge = +1 M.W. = 723.86 g/mol log <i>P</i> = −9.1	Glutathione Net charge = −2 M.W. = 307.32 g/mol log <i>P</i> = −4.5
Homologies and differences in the residues of the binding domains	NADP site Met333, Gly196, Arg221 Arg223, Phe199 FAD site No differences (residues 317–327) Catalytic site Cys53, Cys58, His461 Ala344, Glu19, Trp22, Ser110, Met114	NADP site Met330, Gly195, Arg223, Arg229, Tyr197 FAD site No differences (residues 321–329) Catalytic site Cys52, Cys58, His467 Arg347, Ala34, Arg38, Ile113, Asn117
Overall structural homologies and differences	Large catalytic site Additional lipophilic cleft (also called Z-site) at the catalytic site (Phe396, Pro398, Leu399)	Small catalytic site No additional lipophilic clefts

active site pocket of TR wider, more hydrophobic, and negatively charged than that of GR [60]. Moreover, an additional lipophilic pocket (named the Z-site) enclosed by Phe396, Pro398, and Leu399 at the catalytic site of TR has prompted the rational design of selective inhibitors [61].

As second step, we searched for molecular frameworks to be combined in the new hybrid GAPDH/TR inhibitors. In this respect, we considered the previously identified hit compound B6 (**4**) as a suitable one. In fact, it was shown to inhibit both enzymes in the micromolar range ($IC_{50} = 7.2$ and $9.0 \mu M$ for GAPDH and TR, respectively) [30, 62]. Despite that the activities were not spectacular, **4** can be actually considered a fragment, based on the criteria of the “rule of three” [63]. In fact, it shows a molecular weight of 250.2 Da, fewer than three hydrogen bond acceptors/donors, a *c log P* value of 2.92, a polar surface area (PSA) of $43.37 \text{ \AA}^2$, and ligand efficiencies for GAPDH and TR of 0.31 and 0.24, respectively. Thus, given the balanced activity of **4** toward both TR and GAPDH, we envisaged a target-based approach focused on the molecular framework of **4** and aimed to increase affinity and selectivity toward both targets while concomitantly maintaining balanced activities. By following the previously mentioned approach [44], the 2-phenoxyquinones **4** and **5** were combined, through merging and fusing strategies, with aminoalkoxy coumarin scaffolds (Figure 6.5). In details, the coumarin core of the well-known GAPDH inhibitor chalepin ($IC_{50} = 64 \mu M$) has been chosen to potentially improve the anti-GAPDH profile, while the amino tail, protonable at physiological pH, should allow a TR/GR selective recognition. A small library of eight quinone–coumarin hybrids was then characterized for their multitarget anti-trypanosomal profile, both in enzyme assays and in *in vitro* parasite cultures.

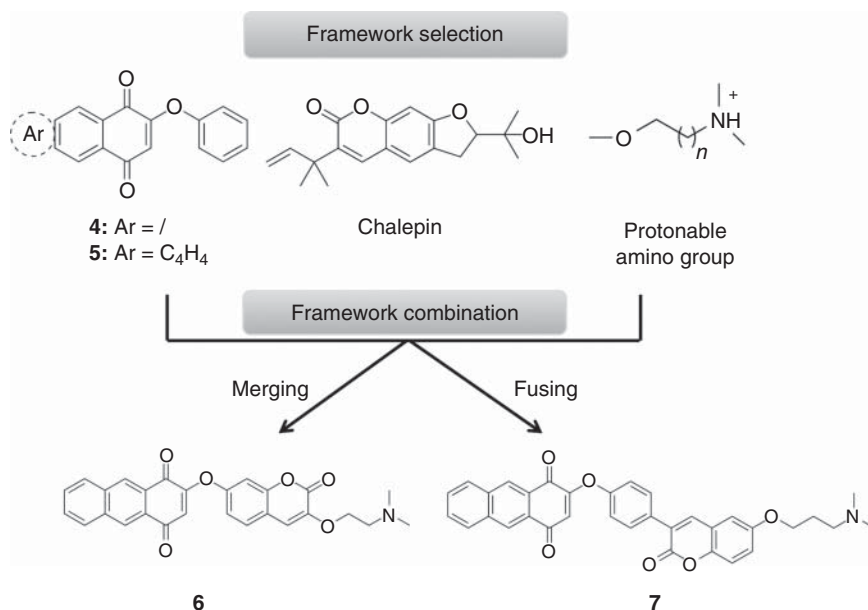


Figure 6.5 Design strategy toward quinone–coumarin hybrids **6** and **7**.

Among the merged derivatives, **6** showed the desired balanced profile with an IC₅₀ value of 5.4 μM against *Tb*GAPDH and a concomitant K_i of 2.3 μM against *Tc*TR. Thus, this molecule is the first rationally designed derivative reported showing an optimal dual activity against trypanosomatid enzymes. However, it should be pointed out that the activity of **6** against *T. brucei* parasites was lower (EC₅₀ = 0.14 μM) and not matching the enzymatic inhibitory potencies. This probably may be due to the involvement of other targets in the mechanism of action. Moreover, **6** resulted to be toxic against L6 cells, showing an SI toward *T. brucei* of 32. This might be related to the low TR/GR selectivity observed for **6**. In fact, despite the presence of the protonable amino group as a putative element of selectivity, we could not verify the desired profile. This was a common behavior for many of the synthesized hybrids, except **7**. Intriguingly, compound **7** belonging to the fused series showed only a slight inhibition of *h*GR at a concentration of 40 μM. Interestingly, compound **7** displayed a remarkable EC₅₀ value for *T. brucei* parasites (0.026 μM) combined with a very low cytotoxicity toward L6 cells (7.95 μM), resulting in an exceptional SI of 302. Collectively, these data are promising and warrant further investigation of this series against trypanosomiasis.

6.5 Search for New Anti-Trypanosomatid MTDL Hits: A Drug Targeting Approach

Drug targeting, which is the uptake and accumulation of a drug into a parasite via specific pathways, has been pointed out as an effective chemotherapeutic

approach to selectively modulate parasite drug targets with equally sensitive counterparts in the human host [23, 64].

It is well known that *Trypanosomatidae* depend on their hosts for several nutrients, such as glucose, purines, amino acids, vitamins, and lipids, essential for their replication and survival [65]. To acquire these substances, parasite cells express membrane transporters that mediate and regulate their intake [65]. In principle, these transporters can be used to selectively deliver toxic compounds inside the parasites, resulting in a selective toxicity mediated by specific transport systems [23, 64].

Fairlamb and coworker [66] first recognized the potential of the P2 purine transporter to carry drugs into the trypanosomes [67]. From these findings, membrane transporters have been targeted by several medicinal chemistry programs aiming at antiparasitic agents with improved activity and safety profiles [62, 68]. Although the results have not been always as expected, these investigations have revealed the largely untapped potential of targeting transporters in antiparasitic drug discovery. Additionally, along with the selective accumulation of drugs into parasite cells, the design and development of novel transporter-inhibiting compounds could also find therapeutic application [69]. Furthermore, from a chemical biology viewpoint, such compounds can be employed as chemical probes to investigate the role of transporters and their potential as drug targets in *Trypanosomatidae*. In this respect, transporter-directed chemical probes have led over the years to the identification of many specific membrane permeases and to the understanding of the underlying molecular transport mechanism [70]. In fact, in the absence of crystallographic and NMR structural data, chemical probing has mainly served for functional identification of targeted transport systems.

The concept of *targeted drugs* being able to accumulate selectively in the parasitic cells was first elaborated by Ehrlich to explain chemotherapeutics' mechanism of action [71]. From a drug design perspective, transporter-directed compounds are typically chemical conjugates obtained by linking two moieties: (i) a so-called haptophore, which is recognized by the transporter, and (ii) a fragment that may have antiparasitic activity (*toxophore*). Thus, in principle, conjugation of a transporter recognition motif to any toxic fragments has the potential to add specificity through selective uptake.

Based on these considerations, we have recently investigated the feasibility of targeting *Leishmania* amino acid transporters via appropriately designed chemical probes [72]. Again, **4** with an IC₅₀ value (1.26 μ M) against *Leishmania* axenic amastigotes only four time higher than the reference drug miltefosine (0.31 μ M) and an SI with respect to mammalian cells of 4.7 [26] was identified as a suitable starting point. We envisaged a "targeting" approach as a suitable strategy to generate novel molecules with such an improved profile.

Naphthoquinone analogs **8** and **9** with leishmanicidal and toxicity profiles similar to **4** but potentially better chemical and metabolic stability were selected as *toxophores* to be conjugated with the proper *haptophoric* amino acids (Figure 6.6) [72]. In fact, in *Leishmania* amino acid transporters play a major role in creating and maintaining intracellular pools of their corresponding substrates. Therefore, conjugating toxic framework to parasite nutrients that exploit specific amino acid

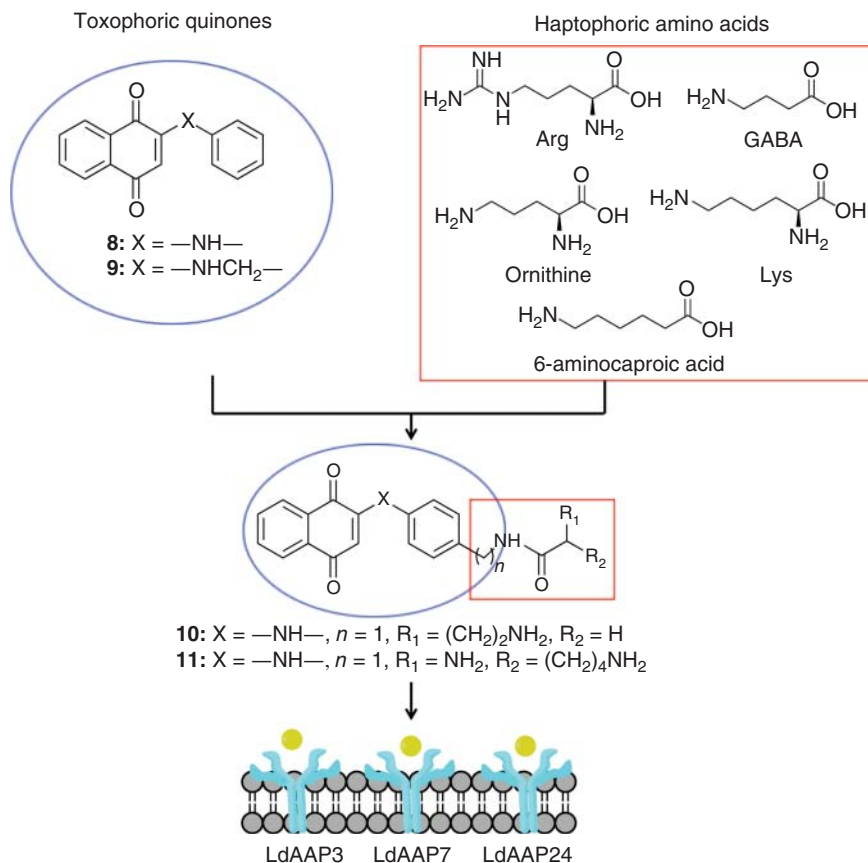


Figure 6.6 Design strategy toward targeted conjugates **10** and **11**.

transporters might result a successful approach for targeted toxicity. However, this applies only when the drug structural features responsible for the antiparasitic activity are preserved in the conjugation process. In our case, this was likely to occur, as the parent compounds and the conjugates shared the same quinone substructure.

L. donovani has specific arginine (LdAAP3), lysine (LdAAP7), and proline (LdAAP24) transporters that translocate the corresponding essential amino acids at high affinity and specificity [73].

Therefore, we envisaged to target LdAAP3, LdAAP7, and LdAAP24 with small molecules by (i) selective delivery of cytotoxic molecules into the parasite or (ii) by alteration of nutrient intake.

Accordingly, 15 conjugates were designed by linking **8** and **9** with several amino acid motifs (arginine, lysine, ornithine, 4-aminobutyric acid (GABA), and 6-aminocaproic acid) (Figure 6.6) [72].

The toxicity of the synthesized conjugates against *Leishmania* extracellular (promastigotes) and intracellular (amastigotes) forms was investigated, as well as the inhibition of the relevant amino acid transporters.

Some conjugates showed toxicity against the parasites; in particular, the GABA conjugate **10** was identified as the most potent derivative (at concentrations of 1 and 2.5 $\mu\text{g/mL}$ residual cell viability was reduced to 15% and 48% in promastigotes and amastigotes, respectively). Notably, the lysine conjugate **11**, while retaining the cytotoxic activity of **9** (at concentrations of 1 and 2.5 $\mu\text{g/mL}$ residual cell viability was reduced to 30% and 50% in promastigotes and amastigotes, respectively), displayed no toxicity against human monocytic THP1 cells.

Interestingly, transport assays indicated that some of the novel conjugates inhibited transport activity of lysine, arginine, and proline transporters. Furthermore, our analyses suggested that the active conjugates were translocated by the transporters into the cells, where they accumulated and the quinone fragment could exert its toxic activity.

Importantly, the lysine conjugate **11**, by exploiting the lysine amino acid transporter LdAAP7, could selectively accumulate and deliver its toxic effects to *Leishmania* cells with respect to the permissive host cell line and resulted as a potential hit for therapeutic application.

This work provided the first evidence that amino acid transporters of the human pathogen *Leishmania* can serve as drug delivery tools, might be modulated by small molecules, and warrant further investigation from drug discovery and chemical biology perspectives.

6.6 Search for New Anti-Trypanosomatid MTDL Hits: A Combined Target/Targeting Approach

From what we have discussed so far, we experienced that both multitarget and drug targeting strategies are viable when searching for small molecules displaying an expanded profile with respect to conventional single-target drugs. Building on the consideration that they are not mutually exclusive, in a further endeavor we exploited both drug design strategies. In particular, we reasoned that conjugation of quinones to a polyamine tail might effectively potentiate their initial antiparasitic profile [1]. With this in mind, we decided to synthesize a combinatorial library of quinone–polyamine conjugates, with eight derivatives bearing the natural polyamines spermine, spermidine, putrescine, and cadaverine, while other two compounds presented an unnatural tetramine, possibly acting as negative controls (Figure 6.7). The idea of conjugating a polyamine tail to the quinone moiety seemed very promising based on the following points: (i) numerous naturally and synthetic polyamines and their conjugates have been reported to be active against trypanosomatid parasites, (ii) enzymes involved in polyamine synthesis and metabolism are validated targets for drug development, (iii) the polyamine tail could exploit the parasite's polyamine transporters to improve the intracellular uptake of the conjugate, and (iv) polyamine conjugation may promote the accumulation of the molecule into the mitochondrion by increasing its cationic character [62].

After the synthesis, we evaluated the activity of the library against *T. brucei rhodesiense*, *T. cruzi*, and *L. donovani* in a phenotypic screening. Then, we assessed their mammalian toxicity using L6 cells [62].

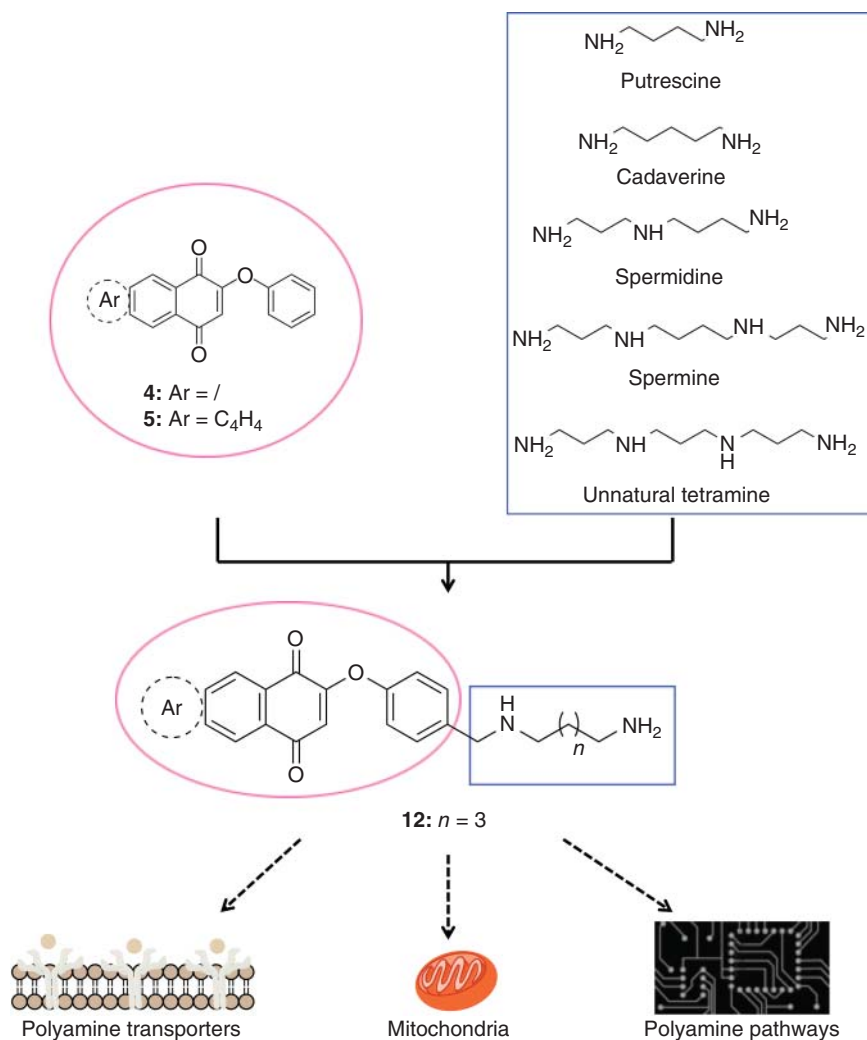


Figure 6.7 Design strategy toward quinone–polyamine conjugate **12**.

Among all the tested compounds, cadaverine derivative **12** exhibited the best IC₅₀ value against all the three parasite strains (*T. brucei rhodesiense* 0.07 μM; *T. cruzi* 17.43 μM; *L. donovani* amastigote stage 2.44 μM; *L. donovani* promastigote stage 3.62 μM). Moreover, some conjugates were selected to evaluate their activity toward *T. brucei* TR. They proved to be more effective inhibitors of TR than the unconjugated starting compounds **4** and **5**, supporting the hypothesis that the presence of a polyamine chain is critical for binding TR. Finally, we tested the effects of all conjugates on mitochondrial functionality, as for both the quinone moiety and the polyamine chain a peculiar mitochondrial tropism has been previously envisaged. The results showed that **12** caused a privileged mitochondrial damage, probably because of the presence of the polyamine

chain, through dissipation of the electrochemical gradient across the inner mitochondrial membrane (Figure 6.7) [62].

Notwithstanding these positive results, as a general trend, most of the new conjugates were less potent than the starting hits (**4** and **5**) against the parasites. This suggests that the introduction of a polyamine chain did not always lead to the expected improvement of the parasitic activity [62].

For instance, we cannot assume that the biological profile of a conjugate exactly corresponds to the sum of those of the starting fragments. This would happen only if the conjugate integrally preserve the initial fragment functions [74]. Otherwise, the combination will give rise to a completely new structure with novel chemical and physicochemical features and, thus, an individual biological profile. Thus, the conjugation approach proved not to be as successful as anticipated; however, the cadaverine derivative **12** seems very promising as hit compound toward *T. brucei*. We speculated that **12**'s improved antiparasitic activity may be due to a fine-tuning between hydrophobicity and charge, as the first one is required for the passive translocation across the membranes, whereas the second one is responsible for the mitochondrial localization and the interactions with intracellular targets.

6.7 Conclusions

Drug selectivity has been a pillar of drug discovery starting from the 1970s. Over the years, this concept has inspired the development of several blockbuster drugs directed against a single receptor or enzyme target (e.g., cimetidine and coxibs).

It would be naïve to say that these concepts are not valid anymore. Undoubtedly, selectivity remains the major focus when looking for chemical probes. These, alike to any other protein-targeted reagent, are only valuable if they, in addition to high affinity, have a proven selectivity. During the past decade, we have gained a greater appreciation that probes of this quality are difficult to generate and that many of the chemical probes available today have been proven to be nonselective and thus not useful [75].

Conversely, the concept of selectivity in drug discovery has changed, driven by the need of more effective and affordable medicines against the most complex diseases on the one hand and the high attrition of current drug discovery on the other. In principle, a molecule able to modulate more than one target has more chances for an effective therapeutic outcome. A field where such molecules seem particularly promising is that of NTDs. However, from what we have discussed in this chapter, it is clear that the discovery of multitarget compounds remains an exceedingly puzzling task.

How could we progress this promising concept forward? Certainly, the final goal is to develop a selectively nonselective small molecule that could be translated from bench to bedside. Most of the basic work in creating new strategies and frameworks for achieving high-quality candidates has been done; companies and nonprofit organizations must now take steps to successfully apply this to their discovery programs and ultimately win the battle against these fightable diseases.

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7

Designing Approaches to Multitarget Drugs

Luca Costantino¹ and Daniela Barlocco²

¹ University of Modena e Reggio Emilia, Dipartimento di Scienze della Vita, Via Campi 103, 41100 Modena, Italy

² University of Milano, Dipartimento di Scienze Farmaceutiche, Via L. Mangiagalli 25, 20133 Milano, Italy

7.1 Introduction

Nowadays it is known that many clinically available compounds, designed to act on a single target, act actually on multiple targets in order to exert their therapeutic or side effects [1]. At the same time, it has been discovered that several diseases (cancer, diabetes, neurodegenerative pathologies, etc.) have a multifactorial origin, and compounds designed to act on multiple targets (designed multitarget compounds) can represent a valuable alternative to the use of drug associations; some of these formulations are already present on the market [2]. The use of designed multitarget compounds can overcome concerns linked to pharmacokinetics or drug interactions; thus, there is a growing interest in the development of this class of compounds and, correspondingly, in the strategies for their design.

Several years ago, Muller proposed, in analogy with the lock and key model, in which a drug is able to fit selectively its receptor, the existence of master keys, privileged structures frequently employed in medicinal chemistry, as compounds able to hit targets from a gene family [3]; if a small molecule binds to a given protein, there are chances for it to bind also to other gene-family proteins. Structural bases for these so-called privileged structures have been found among class A G-protein coupled receptor (GPCR), where a subset of amino acid residues, highly conserved, are able to bind privileged structures [4, 5]. Even if this theory was formulated for the use of privileged structures as starting point for target-centric approach (selective compounds), a logical deduction is that it would be easy to find multiple ligands within a given protein family, following the strategies usually employed in the discovery of targeted compounds. As an example, the discovery of dual inhibitors of HIV protease and renin can be reported, two enzymes that belong to the family of aspartic proteases, a small group of proteins that share common features [6].

In addition, there are also examples of compounds acting at the same time on unrelated proteins. Screening showed that the protein tyrosine kinase inhibitors (TKIs) imatinib and nilotinib strongly inhibit several mammalian α -carbonic anhydrase (CA) isoforms, a family of metalloenzymes, even if they do not contain

moieties such as the sulfonamide group, normally associated with CA inhibition [7]. Moreover, chemical proteomics showed that these compounds inhibit also NAD(P)H:quinone oxidoreductase (NQO2 oxidoreductase) acting probably as FAD-competitive drugs [8]. Many other examples can be found [9] that represent the basis of the selective optimization of side activities (SOSA) [10] drug discovery process. Thus, it seems to be possible the design of multitarget drugs even for unrelated proteins.

Among the possible protein classifications available, protein classifications based on similarities of the putative ligand binding sites regardless of their phylogenetic relationship and on ligand similarities that reflect binding site similarities seem to be better suited than those based on the belonging to the same target family or class (e.g., GPCRs) or overall sequence similarity of the receptors (phylogenetic tree) [11, 12]. In fact, the overall sequence similarity does not need to correlate with the similarity in ligand binding; instead, the similarity of the binding site regions plays the key role [13]. Accordingly, Receveur *et al.* [14] identified a surprising binding site resemblance of the melanin-concentrating hormone receptor 1 (MCH1 receptor) to the phylogenetically unrelated D2/D3 receptors using physicochemical features of the residues in the putative ligand-binding site.

Three examples on the importance of binding site similarities among unrelated proteins can be reported. The discovery of the carbonic anhydrase inhibitory activity of celecoxib (a marketed COX-2 selective inhibitor) followed the assessment of the similarity in binding sites [15]; in this case the algorithm Cavbase was used to screen and match the protein binding pockets in terms of surface-exposed physicochemical properties. The activity of celecoxib against STAT-3, a member of the STAT protein family that acts as transcription activators, was disclosed applying an *in silico* strategy (multiple ligand simultaneous docking (MLSD)). After the creation of a library of fragments from a collection of small molecule STAT-3 SH2 inhibitors, MLSD of the privileged drug scaffold followed by fragment linking led to the discovery of a virtual template that, by means of a similarity search of virtual hits on drug database, identified celecoxib as STAT-3 inhibitor [16]. Another example of a significant cross-pharmacology relationship between distantly related gene families, such as aminergic GPCR and protein kinases, has been recently reported; virtual screening led to the discovery of the kinase inhibitor sorafenib as a nanomolar antagonist of 5-HT receptors [17].

As will be shown in this chapter, it is possible to design multitarget agents irrespective of binding site similarity on the basis of a target-based (based on the active site structures) or ligand-based processes (the use of pharmacophores; compounds able to interact with a single target must be known); an example of the design strategy will be given for each case, with a view of drug-like properties that have been obtained for the final compounds.

All these strategies are based on the prior knowledge of the targets that have to be modulated in order to obtain the desired pharmacological effect. Recently, a new approach is emerging, based on modern phenotypic assays, that allows at the same time the discovery/optimization of multitarget drugs and target selection, a process reminiscent to *in vivo* assays that led to many clinically useful drugs in the past; a separate section has been reserved for this approach.

7.2 Target-Based Approaches for Multitarget Drug Design

7.2.1 Designing Approaches for Structurally Related Targets

7.2.1.1 Fragment-Based Approach

The lead generation based on fragments relies on the hypothesis that compounds provided with a molecular weight lower than that of drug-like molecules (usually around 300 Da) called fragments can be screened, which, even if they are able to bind to a target weakly, fit well into the binding pockets. These compounds can subsequently be elaborated, on the basis of structural information about the target, through X-ray crystallography into lead series. Many drug candidates that originated from fragment-based lead discovery are in the clinic; thus, this strategy seems to be a useful drug discovery approach [18]. Since, in general, fragments are less selective than the elaborated compounds [19, 20] and are able to interact with multiple unrelated protein environments [20], this strategy can be applied also in the field of multitarget drug discovery.

Interestingly, although selective ligands are believed to be more complex in terms of pharmacophore features and molecular shape with respect to fragments [20, 21], the results obtained in a fragment library screening that led to the discovery of dual histamine H₄/5-HT_{3A} receptors (a GPCR and an ion channel, respectively) [22] showed that there is indeed a delicate balance between ligand complexity (a descriptor of the size and shape of the scaffold, taking into account the smallest set of smallest rings, the number of heavy atoms, the number of bonds between the heavy atoms, and the sum of heavy atoms' atomic number) and target selectivity; multitarget fragments should possess complexity that is high enough to hit several targets (in this case H₄R and 5-HT_{3A}R), but low enough to be too specific for a single target. Moreover, high complexities of the discovered dual-acting fragments indicate not only a similarity in the recognition profiles of these receptors but also differences between the properties of selective receptor ligands [22].

Plexxikon adopted a fragment-based drug discovery strategy (the scaffold-based drug discovery approach) for the design of multitarget drugs (kinase inhibitors and proliferator-activated receptor (PPAR) activators) that represents an evolution of the procedure followed for the discovery of a family of selective phosphodiesterase (PDE) inhibitors.

Cyclic nucleotide PDEs, enzymes that hydrolyze cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are classified into 11 families; in particular, PDE_{4B} subfamily members are involved in the inflammatory responses of lymphocytes. In this case, a family of potent PDE₄ inhibitors (the compounds do not discriminate between the two isoforms tested, PDE_{4B} and PDE_{4D}) was discovered using the scaffold-based drug design. The process started with the design of a library of low molecular weight compounds, aimed at finding new scaffolds following an *in vitro* assay; this screening process was conducted against the target protein as well as several other members in the protein family. Only compounds that inhibit multiple members of the target family have been selected for the next steps. The scaffold thus obtained (compound (1), Figure 7.1)

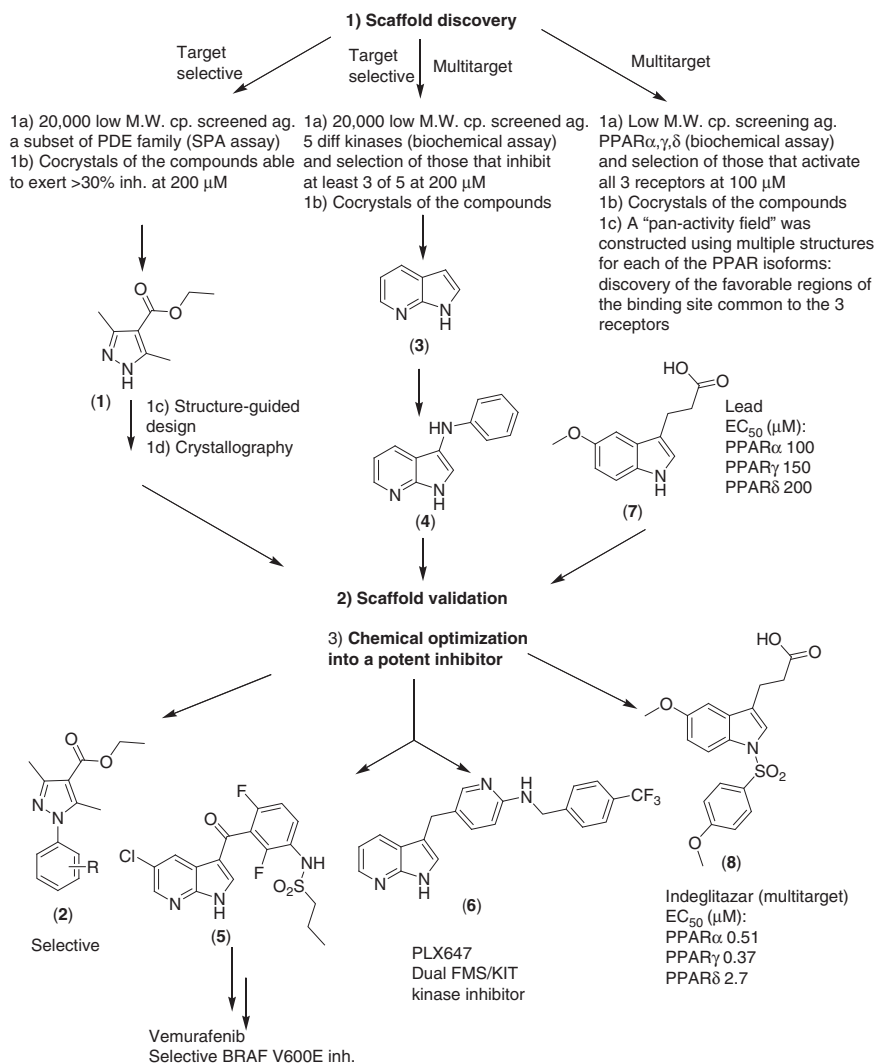


Figure 7.1 Fragment (scaffold)-based drug discovery at Plexxikon.

were subsequently developed into more potent and selective inhibitors by an iterative process involving cocrystallography of the compounds with the enzyme, followed by structure-based drug design (compound (2), Figure 7.1). Very important in this strategy is the control of the presence of a conserved binding mode during scaffold evolution; in the scaffold validation step, it was checked by cocrystallography if the binding mode of the compound was retained with respect to the scaffold and substitutions on it were allowed; only in this case the scaffold is considered as validated. In other words, a scaffold was considered validated only when the conserved portion of the derivatives make the same interactions with the target protein as the scaffold itself [23]. This drug discovery strategy was

then applied to different protein families to discover kinase inhibitors and PPAR activators for the treatment of cancer and type 2 diabetes, respectively.

Kinases are a family of enzymes that catalyze the transfer of a phosphate from donor molecule, ATP, to their specific substrates; this class of enzymes is actively studied for the development of antitumoral compounds. Due to the sequence and structural similarity of these enzymes in the kinase domain and in particular in the ATP binding site, it is difficult to obtain selective inhibitors. Even STI-571 (Gleevec), the first kinase inhibitor that entered the market, was believed to be selective, showing ability to inhibit other kinases during a kinase profiling assay. This finding led to its exploitation in different cancer types, showing clinical activity against malignancies in which the kinases affected by STI-571 were involved.

Then, it was discovered that other clinically successful kinase inhibitor drugs are not selective as previously thought and that, at the same time, a proper balance of multikinase inhibition is required for an optimal antitumoral activity. Obviously, target selection should be performed before the drug design process. Very often, however, the multikinase inhibitory profile has been performed only after the *in vivo* optimization of the compounds [24].

Multikinase profiling of a kinase fragment set of compounds was performed in order to see if selectivity can be linked to specific fragments that address this class of enzymes. While some of the fragments considered inhibit numerous kinases, others are selective, but selective lead-sized compounds can be obtained from unselective fragments, and unselective lead-like compounds are frequently based on selective fragments [25]; thus, it seems that the property of selectivity does not need to be maintained between fragments and their related lead-sized molecules.

In the case of Plexxikon kinase inhibitors, a library of low molecular weight compounds (150–350 Da) was initially screened against a panel of structurally related targets in a biochemical assay in order to select weakly active ligands as hits for antitumoral compounds; 7-azaindole scaffold (compound (3), Figure 7.1) was identified among 20,000 through a low-affinity, low-selectivity, low molecular weight screen against multiple kinases. This compound showed multiple binding poses when it was crystallized with PIM kinase, but it assumed a single binding mode once substituted at 3-position (compound (4), Figure 7.1). This fragment then represented the starting point for the development of selective v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutant V600E (the oncogenic form of the protein) (compound (5), Figure 7.1) and also for dual FMS/KIT kinase inhibitors (compound (6), Figure 7.1) [26–28].

The development of these compounds was guided by cocrystallography; in particular, a step of scaffold validation was performed during this design process in order to assess if the derivatives are able to give interactions similar to those of the selected scaffolds with the target protein. Afterward, structure-based chemical optimization leads to optimized compounds. Vemurafenib was approved by FDA in 2011 (it was the first drug discovered by means of fragment-based drug design), and a dual inhibitor (PLX-647, (compound (6), Figure 7.1)) was obtained starting with the same scaffold [26, 27].

Another field of research at Plexxikon for multitarget agents deals with PPAR receptor activators. PPAR α and PPAR γ activators are on the market as lipid-lowering agents (fibrates) and insulin-sensitizing drugs (glitazones),

respectively, while PPAR δ activators are in preclinical research for the treatment of dyslipidemia. Thus, on these bases, agents able to activate all the three receptor subtypes have been suggested as a valuable treatment for type 2 diabetes.

These receptor subtypes possess binding sites that show remarkable similarities, and pan-receptor activity cliffs were found recently *in silico* by means of structure–activity relationship (SAR) “activity landscape modeling” of 168 known PPAR agonists [29], suggesting the possibility to develop easily pan-agonists. The strategy followed by Plexxikon for the development of the multitarget indeglitazar was based on fragment biochemical screening. Active hits were then cocrystallized; at the same time, a blueprint of the binding site compatible with pan-activity was constructed computationally. The initial scaffolds were then evaluated for their ability to interact *in silico* with the energetically favorable regions of the binding site that are common to the three receptors. Compound (7) (Figure 7.1) was selected as a lead and optimized to give compound (8) on the basis of its crystal structure with the ligand-binding domain of PPAR γ and superimposition on the pan-activity field. Indeglitazar (compound (8), Figure 7.1) entered phase 2 clinical trials [30].

Some other compounds in clinical trials (chiglitazar, aleglitazar), possessing the same multitarget characteristics, have been discovered during activity profiling of optimized PPAR γ activators. The only pan-agonist present on the market is bezafibrate, introduced in 1997, which simulates all of the three PPARs with the same efficacy and shows a good safety profile [31]. Interestingly, bezafibrate was developed as a hypolipidemic agent through the optimization of its lipid-lowering activity in rodents before the discovery of the PPAR receptors, underlining the importance of phenotypic assays in drug discovery.

7.2.2 Designing Approaches for Structurally Unrelated Targets

7.2.2.1 Crystallography/SAR

Phosphoinositide 3-kinase (PI3K)/tyrosine kinases dual inhibitors were obtained by means of crystallography/SAR.

Tyrosine kinases promote cell growth and proliferation and are validated targets for antitumoral compounds. At the same time, most parts of these kinases activate PI3K, and reactivation of PI3K signaling is a common mechanism of resistance to TKIs. Accordingly, the combination of inhibitors of these two families of proteins showed efficacy in preclinical studies.

Protein kinases and PI3K, on the other hand, diverged early in evolution and therefore lack significant sequence similarity; they, however, share several short motifs, the architecture of their kinase domains and a set of residues involved in catalysis. This situation is mirrored by the limited overlap among known inhibitors of protein kinases and PI3K, making it difficult to design dual inhibitors. This problem was faced by screening a library of TKIs for activity against the catalytic subunit of PI3K, p110 α , leading to compounds (compounds (9) and (10), Figure 7.2) provided with a pyrazolopyrimidine scaffold, a well-characterized nucleus for Tyr kinase inhibition [32–34]. The two leads thus obtained were crystallized with human p110 γ , an isoform available among the highly homologous p110 subunits, and profiled against a panel of

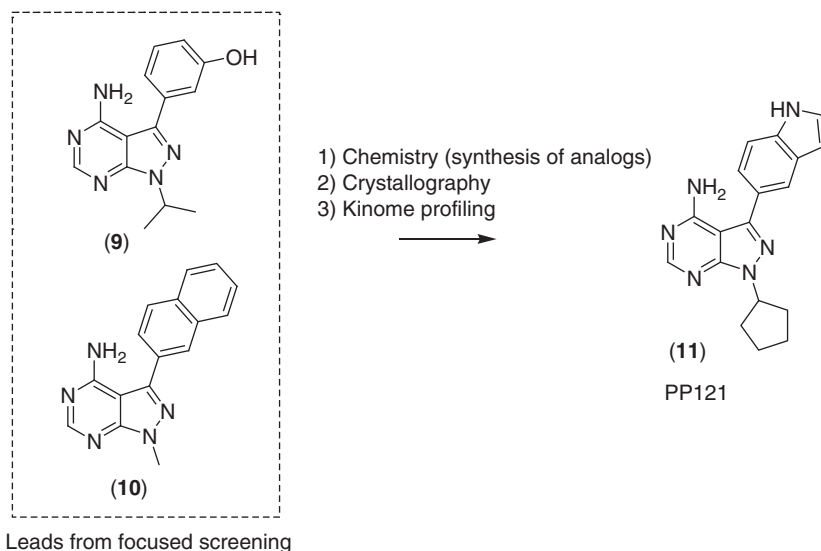


Figure 7.2 Development of dual PI3K/Tyr kinase inhibitors.

kinases, giving the starting point for the iterative synthesis of analogs, designed on the basis of SAR against a panel of 14 tyrosine kinases and PI3K.

Structural basis of the dual inhibition exerted by the drug-like, dual-target compound PP121 thus obtained (compound (11), Figure 7.2), active *in vitro* and also on tumor cells, which targets the conserved catalytic residues, has been established by solving the structure of its complex with the tyrosine kinase c-Src and comparing it with that hypothesized in the presence of p110 γ [35].

Fusion of compounds able to interact with different targets was applied also in the discovery of ligands targeting different regions of the binding site of the same protein. As an example, the structure-based discovery of a type II inhibitor of the kinase c-Src (that binds to both the allosteric and the ATP binding site, compound (14), Figure 7.3) by fusion of a pyrazoleurea scaffold (type III, allosteric inhibitor, compound (13), Figure 7.3) with a quinazoline compound (type I, ATP-competitive inhibitor, compound (12), Figure 7.3) is reported here. The compounds, designed by the superposition of the crystal structures of the compounds bound to the protein, were optimized for mutated c-SRC-T338M and, further, for gatekeeper drug-resistant mutations in Kit-T670I and Abl-T315I [36, 37].

7.2.2.2 Molecular Docking/Pharmacophore Matching

Molecular docking is a virtual screening method that uses geometrical matching to dock small molecules to the target site's 3D structure. Molecular docking and pharmacophore matching methods have been used for the identification of dual inhibitors of two targets involved in inflammation: phospholipase A2 (PLA2) that hydrolyzes membrane phospholipids to release arachidonic acid, a precursor of proinflammatory eicosanoids, and leukotriene A4 hydrolase

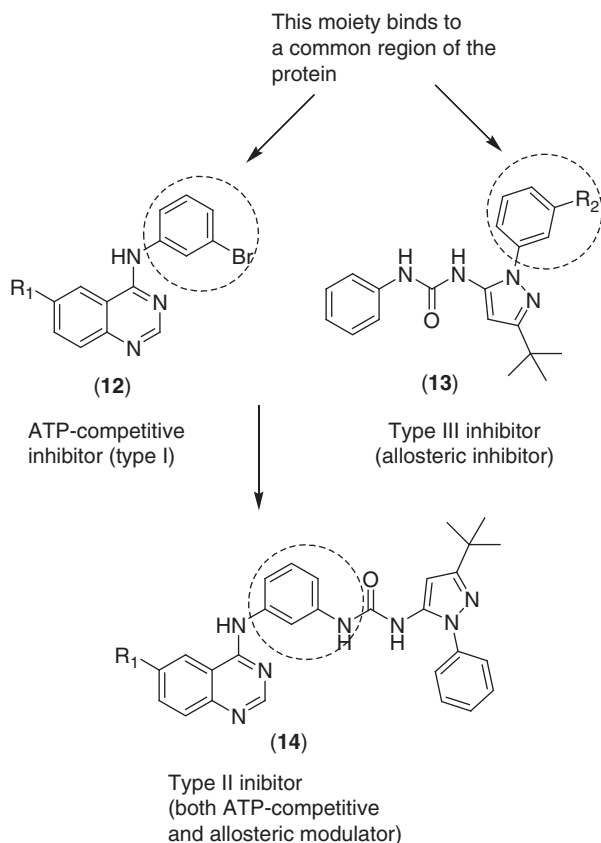


Figure 7.3 Design of type II hybrid inhibitors for c-Src kinase.

(LTA4H) that catalyzes the hydrolysis of the epoxide LTA4 to generate the leukotriene B4 involved in inflammation [38].

The selection of these two targets for the development of dual-acting anti-inflammatory compounds was guided by systems biology; the computational model for the arachidonic acid metabolic network and its dynamic properties designed the two targets, PLA2 and LTA4H, as therapeutically relevant for the development of anti-inflammatory compounds [39]. The strategy started with the *in silico* generation of a pharmacophore model based on the protein structures; then, compounds were docked on common pharmacophores in order to select the most suitable candidates for tests (compounds (15) and (17), Figure 7.4) [38]. A subsequent optimization step led to other compounds (compound (18), Figure 7.4) [40]. Notable is the close similarity of the compound (15) obtained following this procedure with respect to the compound SC-22716 (16) (Figure 7.4), disclosed by Monsanto in 2000 as LTA4H inhibitor by screening; activity on PLA2 was not determined at that time [41].

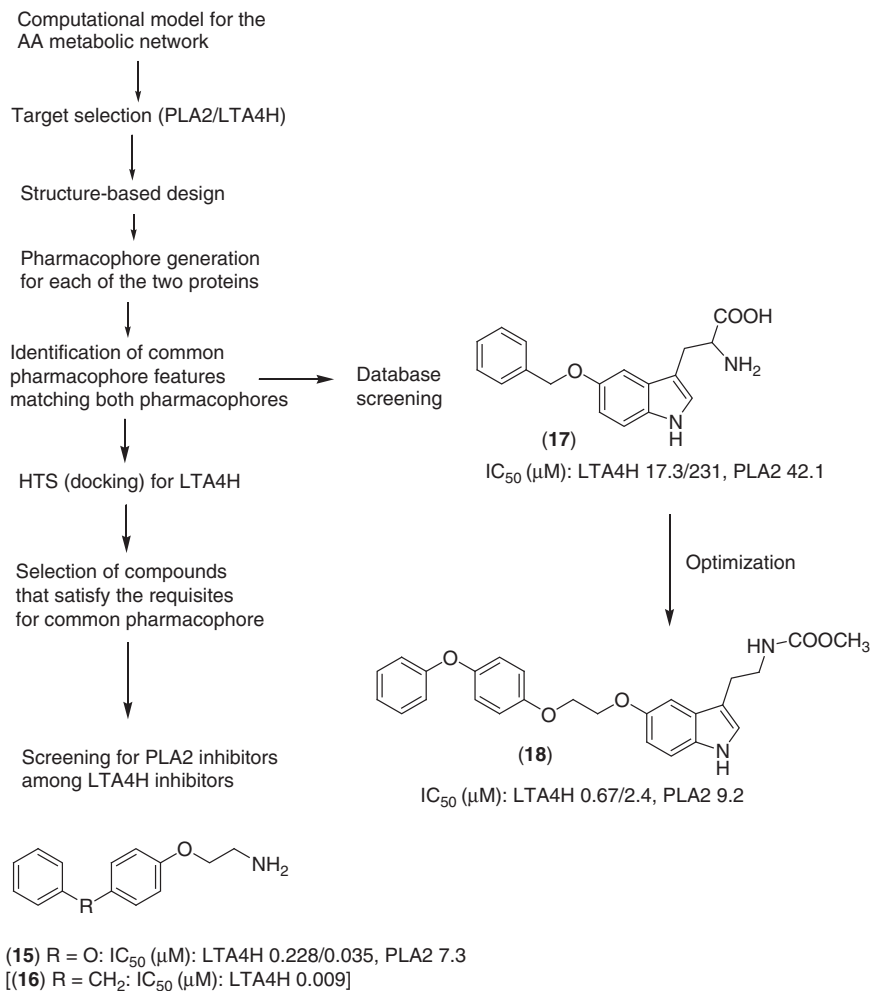


Figure 7.4 Multitarget anti-inflammatory drugs. LTA4H possesses both intrinsic aminopeptidase activity and epoxide hydrolase activity; the inhibition values for the two activities were reported, respectively.

Another *in silico* procedure, based on docking into the structure of fatty acid amide hydrolase (FAAH, an intracellular serine hydrolase that hydrolyzes a family of naturally occurring ethanolamides, such as anandamide, an endogenous cannabinoid agonist) of COX inhibitors, led to the selection of carprofene as a dual-acting compound (mixed FAAH/COX inhibitor). In this case, the selection of the targets to be affected was performed on the basis of *in vivo* studies that indicated that the simultaneous inhibition of COX and FAAH activities by means of selective inhibitors produced superadditive pharmacological effects and lowered toxicity in animal models of the disease [42].

7.3 Ligand-Based Approaches for Multitarget Drug Design

7.3.1 Designing Approaches for Structurally Related Targets

7.3.1.1 Fragment-Based Approach

A fragment-based drug discovery strategy was used in order to design the multikinase inhibitor sorafenib (compound **(21)**, Figure 7.5). High-throughput screening (HTS) resulted in a fragment-like hit (compound **(19)**, Figure 7.5) that was subsequently optimized by means of biochemical assays as cRaf1 inhibitor [43, 44]. A subsequent biochemical profiling showed that the compound suppresses both wild-type and V599E mutant BRAF activities *in vitro* and demonstrated significant activity against several receptor tyrosine kinases involved in neovascularization and tumor progression [45]. This approach was followed for many multikinase inhibitors discovered to date, and it is grounded on the high target promiscuity of kinase inhibitors. This promiscuity is greater than in the presence of other type of drugs, owing to the ability of the kinase superfamily to bind to a common substrate, the ATP.

Very recently, fragment-based drug design was successfully applied for the discovery of multitarget protein–protein interaction inhibitors. The discovery of compounds provided with low molecular weight able to disrupt these interactions is a huge challenge, owing to the characteristics of these interfaces: the contact surfaces involved are larger than those involved in protein–small

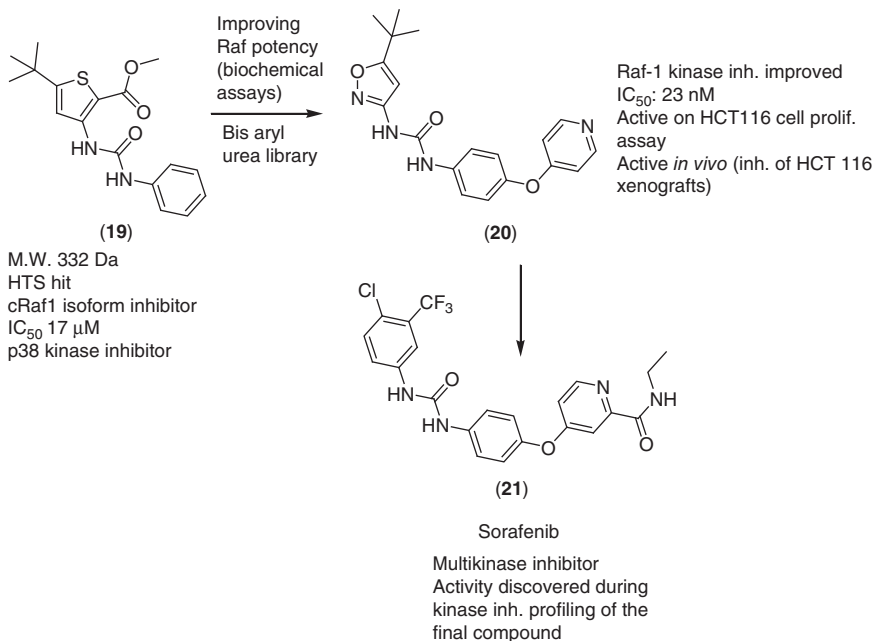


Figure 7.5 Development of the multitargeted inhibitor sorafenib.

molecule interactions, and the contact surfaces are generally flat and often lack the grooves and pockets present at the surfaces of proteins that bind to small molecules. Moreover, protein–protein interactions do not have natural small molecule partners, not allowing the design of compounds on the basis of the structure of a small natural substrate or ligand.

Screening of fragments by NMR together with X-ray crystallography allowed the identification of fragments able to bind to BIR domains of cellular inhibitor of apoptosis protein 1, cIAP1, and X-linked inhibitor of apoptosis protein (XIAP), then optimized by NMR and X-ray spectroscopy. Remarkably, the resulting compounds were active not only in cell-based assays but also in a mouse xenograft efficacy model [46].

Another drug, ABT-263, was discovered by means of “SAR by NMR” (a technology based on the linkage of proximal fragments to achieve high-affinity binding) screening of a chemical library to identify small molecules that bind to the hydrophobic BH3-binding groove of Bcl-X_L, followed by optimization on Bcl-2 by NMR, inhibiting the interaction of Bcl-X_L and other members of the Bcl-2 family with the helical portion of another protein. This compound is in clinical trials for the treatment of lung cancer and other malignancies [47].

7.3.1.2 Machine Learning

Machine learning is a branch of computer science that is based on algorithms that can learn complex patterns from input data and a set of responses (training data) to predict the response of new data. A machine learning method, support vector machines, combined with molecular docking, was successfully used for the discovery of 2-arylbenzimidazole compounds as multitarget (endothelial growth factor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR-2), and platelet-derived growth factor receptor (PDGFR)) inhibitors [48] and acridine scaffold for multitarget VEGFR2 and Src kinase inhibitors [49].

Recently, compounds with a polypharmacology profile against aminergic (class A) GPCRs potentially useful for the treatment of psychiatric disorders were designed following a machine learning system [50].

The process started with the discovery, by means of the algorithm, of the off-target activity of donepezil (compound (**22**), Figure 7.6), an Acetylcholinesterase inhibitors (AChEi), on D4 receptors and a low affinity at D2 receptors; this prediction was based on the Bayesian probabilistic activity models built from ChEMBL database (it contains 5.4 million bioactivity measurements for more than 1 million compounds and 5200 protein targets [51]). Then, this compound was used as a starting point, on the basis of medicinal chemistry literature, for a process of adaptive design, to improve D2 activity together with achieving blood–brain barrier (BBB) penetration.

Computationally generated chemical structures were then prioritized on the basis of novelty, rule-of-five compliance, and synthetic accessibility. The isoindoles thus obtained (e.g., compound (**23**), Figure 7.6) were synthesized and tested; they were found to be ligands for D2 receptors and were able to cross the BBB, but resulted to be provided also with affinity to 5-HT_{1A} and α 2 receptors. The next step of machine learning was performed in order to reduce the off-target activity on the α 1 receptor, while maintaining the polypharmacological profile achieved,

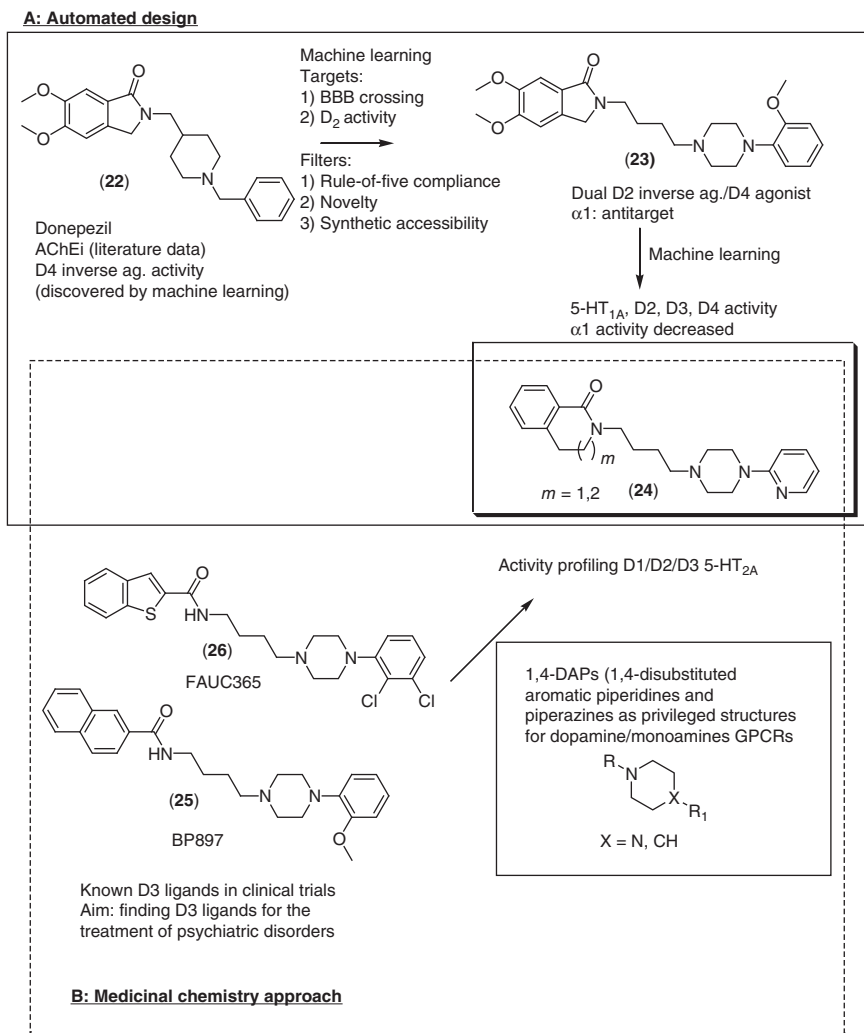


Figure 7.6 Two different approaches led to the discovery of the same class of compounds that are potentially useful for psychiatric disorders: The first strategy involved an automated design (a machine learning approach) for the obtainment of compounds with the desired pharmacological profile, based on a training set of active compounds. The second was based on a medicinal chemistry approach (privileged structures, isosteric substitutions, etc.). The pharmacological activity of the compounds thus obtained was determined as the last step.

in order to obtain a compound with a desired characteristic as an antipsychotic; the resulting compounds (24) are reported in Figure 7.6 [50].

Interestingly, the same compounds have been synthesized in a project aimed to find D3 ligands, without *in silico* procedures, by a medicinal chemists' team [52], on the basis of the structures of known compounds (D3 ligands in clinical trials for psychiatric disorders (compounds (25) and (26), Figure 7.6)) and on the privileged structures 1,4-disubstituted aromatic piperidines and

piperazines (1,4-DAPs) for the monoamine GPCRs; these substructures are in fact present in most of the highly potent and top-selling antipsychotic drugs [53]. Compounds thus obtained were profiled against D2, D3, and 5-HT_{2A} receptors [52].

7.3.1.3 SAR around a Lead

Cancer is the therapeutic area that showed the greatest increase in the number of projects from 2001 to 2010 [54]. The newest class of multitarget drugs that entered the market is represented by kinase inhibitors for cancer treatment. Protein kinase enzymes regulate the biological activity of proteins by phosphorylation of specific amino acids, using ATP as a substrate.

Most of these multitarget drugs exert their action by binding to the highly conserved ATP binding site of the enzyme, and, owing to this, they, very often, are not selective for a given kinase; this multitarget activity was found usually in the late stage of the drug discovery process, during kinase inhibition profiling of the final, *in vitro* (cellular cultures), or *in vivo* optimized compounds [24].

As an example of the multitarget drug design strategy based on SAR around a lead, we report the discovery of PI3K/mTOR (mammalian target of rapamycin) kinase inhibitors.

PI3Kinase-related kinase (PIKK) is a family of enzymes that includes PI3K, DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and mTOR. The PI3K family of lipid kinases in turn consists of three classes based on their substrate specificity and sequence homology. In class I PI3K, four isoforms of the catalytic subunit p110 have been identified. Class I PI3K is comprised of p110 α (PI3K α), p110 β (PI3K β), p110 δ (PI3K δ) (class 1A), and p110 γ (PI3K γ) (class 1B). As a key component of the PIKK family, mTOR shares the highly conserved ATP binding pockets of the PI3K family with 25% sequence similarity in the kinase catalytic domain [55, 56].

PI3K–AKT–mTOR pathway is one of the most dysregulated in cancer. In particular, the PI3KA gene encoding the p110 α catalytic subunit is frequently mutated in many cancers, and it has been identified as an important chemotherapeutic target. Inhibitors of mTOR are available, but dual inhibitors, which may be better suited to overcome the mutability of cancer cells that reduced the efficacy of the first mTOR inhibitor, are under development. Such dual inhibitors would be advantageous, based on the evidence that mTOR is a pivotal downstream target of PI3K and that mTOR can be activated by alternative signaling pathways. The potential synergistic effect of concomitant inhibition of PI3K and mTOR has also been exploited by preparing hybrid molecules (17-hydroxywortmannin analogs conjugated to rapamycin derivatives via a diester prodrug linker [57]).

Research of compounds able to target this pathway started with the discovery of a pan-active class 1 PI3K inhibitor, LY294002 (compound **(28)**, Figure 7.7) [58].

The synthetic flavone LY294002 (**(28)**) (Figure 7.7) was synthesized on the basis of the structure of the natural product quercetin (compound **(27)**, Figure 7.7), a pan-kinase inhibitor; following *in vitro* studies, it was found to be a selective PI3K inhibitor [58]. This compound was the first synthetic PI3K inhibitor to have its complex with PI3K γ structurally elucidated. According to the SAR of this compound that highlighted the importance of the oxygen atom of the morpholine

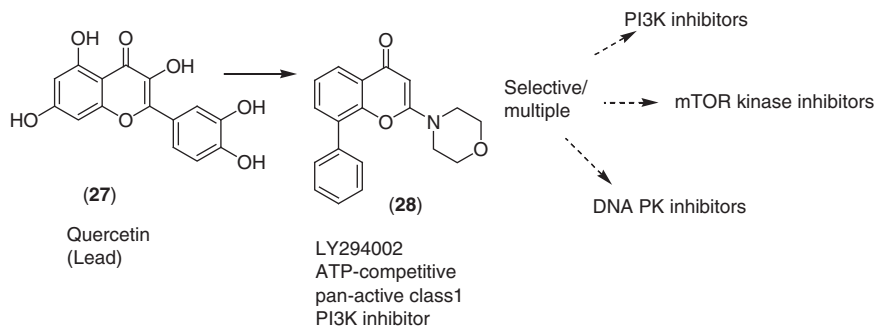


Figure 7.7 Development of LY294002 starting from the structure of the natural compound quercetin.

ring, it was observed that it forms a key hydrogen bond interaction with the hinge region of the kinase [59].

LY294002, which contains the morpholine-privileged structure for compounds acting on the PI3K/mTor pathway [60], represented the starting point for the development of selective or multiple kinase inhibitors (Figure 7.7).

Afterward, two new leads – compound (29) (Figure 7.8) [61] and compound (31) (Figure 7.9) [62] – containing the morpholine moiety, were discovered by Astellas Pharma through HTS conducted on *in vitro* assays for p110 α inhibition. Compound (29) evolved into PI103 (30) (Figure 7.8), a compound tested for p110 α inhibitory activity and as antiproliferative against A375 melanoma cells *in vitro*; selectivity against other kinases was observed, but the activity on mTOR was not assessed [61].

Afterward, during a screening (cellular assays) of potent and structurally diverse compounds that target the PI3K enzyme family, it was discovered that only PI103, among the tested compounds, showed to be able to cause a proliferative arrest in glioma cells. In order to explain the cellular activity of

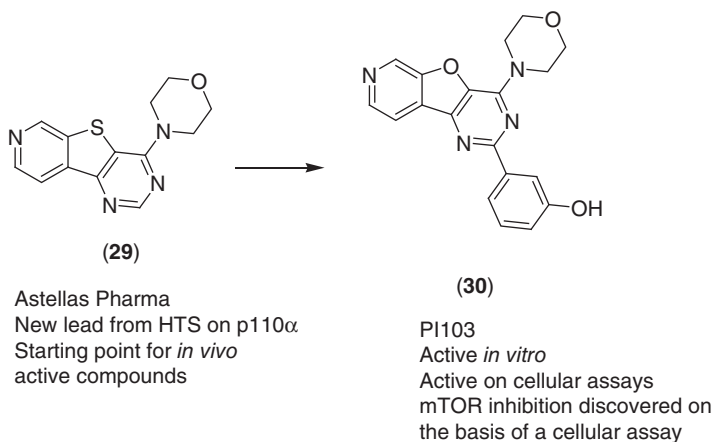


Figure 7.8 Discovery of PI103.

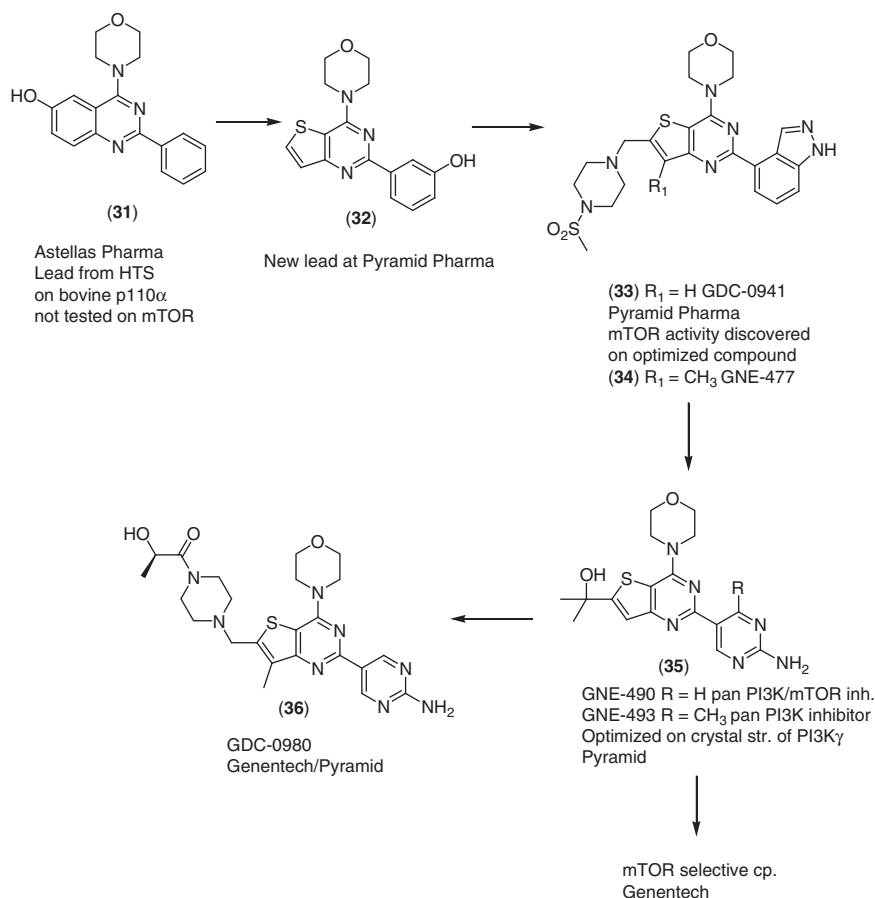


Figure 7.9 Evolution of PI3K/mTOR dual inhibitors containing a morpholine moiety.

PI103, the compound was profiled against a panel of kinases, and its unique cellular activity was traced directly to its ability to inhibit both PI3K α and mTOR. The compound showed also activity in xenograft tumors with no observable toxicity, suggesting for the first time a potentially effective strategy for cancer therapy based on dual inhibition of these two kinases [63]. Structural bases for the interaction between PI103 and mTOR have been recently elucidated by means of X-ray crystallography [64].

The other lead (31) evolved in compound (32) by means of SAR [62]; this compound was selected as starting hit at Pyramid Pharma for the development of PI3K p110 α inhibitors. It demonstrated encouraging activity against several tumor xenografts [65], owing to a lower molecular weight with respect to compound (30) and the opportunity to substitute the 6- and 7-positions of the thieno[3,2-d]pyrimidine, thus offering a greater potential for further optimization, despite its *in vivo* inactivity, owing to a poor PK profile (half-life less than 10 min after ip administration in mice) [62]. Compound (32) was then docked into a homology model of p110 α based upon the crystal structure of p110 γ to

obtain the optimized compound (**33**) (GDC-0941) [66]; this compound showed to be able, during kinase profiling, to inhibit also mTOR [66], an activity that has to be expected on the basis of the similarity of the ATP binding site of PI3K and mTOR [64, 67].

Starting from this discovery, several compounds acting as selective mTOR [68] or PI3K α [69], dual PI3K/mTOR [69–71], or other dual PI3K/mTOR inhibitors that originated in other ways but containing a morpholine moiety [72–77] have been developed; either their design was structure based, or the dual activity was discovered during kinase profiling of the final compounds. Since the crystal structure of the apoprotein of PI3K α was deposited in the PDB only in 2007 [78], the design strategy that was followed for the development of the compounds reported in Figure 7.9 was based on homology models of PI3K α or of mTOR based on the available crystal structure of PI3K γ , a structure that was used as a template to build a homology model. Alternatively, the activity against the two enzymes was determined simply after the optimization of the compounds against PI3K α , given the structural similarity of the ATP binding sites of mTOR and PI3Ks. Overall sequence similarity between mTOR and PI3K γ and between PI3K α and PI3K γ is 25% and 43%, respectively, in the kinase catalytic domain; however, the ATP binding sites are significantly more conserved with a sequence similarity of 68% [67] and with 81% of the residues being identical [73].

LY-294004 showed also to possess a modest ATP-competitive inhibition of DNA-PK. This enzyme is a member of the PIKK family, and it is involved in DNA double-strand breaks repair; thus, it is an attractive target for cancer therapy. Small molecule library approach [79], SAR [80], or homology model of DNA-PK based on PI3K γ [81] led to potent DNA-PK inhibitors with a concomitant activity against other PI3K family members.

Recently, PIQUR [82] claimed to possess in its pipeline finely tuned mTOR inhibitors paired with strong PI3K inhibition [83–85] (see, e.g., PQR-309, phase 1 successfully concluded in 2015, structure not disclosed), a clinically very useful property since an excessive inhibition of mTOR is able to reactivate Akt through alternative pathways, generating resistance. This finding underlines the importance of the relative activities in dual inhibitors.

7.3.1.4 Pharmacophore-Based Approach

Attempts were made to design multitarget compounds by linking pharmacophores responsible for the activities. This strategy, however, led very often to compounds that violate the Lipinski “rule of 5.” This is particularly evident for the design of compounds able to interact with structurally unrelated targets, in which it is likely that the pharmacophores will be very different. Two examples will be reported: the design of triple monoamine reuptake inhibitors (antidepressants) and thymidylate synthase (TS)/dihydrofolate reductase (DHFR) antitumoral dual compounds.

Plasma membrane monoamine transporters are members of the group of Na⁺/Cl[−]-dependent substrate-specific neuronal membrane transporters belonging to the SLC6 gene family. These proteins are large integral membrane proteins composed of 12 transmembrane domains connected by intracellular and extracellular loops. Three major classes of monoamine transporters

(serotonin, SERT; dopamine, DAT; norepinephrine, NET) are responsible for the reuptake of monoamine neurotransmitters. The identification of residues and domains that participate in substrate binding and translocation has been an area of intense research. To date, a clear picture of the site responsible for the binding of the neurotransmitter is not yet available; it has been determined that an Asp in TMD1 is crucial for substrate recognition, presumably through an ionic interaction with the positively charged amines of the neurotransmitter [86–88].

The idea to develop broad-spectrum antidepressants able to inhibit amine uptake of the 5-HT, NE, and DA transporters comes from both preclinical and clinical studies [89]; thus, the research at GSK was addressed to the discovery of SERT, NET, and DAT triple reuptake inhibitors (TRUIs). A pharmacophore model was developed on the basis of structurally rigid and selective derivatives shown in Figure 7.10 (compounds (37)–(39)). A single-ligand pharmacophore was then built over these structures, and the three pharmacophores were finally merged together to create the TRUI pharmacophore. This model was in turn used as a guide for the development of new TRUI, namely, the most suitable position for the functionalization of the azabicyclo[3.1.0]hexane scaffold by means of an alkoxyalkyl side chain (compound (40), Figure 7.10 [89] and then (41) [90] and its derivatives [91]). Likely, the development of these compounds was based on the structures of the previously disclosed TRUI compounds (see compounds (43) and (44) that originated from SAR around analgesic agents [92–94] and (46), Figure 7.10 [95], a tropane derivative described in 1997). Amitifadine (DOV-21,947, EB-1010) disclosed in 2003 by DOW, now developed by Euthymics Bioscience Inc. (that bought DOV Pharmaceuticals), is at present in phase II/III clinical trials for the treatment of depression. EB-1020 is entering phase 2. TRUI compounds (42) [96] and (45) [97] were obtained during SAR around the respective leads. In turn, SAR around compound (42) led to dual SERT/NET reuptake inhibitors [98].

Another strategy, molecular docking coupled with common pharmacophore mapping, allowed the design of TS/DHFR dual inhibitors (compounds of general formula (47), Figure 7.11); the activity of the designed compounds has not been experimentally tested yet. These two enzymes of the folate metabolic pathway, which are key factors for the biosynthesis of RNA, DNA, and proteins, are clinically useful targets in cancer chemotherapy and are selected for the design of multitarget compounds on the basis of their antitumor synergistic activity in coadministration preclinical assays. These proteins belong to two different families (SCOP classification), and antifolate inhibitors of DHFR generally are 2,4-diaminopyrimidine, while TS inhibitors are 2-amino-4-oxopyrimidine derivatives.

These compounds were designed by a novel computational approach (upper part of Figure 7.11) that integrates the affinity predictions from structure-based virtual screening with dual ligand-based pharmacophore. This strategy involves docking experiments as starting point using a drug-like database; selected hits were then mapped over a dual pharmacophore that was developed from experimentally known dual inhibitors of TS and DHFR, then, an *in silico* optimization step followed [99].

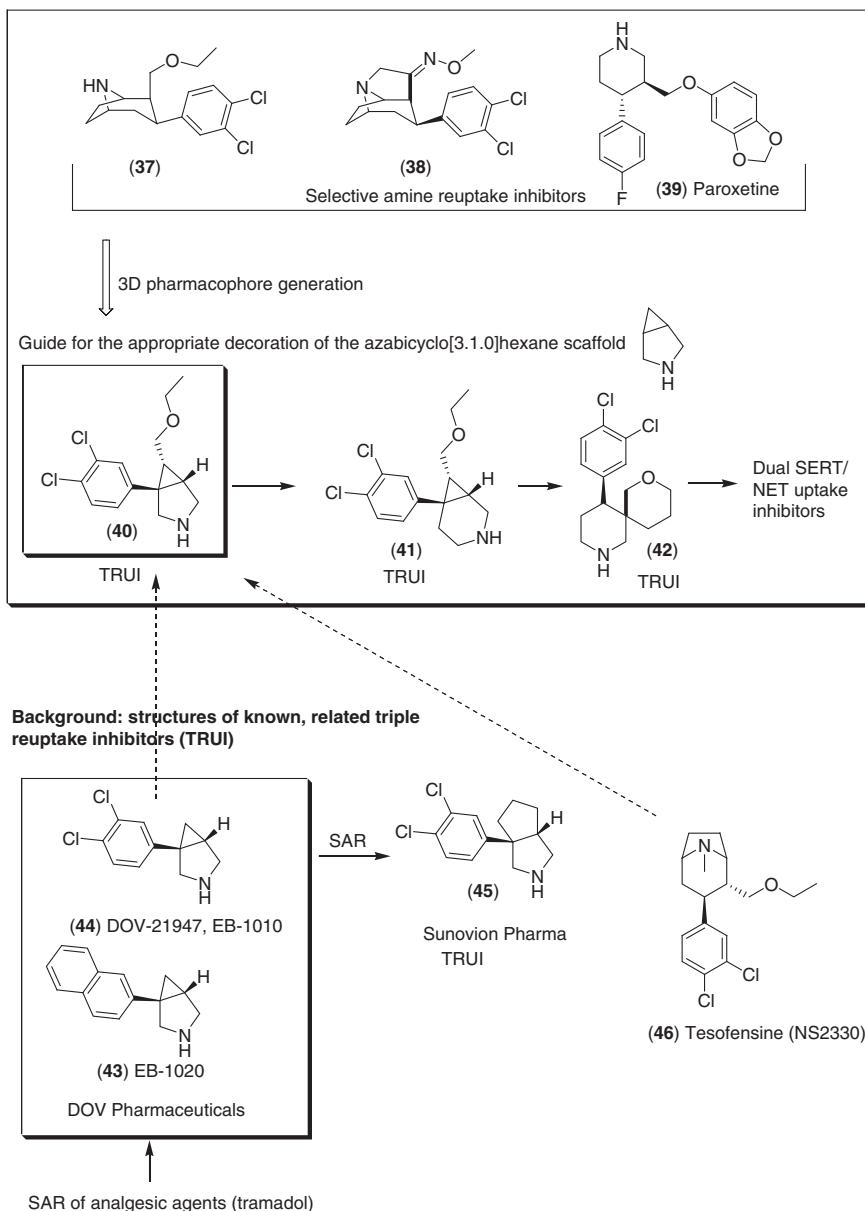


Figure 7.10 Pharmacophore-based design of triple reuptake inhibitors developed at GSK, together with compounds triple reuptake inhibitors with similar structures present in the previous literature.

Dual-acting compounds (lower part of Figure 7.11), necessary for the pharmacophore construction, have been in turn discovered starting from the activity profile determination of the clinically used antifolate TS inhibitor pemetrexed (compound (48), Figure 7.11) that showed also activity against DHFR [100]. This dual activity was allowed by the possibility of the 6,5-fused ring system

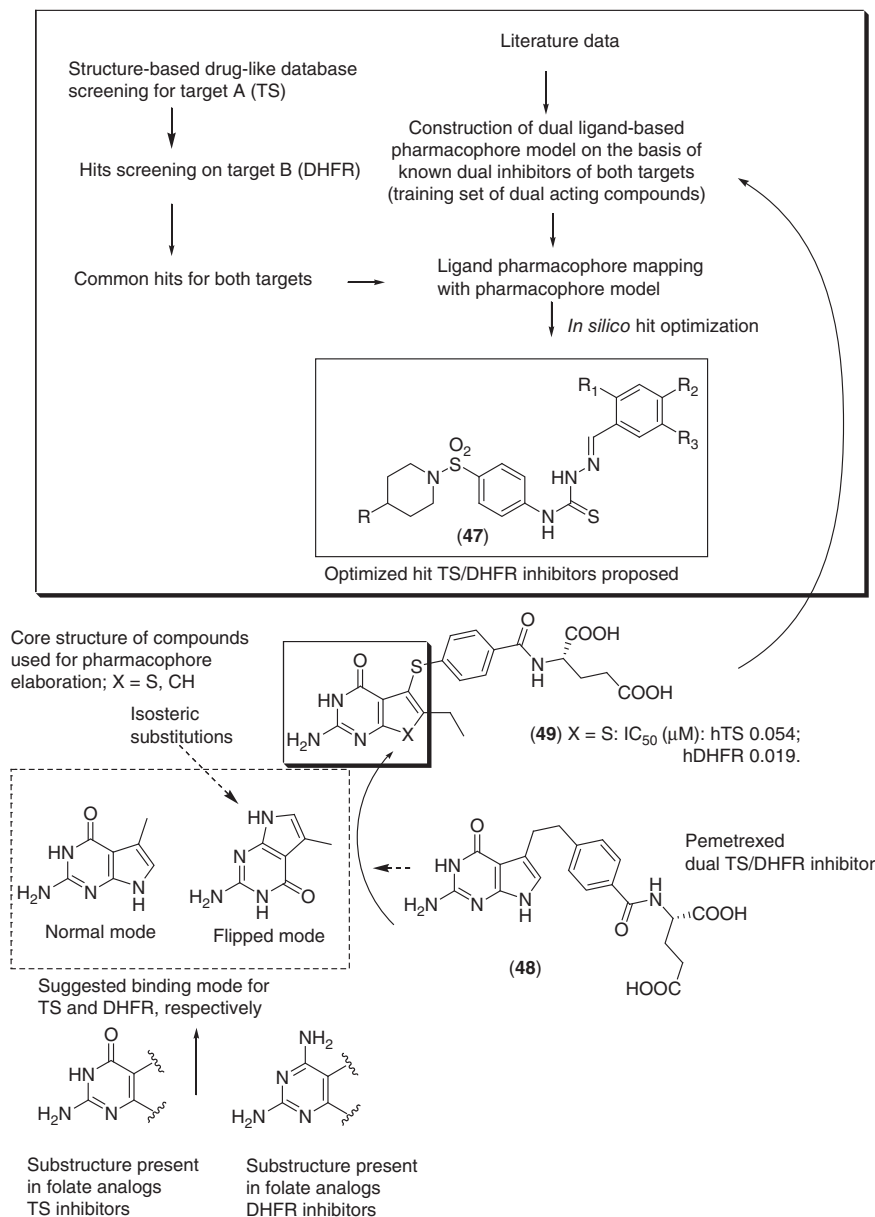


Figure 7.11 (Upper part): *In silico* design of dual TS/DHFR inhibitors. (Lower part): Design of experimentally validated dual TS/DHFR inhibitors, starting point for the generation of a pharmacophore model used in the virtual approach described above.

to bind to their targets in a normal or flipped mode, with the pyrrolic NH mimicking the amino group present at position 6 in DHFR inhibitors. Then, isosteric substitution of NH led to different dual-acting compounds (compounds of general formula (49), Figure 7.11) that were used as a training set in the *in silico* strategy [101–106]. Moreover, on the basis of the wide presence of

substituted 2,4-diaminopyrimidine scaffold in the field of kinase inhibitors, 6–5-fused 2,4-diaminopyrimidines were modified to originate also receptor tyrosine kinase (RTK, enzymes involved in tumor angiogenesis)/DHFR and RTK/TS dual inhibitors, able to bind to the adenine cavity of the ATP binding site of these enzymes [107–109].

7.3.2 Designing-In Approaches for Structurally Unrelated Targets

7.3.2.1 Fragment-Based Approach

This approach has been recently considered also for the design of ligands for multiple targets with large differences in their binding site. Before an iterative growing strategy (implemented by docking experiments) follows, these methods are based on the extraction of fragments from known ligands for a given target: COX-2/LTA4H [110], BACE-1/glycogen synthase kinase 3 β (GSK-3 β) [111], 5-LO/soluble epoxide hydrolase (sEH) [112], BACE-1/AChE [113], and AChE/semicarbazide-sensitive amine oxidase (SSAO)/vascular adhesion protein-1 (VAP-1) [114]. These procedures are based on *in silico* experiments [110, 112, 113] or visual inspection of the pharmacophoric features of known ligands [111]; moreover, the development of a computational *de novo* method for the design of combinatorial libraries able to obtain compounds provided with the desired polypharmacological profile (dopamine D4/sigma 1 receptor ligands) has been recently reported [115].

7.3.2.2 Pharmacophore-Based Approach

In the first case reported here, the design of dual-target compounds useful in metabolic disease, was performed by Prosidion Ltd, with the development of dual MHC-1R antagonist/DPP-IV inhibitors and GPR119 agonists/DPP-IV inhibitors as antidiabetic agents.

Thus, by merging suitable ligands (compounds **(50)** and **(51)**, Figure 7.12) for two targets belonging to different protein classes, it was possible to obtain potent dual MHC-1 (a class A GPCR) antagonists and DPP-IV (a peptidase) inhibitors. MHC-1R is the receptor for melanin-concentrating hormone (MCH); this is a cyclic orexigenic peptide expressed in the lateral hypothalamus, which plays an important role in regulating energy balance, while DPP-IV hydrolyzes GLP-1. These multiple ligands were designed on the basis of the common structural motifs between drug classes that act on the targets considered here, making a partial overlap between these compounds. The examination of *in vitro* data for about 1500 MHC-1R antagonists and about 2500 DPP-IV inhibitors identified a number of cases where MHC-1R and DPP-IV ligands shared common structural motifs; potential dual ligands were then constructed virtually, and the lead (compound **(52)**, Figure 7.12) is optimized on the basis of known MHC-1R ligand data and filtered for drug-like properties [116].

In the design of multiple ligands for the treatment of type 2 diabetes, combining the inhibition of DPP-IV (GLP-1 has a half-life of less than 3 min, undergoing rapid cleavage by the serine protease DPP-IV) and agonism of GPR119 (the GPCR GPR119 agonists promotes the secretion of GLP-1 from intestinal L-cells), the crystal structure of the DPP-IV active site was used to assess the tolerance of incorporating the GPR119 pharmacophore [117]. In this case, target selection,

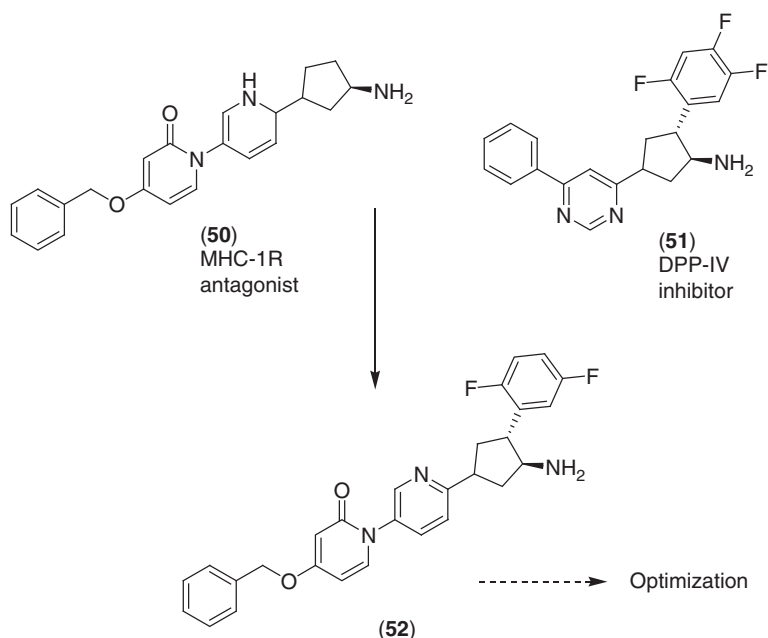


Figure 7.12 Generation of a multitarget MCH-1 antagonist/DPP-IV inhibitors.

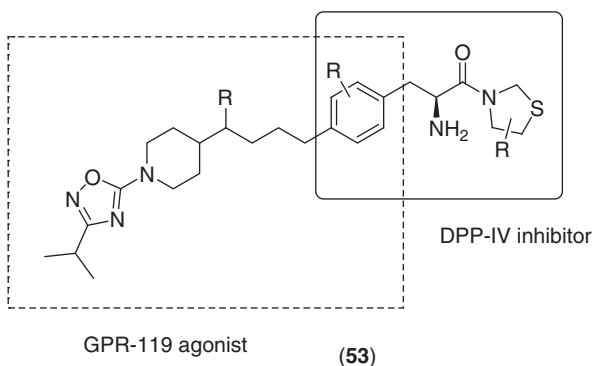


Figure 7.13 Generation of a multitarget GPR119 agonist/DPP-IV inhibitors.

namely, the examination of the effects of the simultaneous modulation of multiple targets, was checked in studies carried out on animal models of diabetes, since the individual targets are expressed in different tissues of the body, using a coadministration of a GPR119 agonist and a DPP-IV inhibitor to modulate each target individually. Clinical candidate is represented by PSN491 (general formula (53), Figure 7.13). These compounds are active *in vivo* in a rodent model of diabetes [118].

7.3.2.3 SAR around a Lead

Based on the results of clinical research demonstrating that the antidepressant activity of selective serotonin reuptake inhibitor (SSRI) may be augmented

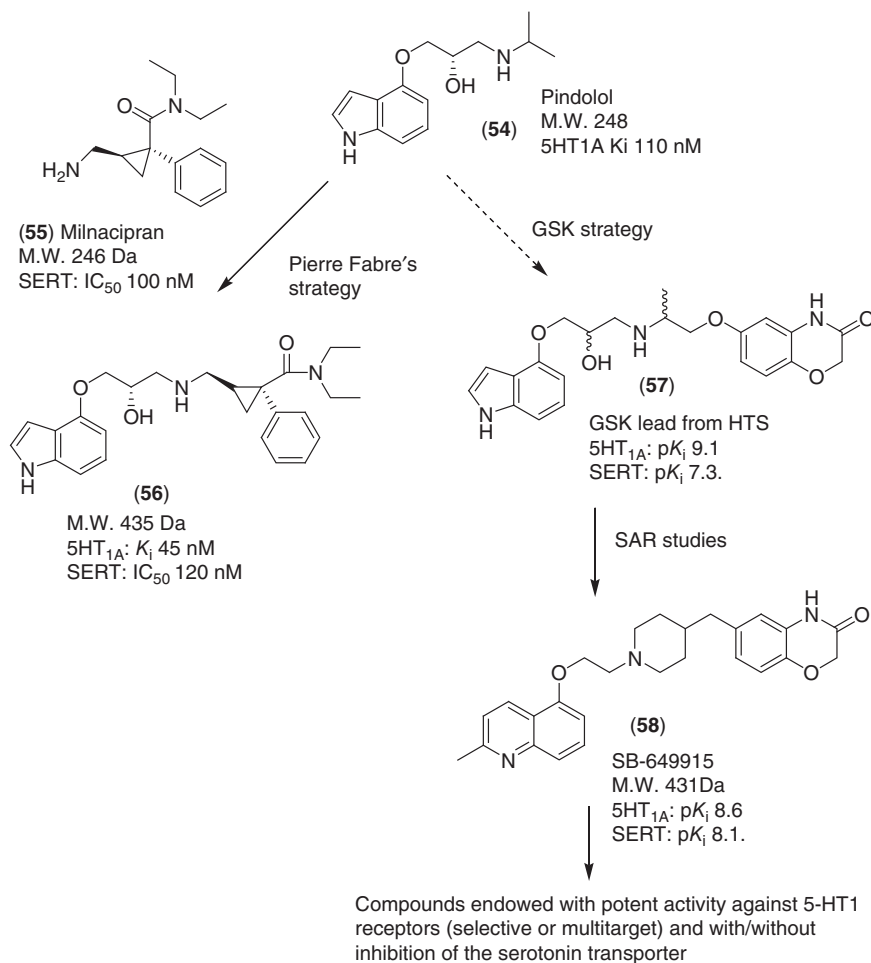


Figure 7.14 Development of multitarget 5-HT_{1A}/SERT inhibitors as antidepressants, following a pharmacophore strategy (merging the two pharmacophores) and an SAR optimization process.

by coadministration of pindolol (compound **(54)**, Figure 7.14), an effect that has been attributed to pindolol partial agonistic effect at the 5-HT_{1A} receptor [119, 120], two strategies have been used for the design of dual 5-HT_{1A}/SERT inhibitors for the treatment of psychiatric disorders. The first is represented by the fusion of the two pharmacophores (pindolol and the SERT inhibitor milnacipran, compound **(55)**, Figure 7.14) [121] to originate **(56)**; the second was based on HTS using an *in vitro* binding assay ([³⁵S]GTPγS binding assay with CHO cells expressing h5-HT_{1A} receptors). Compounds with good antagonistic potency were then tested for blockade of [³H]5-HT reuptake into rat cortical synaptosomes. Compound **(57)** (Figure 7.14) was thus discovered [122]; subsequent SAR led to the development of compound **(58)** [122, 123] and other derivatives [124–133]. In this case, the compounds obtained following both

strategies possess a molecular weight of the same order of magnitude; however, as can be seen below, pharmacophore linking or fusion can lead very easily to high molecular weight (greater than 500 Da) compounds.

Multitarget compounds have been designed for the treatment of Alzheimer's disease, on the basis of the targets structurally unrelated but relevant for this pathology, against acetylcholine esterase, the enzyme that hydrolyzes acetylcholine, and BACE (β -secretase), a protease that cleaves the amyloid precursor protein (APP) into A β peptides, which play a pivotal causal role in the pathogenesis of AD.

Two classes of compounds that originated from two different strategies acting on these targets, have been reported. The first class of compounds that led to the discovery of the orally active memoquin (compound (61), Figure 7.15) were designed starting from caproctamine (compound (59), Figure 7.15), a polyamine able to interact with muscarinic M2 receptors (it antagonizes selectively presynaptic muscarinic M2 autoreceptors facilitating ACh release) and to inhibit AChE, interacting with both the active and peripheral sites of AChE; the interaction of A β with peripheral sites in its non-amyloidogenic form induces the development of an amyloidogenic conformation.

This lead was then modified not on the basis of the target or ligand structures, but simply by implementing the compound with the substructure, the quinone moiety, present in idebenone (compound (60), Figure 7.15), the synthetic form of coenzyme Q10, on the basis of the *in vitro* (the antioxidant activity) and the clinical activity of this compound (improvement of cognitive functions and behavioral deficits in patients with mild to moderate AD). Memoquin, the compound that resulted following this design strategy, is able to be absorbed after oral administration and to access CNS despite its molecular weight that violates the Lipinski "rule of five" [134]. Its simplified monovalent ligand (compound (62), Figure 7.15; possessing a molecular weight of 420 Da) showed to inhibit AChE, amyloid aggregation, and BACE-1, an aspartic acid protease involved in Alzheimer's disease through the generation of amyloid β peptides, an activity not involved in the design of the compound but that appeared serendipitously; *in vivo* activity has not been studied yet [135].

Another approach [136] combined the structures of a BACE-1 inhibitor with that of an AChE inhibitor (donepezil) by means of a linker (compound (63), Figure 7.15); in this case, the dual inhibitor obtained possesses a high molecular weight (738 Da) reflecting its design strategy and has to be administered in the *in vivo* assays by means of an intracerebroventricular (icv) administration, but showed interesting *in vitro* and *in vivo* activities, comparable with those obtained following the strategy reported above.

7.3.2.4 Mining Literature Data

In the previous section it was shown that an *in silico* procedure, a machine learning approach based on the activities of known compounds, can lead to the discovery of multitarget agents with a desired biological profile. A new, more pragmatic procedure that allows a systematic exploration of dual-acting modulators has been recently proposed based on the combination of the knowledge of the ability of most drugs to modulate more than one target and

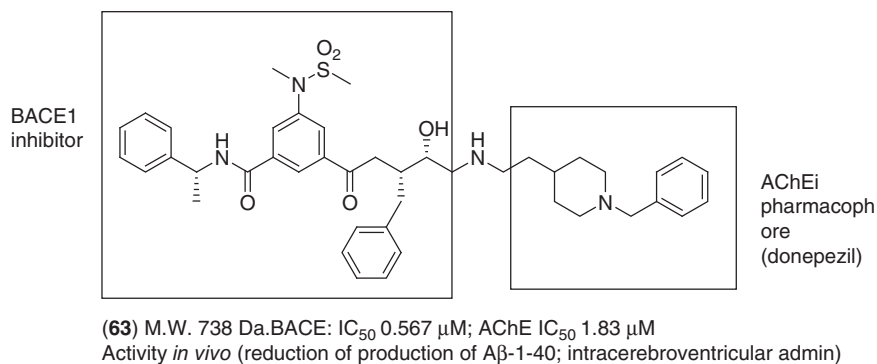
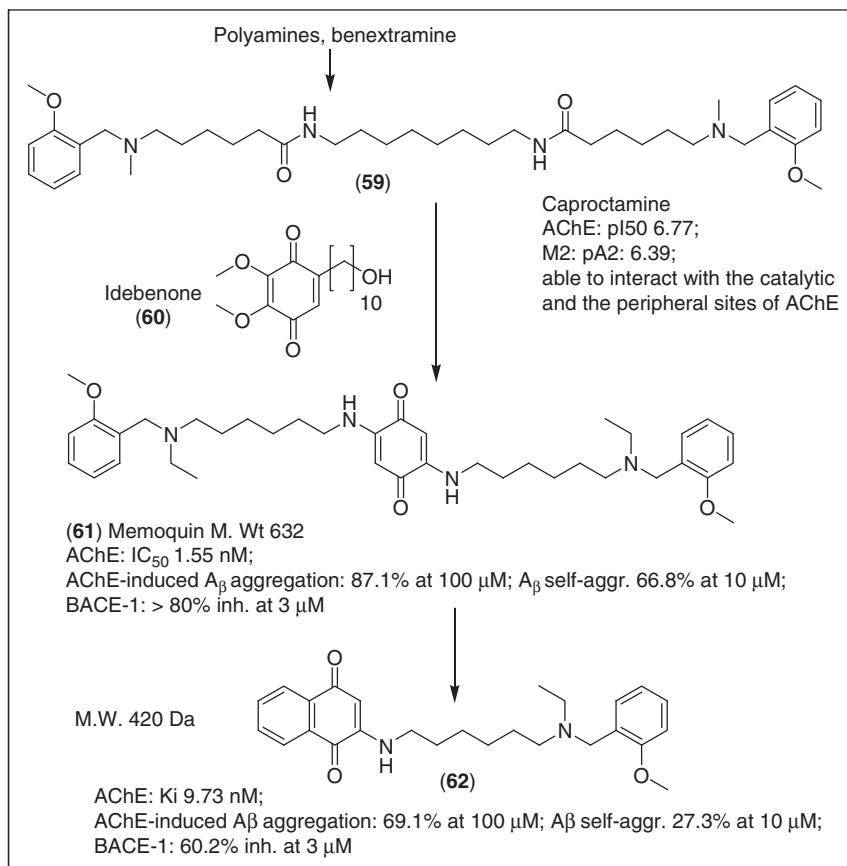


Figure 7.15 Discovery of dual AChE/BACE inhibitors following two strategies: SAR around a lead and pharmacophore linking (BACE-1 inhibitor with the AChEi donepezil).

the increased availability of relevant chemical and biological informations to rapidly identify dual-acting modulator opportunities in a given disease area. The exploration of dual-acting modulators within the gastrointestinal disease area has been reported as an example (see Figure 7.16). The methodology is based on a workflow, able to identify opportunities for rationally designing dual-acting modulators, reported below; even if it does not make novel predictions of suitable target combinations, it collects all the available data to decide easily

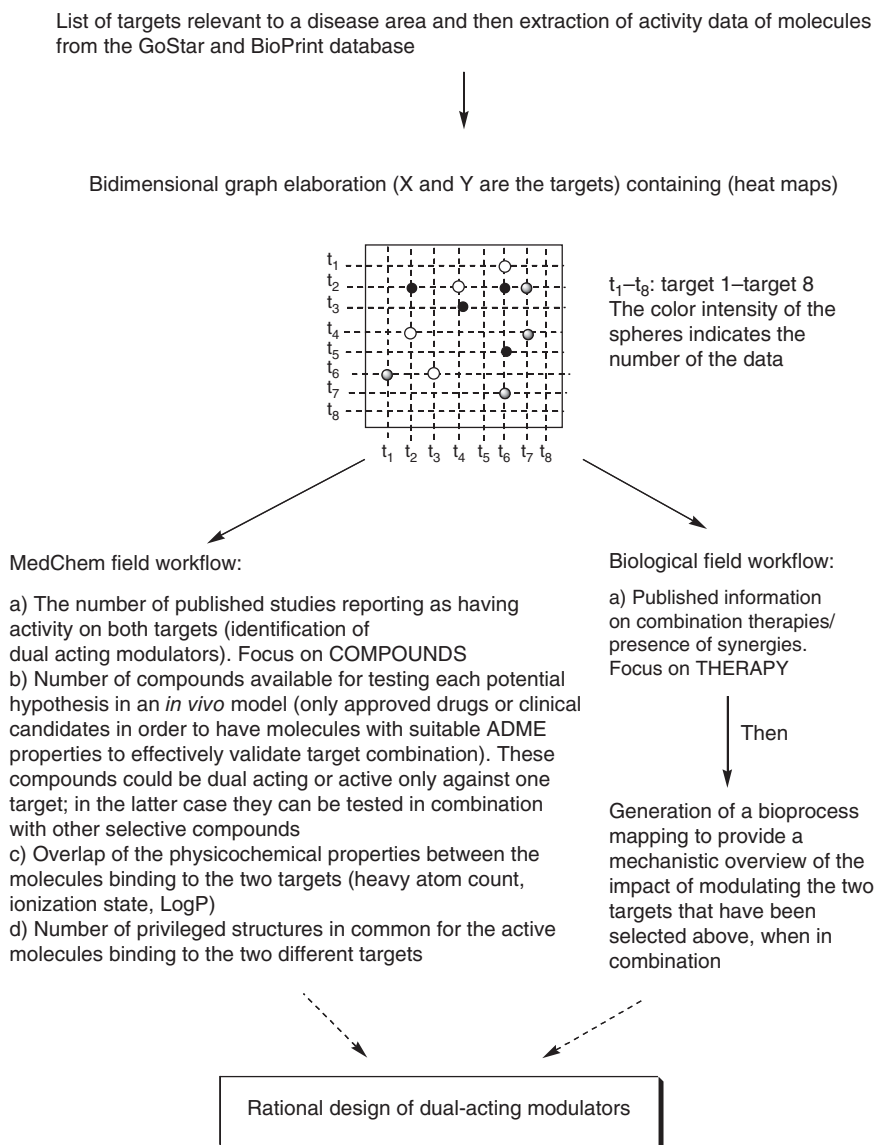


Figure 7.16 Workflow of the discovery of dual-acting modulators based on literature data.

which options, among those that are available, should be prioritized for further validation of *in vitro* or *in vivo* disease model [137].

Given the complexity of Alzheimer's disease, which involves functional modulation of multiple proteins, efforts to design multitarget agents acting on this disease have been recently performed, including a multitarget-quantitative SAR (QSAR) approach predicting chemical–protein interaction (25 key targets) for Alzheimer's disease, based on two machine learning tools [138], and an *in silico* automatic extraction of fragments able to interact with five targets related to Alzheimer's disease [139] used as a tool for the design of new multitarget drugs. Interestingly, an *in silico* system that allows target identification and polypharmacological analysis for Alzheimer's disease research, on the basis of available chemogenomics data, activity profile of marketed drugs, and bioactive chemicals (928 genes, 320 proteins, 194 drugs, 405,188 chemicals related to Alzheimer's disease), leading to the discovery of highly potent multitarget ligands [140] was also reported.

7.4 Designing Approaches Based on Phenotypic Assays

FDA-approved discovery strategies for new molecular entities between 1999 and 2008 showed that the contribution of phenotypic screening to the discovery of first-in-class small molecule drugs exceeded that of target-based approaches in an era in which the major focus was on target-based approaches [141]. The following scheme shows two different approaches that have been followed for the discovery of multitarget RET inhibitors as antitumoral compounds; one strategy is based on kinase inhibitory activity optimization following *in vitro* assays, while the other is based on phenotypic screening.

Vandetanib (compound (65), Figure 7.17), a kinase inhibitor, was approved in 2011 by FDA for the treatment of certain tumors of the thyroid gland (medullary thyroid carcinoma (MTC)); most multiple endocrine neoplasia type 2 (MEN2) patients have an autosomal-dominant activating germ line mutation in the RET RTK that is necessary for the development of cancers such as MTC. Compounds able to interact with this kind of tumors were developed in two ways: the first that started before 1999 (the date of publication of the first report of a lead) with the screening of compounds able to inhibit VEGF signals to prevent tumor growth (compound of general formula (64), Figure 7.17); the compounds were optimized as VEGFR inhibitors (biochemical assays) and tested for cellular activity (inhibition of the incorporation of tritiated thymidine during the growth of human umbilical vein endothelial cells (HUVECs), stimulated by VEGF *in vitro* and *in vivo* (increase in uterine weight in immature rats following administration of estradiol, an effect due to tissue edema induced by the expression of VEGF in the uterus, then on a number of human tumor xenografts grown subcutaneously in athymic mice). Its activity on RET (*in vitro* and as an inhibitor of RET/PTC3-induced tumor formation in nude mice) was highlighted after compound optimization during the kinase inhibition profiling (screening) in 2002; these results suggested its potentiality in the treatment of RET-related tumors

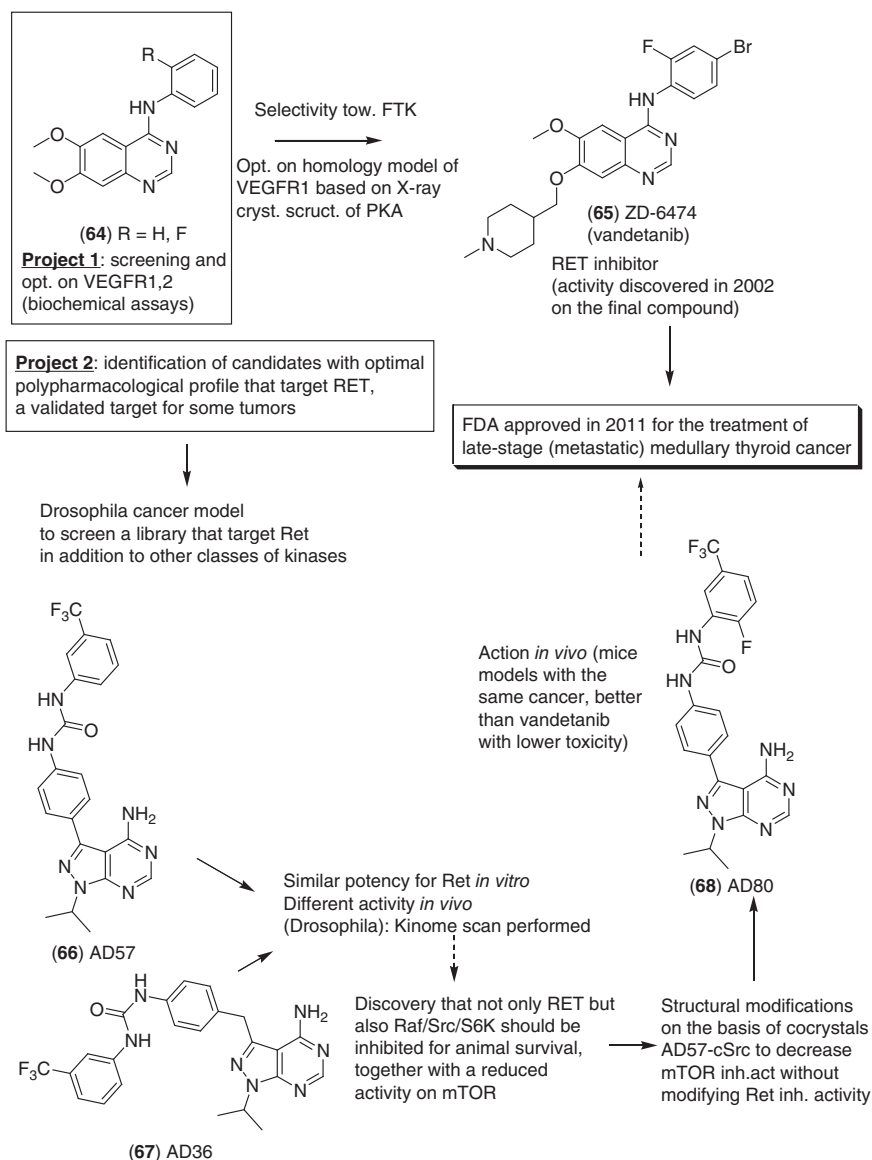


Figure 7.17 Strategies followed for multikinase antitumoral drug discovery based on RET targeting.

acting both as an antiangiogenic and antineoplastic agent [142–144]. In 2011, FDA approved vandetanib for MCT.

The second project, published in 2012, was aimed at finding candidate compounds with optimal pharmacological profiles that target RET for the treatment of MEN-2 patients, and distinct downstream kinases, by means of a systems pharmacology approach. Strategy in this case relied on whole-animal screening (Drosophila) to both explore the mechanism of the compounds and identify

an optimal polypharmacological profile for suppressing tumors *in vivo*. Thus, a cancer form of RET was engineered into *Drosophila*, and compounds that target RET in addition to other classes of kinases were screened with the goal of curing the tumor. Even in this case the scaffold of the compounds, as in dual Tyr/PI3K inhibitors, was a pyrazolopyrimidine (compounds (66) and (67), Figure 7.17). In this way, it was discovered (by means of a kinomescan) that drug inhibition of RET plus Raf, Src, and S6K was required for optimal animal survival, whereas TOR inhibition led to toxicity, owing to the release of negative feedback. Rational design to reduce TOR binding afforded AD80 (compound (68), Figure 7.17), which was shown to be much better on human cell lines and in mice models with the same type of cancer than vandetanib [145].

Figure 7.18 reports the approach that has been followed for the development of antinociceptive multitarget drugs, which could be a successful strategy in order to find new and better therapeutic options for pain treatment, given the failure of analgesic developments derived from the logic of targeting a single receptor. The “bait-target” approach is based on the selection of a target relevant for antinociceptive multitarget drugs, in this case the glycine transporter type II, GlyT2; inhibitors of this transporter are already being studied in pharmaceutical companies as analgesic candidates. Then, an *ex vivo* system for efficacy screening using optically imaged long-term potentiation in rat spinal cord has been set out, a system that could be used for HTS that reflect the complexity of neuronal systems in alternative to animal models of pain, which are notorious for low throughputs. Following this approach, it was possible to select compounds that have been tested in *in vivo* assays; while the most *in vitro* active compounds showed serious side effects, the moderately potent ones showed good *in vivo* and *ex vivo* efficacy without any serious side effects. Implementation with activities on other targets relevant for this pharmacological action could overcome the low activity against the primary selected target (the bait target). This hypothesis

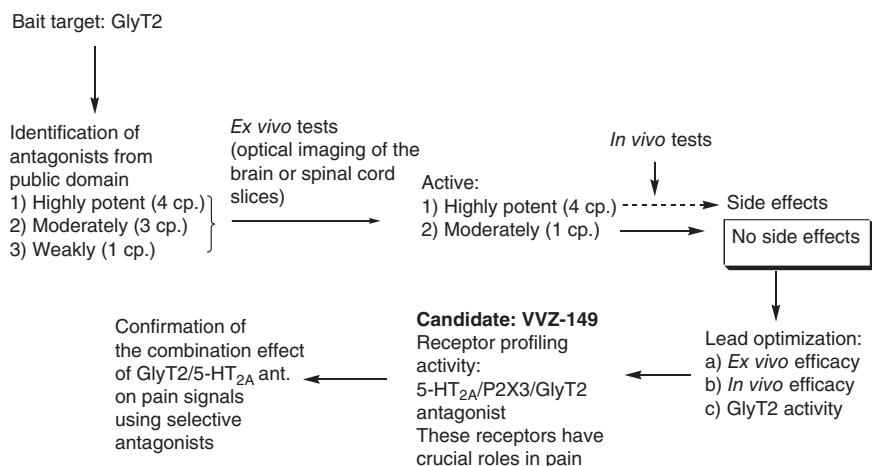


Figure 7.18 Development, by means of phenotypic assays, of multitarget antinociceptive drugs.

was already formulated for low-dose drug combinations acting along molecular pathways that could maximize therapeutic effectiveness while minimizing collateral adverse effects [146]. This lead was then optimized and profiled; the final compound VVZ-149 showed activity, besides on GlyT2, also on other two receptors, P2X3, a purinergic receptor subtype ligand-gated ion channel, and 5-HT_{2A}, a GPCR relevant for pain [147]. In 2015, phase 1 studies have been terminated (no data available); phase 2 studies are ongoing.

7.5 Conclusions

While many clinically useful multitarget drugs have been obtained serendipitously, nowadays several strategies are available for the design of multiple ligands, even if the problem of designing for a particular selectivity profile is significantly more complex than the designing for high affinity to a single target. These strategies are either target-based (irrespective of the structural similarities of the targets considered) or ligand-based processes that are the logical extension of drug design for selective compounds.

A survey of the literature showed that the most common strategy followed for the design of multitarget drugs is that based on the use of pharmacophores or SAR around a lead (especially for drugs acting on psychiatric disorders and kinase inhibitors). Very often, the compounds that have been obtained following the first strategy do not possess drug-like characteristics, and only preclinical activity data are reported (*in vitro* assays). Only if the starting pharmacophores possess a low molecular weight or the merging of pharmacophores has been performed, compounds with favorable characteristics can be obtained. Ziprasidone (compound (71), Figure 7.19) [148] and ladostigil (compound (74), Figure 7.19 [149]) were reported as successful examples. The clinically available antipsychotic drug ziprasidone originated from the fusion of dopamine (69) and a 5-HT₂ ligand, while ladostigil (74) originated from the fusion between the AChE inhibitor rivastigmine (72) and the monoamine oxidase MAO inhibitor rasagiline (73); at present it is on phase IIb of clinical studies.

Several compounds obtained following the second strategy are on the market; very often, the multitarget activity of these compounds was assessed only after their *in vitro* (cellular assays) or *in vivo* optimization during their pharmacological profiling.

Today, tools are available for the design of multitarget compounds, given the knowledge about the targets that have to be modulated for achieving a therapeutic effect; fragments, successfully considered for “selective” drug discovery, started to be considered as seeds to the development of multitarget compounds and to be optimized by computational or structure-based approaches. Even multiple protein–protein interaction inhibitors can be designed on the basis of fragments. Moreover, there has been an increase, during the past years, in computational approaches for the design of compounds provided with the desired polypharmacological profile. The problem, at this point, is represented by target selection [150–154] and the extent of the modulation of the multiple targets that are necessary to obtain a therapeutic efficacy [1].

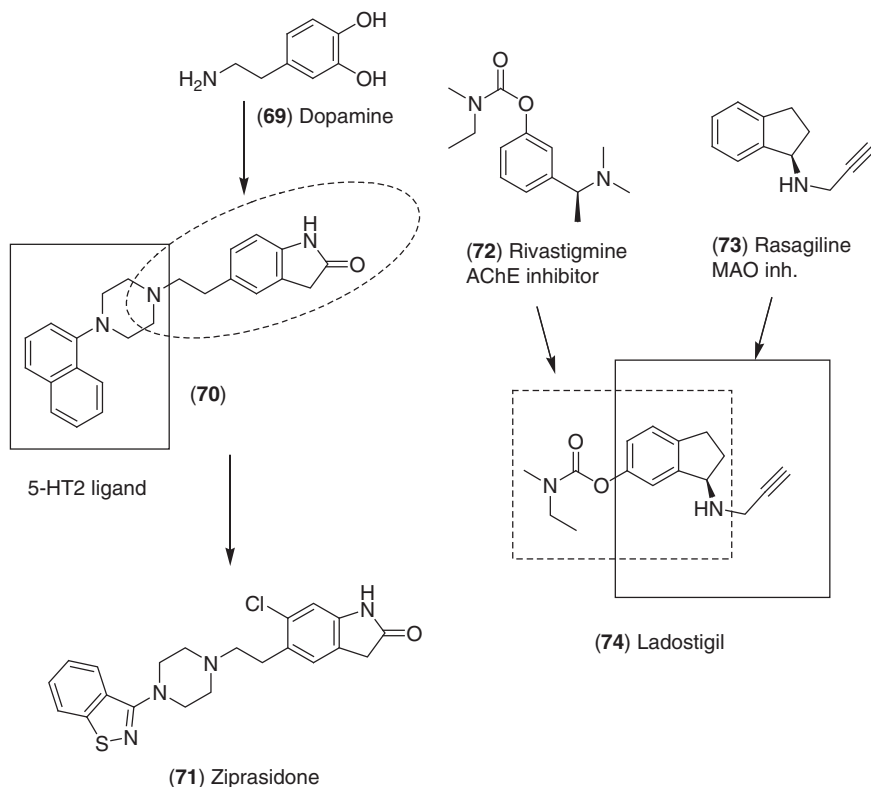


Figure 7.19 Multitarget drugs in advanced clinical trials and their design process.

It is now recognized that among the first-in-class drugs approved by FDA between 1999 and 2008, 37% resulted from phenotypic screening, an assay that looks at the effects or phenotypes that compounds induce in cells, tissues, or whole organisms, whereas 23% were identified by the target-based screening; moreover, the discovery rate of new phenotypic drug was greater than that of the target-directed drug and was invariant over the 9-year study period, an era where the major focus was on target-based approaches [141]. Accordingly, phenotypic screening approach is gaining great interest in the Big Pharma [155, 156]. After screening, chemical proteomics is then used in order to find the target; in this way new therapeutic targets and new disease biology can be found. To date, modern phenotypic screening has been successfully applied for the discovery of antinociceptive multitarget compounds and for RET inhibitors; this kind of approach can be used fruitfully also for drug repurposing (see, e.g., [157]). These strategies could represent the solution for the discovery of clinically effective drugs, in which multitarget selection and optimization are performed at the same time using assays that reflect the complexity of a living organism.

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8

The Linker Approach: Drug Conjugates

Daniel Merk and Manfred Schubert-Zsilavecz

Goethe University Frankfurt, Institute of Pharmaceutical Chemistry, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany

8.1 Introduction

While a majority of drug discovery programs for a long time pursued high selectivity to reduce off-target side effects for several decades, a growing interest is focusing on conjugates of two or more pharmacologically active agents with predominantly the same ultimate goal of lower systemic toxicity. At first glance the concept of lowering side effects with few selective linked agents is based on a targeted delivery that is facilitated by one component of a conjugate. With the availability of drugs and antibodies selectively addressing cell surface proteins, the opportunity of targeted delivery of potent agents to a certain population of cells, that is, cancer cells, is established. The approach of delivering highly toxic compounds to cancer cells without touching healthy tissue hence relies on the linkage of a targeting drug, that is, an antibody, and a cytotoxic drug that is released upon degradation of the drug conjugate.

But targeted delivery is not the only potential advantage of drug conjugates. Drug conjugation might also be successful in polypharmacology where, for example, several active agents can be coupled to a carrier, which might help to improve individual therapeutic regimen containing several drugs. Furthermore, drug conjugates can be used to trigger the stability, release, or activity of drugs. For this purpose polymer-based conjugates have been developed.

For all possible utilizations of drug conjugates, intensive research has been conducted over the last decades and knowledge and experience on these new drug classes is strongly growing. Although it took nearly 40 years from the first experimental peptide drug conjugates to the approval of brentuximab vedotin as first antibody–drug conjugate, future development with drug conjugates will probably yield many valuable agents in considerably less time (Figure 8.1).

In this chapter, we will review and evaluate the concepts of drug conjugation by discussing the different types of drug conjugates as well as the chemistry of their linkers and by gathering available clinical data for drug conjugates.

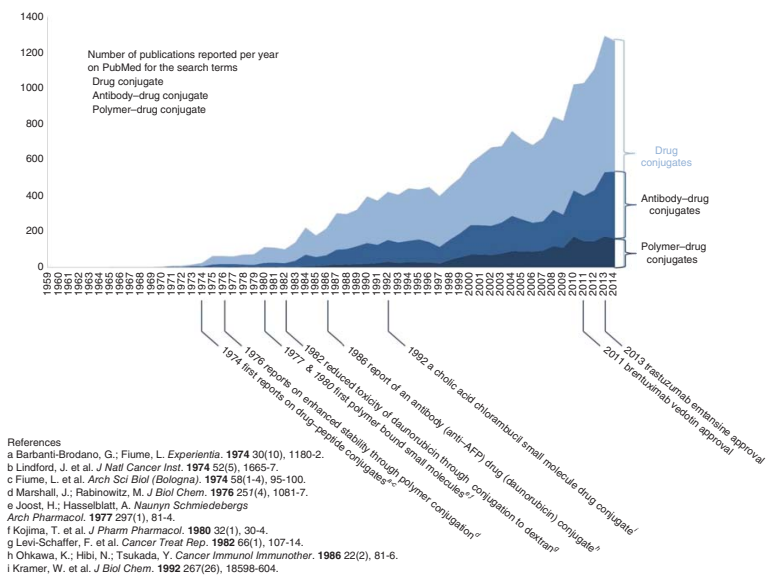


Figure 8.1 Drug conjugates have gained considerable interest over the last decades, and with the approval of the first antibody–drug conjugates in 2011 and 2013, this intensive research started to yield clinical benefit. With several more agents in all stages of preclinical and clinical development, a growing significance of drug conjugates is growing.

8.1.1 Targeted Delivery

The most prominent role of drug conjugates is currently found in targeted delivery in which conjugation facilitates more or less selective uptake in a specific cell type or tissue. For this purpose, one component of the conjugate constitutes a ligand of a structure that is present in the desired tissue. In many cases this structure is a cell surface receptor to which the conjugate binds via its ligand part and then is internalized by receptor-mediated endocytosis. To produce a targeting effect toward a specific tissue, the target structure/receptor should be overexpressed on the desired cell type at least to a certain extent, and must display a sufficient cell surface density in order to deliver a sufficient amount of the conjugate into the cell. Receptors that are found inside the desired cells are significantly less suitable for targeting since the conjugate then would have to enter cells nonselectively, which would contradict the concept of targeted delivery that is supposed to facilitate tissue selectivity and reduce systemic effects. After receptor-mediated endocytosis, the complex of receptor and drug conjugate is taken up into the endosomes/lysosomes from where the conjugate has to escape in order to exhibit its pharmacological activity within the targeted cell. Many conjugates are supposed to be cleaved inside the lysosomes to release their cargo, and sophisticated linkers have been developed to govern this process [1].

8.2 Drug Conjugates

8.2.1 Small Molecule Drug Conjugates

Small molecule drug conjugates link two (or more) small molecules with defined molecular targets in one molecule. Usually, the two components can be separated through the use of a cleavable linker. The resulting conjugate then unites two (or more) pharmacophores and can exhibit more than one pharmacological activity. This dual (or multiple) pharmacological activity can either include pharmacodynamic effects with the activation or inhibition of more than one target or one part of the conjugate can exploit pharmacokinetic advantages through high affinity to a distinct target that leads to targeted drug delivery. Both strategies have successfully been investigated, but the use of small molecule drug conjugates for targeted delivery is more prominent at present.

8.2.1.1 Chances and Challenges

The conjugation of two (or more) small molecules can offer several pharmacological advantages. So far, most reported conjugates are designed for targeted delivery, which means that one of the pharmacophores of the conjugate helps the agent to be especially taken up by cells expressing the target receptor of the pharmacophore. This approach enables the use of highly potent agent that when used alone would exhibit high systemic side effects and toxicity. With the help of a targeted conjugate, these dose-limiting systemic effects can be reduced, while the activity on targeted cells or tissues is retained or even enhanced.

Besides targeted delivery, small molecule drug conjugates can also be used for the application of two agents whose pharmacodynamic effects shall be

combined. In this case, the conjugate might improve the physicochemical and pharmacokinetic properties of the two drugs and could produce synergistic effects when synergistically active agents reach their tissues of action together.

Small molecule drug conjugates offer several opportunities and advantages. Compared with classical small molecules, a conjugate can be superior especially for its selectivity and lower systemic effects. Compared with other drug conjugates that include a macromolecule such as an antibody or a polymeric carrier, small molecule drug conjugates are easier prepared and less expensive. In addition, small molecule conjugates are less capable of evoking immune reactions, and finally they are easily analyzed and characterized *in vitro* and *in vivo* [1].

The design of a small molecule drug conjugate is a challenging task and several factors are very important for the success of such agents. In the development, a suitable linker must be chosen that satisfies the demands on the desired conjugate. When the conjugate shall be cleaved in a specific cell type, the linker must be stable enough that the conjugate reaches these cells but at the same time labile enough to be cleavable after cellular uptake. Additionally, when one of the small molecules of the conjugate is supposed to facilitate targeted delivery through its affinity to a molecular target, the linker has to be attached in a way that does not interfere with the ligand's binding to the target. The same holds true for drug conjugates that are connected by a non-cleavable linker.

8.2.1.2 Examples

Especially for cancer therapy, a number of small molecule drug conjugates have been developed. In most cases one pharmacophore of the conjugate is supposed to facilitate targeted delivery, while the other part often constitutes a potent cytotoxic agent. With growing incidence of cancer and highly cytotoxic agents as dominant treatment options that cause severe systemic side effects, the concept of transporting highly toxic agents selectively to the targeted cancer cells is getting more and more into the focus of drug discovery.

The concept of using a ligand for a specific receptor or transport system linked to the actual drug to generate a targeted drug conjugate with potentially lower systemic side effects is not very new, however. In 1993 a group at Hoechst Pharma reported the first series of drugs such as chlorambucil conjugated with cholic acid as ligand for targeted delivery. The conjugates had high affinity to bile acid transporters, which led to a specific uptake in the liver [2–4]. Similarly, conjugates of estradiol derivatives and mitomycin C have been reported in 1997 as potential targeted antitumor agents, and also in 1997 the concept to overcome multidrug resistance (MDR) in cancer cells by conjugation of cytotoxic agents with glutathione was described [5–7]. However, no such drug conjugate has received clinical significance so far, and research addressing small molecule drug conjugates for targeted delivery remained low for a long time until it significantly increased in the early 2000s potentially because new, more effective, and less toxic antitumor agents were required. Since then several targets and suitable ligands for targeted delivery of cytotoxic compounds to cancer cells were discovered and validated.

As a potentially valuable ligand for targeted delivery of small molecule drug conjugates, folate has been investigated based on the assumption that

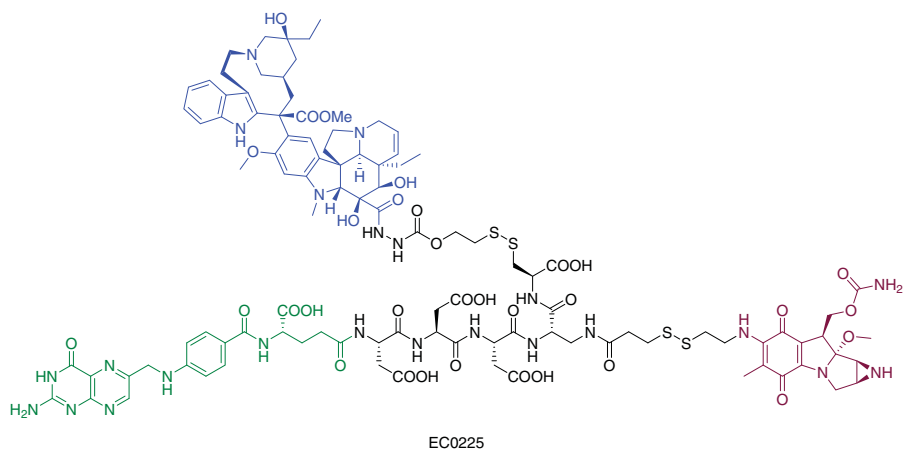
folate-containing conjugates are recognized by folate receptor (FR)-positive cells and only taken up by these cells, leading to selective cytotoxicity. FR α is highly expressed in several epithelial cancers and therefore seems suitable for targeted delivery to various tumors [1].

The trivalent drug conjugate EC0225 [8] (Figure 8.2) contains one folate unit, one moiety mitomycin, and a vinca alkaloid connected by a peptide linker. The cytotoxic components are attached via cleavable disulfide bonds to be released in the targeted FR-positive cancer cells. EC0225 exhibited promising preclinical anticancer activity and selective cytotoxicity for FR-positive cells, confirming the possibility of targeted delivery of folate conjugates. The agent entered clinical development and phase I was completed, but no results have been reported so far and no further trial was announced [9, 10].

The structure–activity relationship of folate–vinca alkaloid conjugates has later been investigated and revealed differences in potency for distinct linkers and different vinca alkaloids. A bio-cleavable linker containing a disulfide or an acylhydrazone bond was crucial for activity. Additionally, the regiochemistry of attaching the alkaloid to the linker had great influence on the conjugates' potency, and finally, some differences were observed for distinct alkaloids [11].

Folate targeting was furthermore investigated as strategy for anti-inflammatory drugs since FR are often present on activated macrophages. In this study, the drug conjugate EC0746 (Figure 8.3) composed of folate and the folate antagonist aminopterin connected by a sugar peptide and a cleavable disulfide bond was used. EC0746 predominantly targets folate receptor β (FR β), which in contrast to the reduced folate carrier (RFC) is mainly present on activated immune cells. Especially in synovial tissue of rheumatoid arthritis (RA) patients, FR β is highly expressed, making the targeted delivery to FR β -positive cells a very promising approach for the treatment of RA. Accordingly, the conjugate showed high specificity for FR β -expressing cells *in vitro*, and the targeted delivery produced by the conjugate drug led to about 40 times lower toxicity in animals. Moreover, the agent was effective in adjuvant-induced arthritis in rats and in animal models of autoimmune uveitis and autoimmune encephalomyelitis, which seemed to be due to inhibition of macrophage infiltration by EC0746 [12, 13].

At present, a number of folate targeting small molecule drug conjugates are investigated in clinical trials. Vintafolide (EC145, Vynfinit[®]) is composed of folate and vinblastine conjugated by a disulfide linker and is undergoing a phase II trial for non-small cell lung cancer as well as a phase I study for various advanced cancers. In addition, EC1456 that is a conjugate of folate and tubulysin B connected via a hydrazine-based linker has entered phase I. As imaging agent to determine the FR status of the tumors to be treated with either of the aforementioned agents, the conjugate etarfolatide (Folcepri[®]) of folate and technetium (^{99m}Tc) is co-developed. Results reported so far are promising and clinical development is ongoing [14–16]. In contrast, the development of the folate-targeted conjugate epofolate (BMS-753493) composed of folic acid and the microtubule-stabilizing agent epothilone was discontinued for lack of clinical efficacy [1, 17, 18].



EC0225

Figure 8.2 The small molecule drug conjugate EC0225 is composed of one unit folate for targeting (green), a vinca alkaloid (blue), and the cytotoxic antibiotic mitomycin (red). Release of the cytotoxic agents inside the targeted folate receptor-positive cells is enabled by the use of disulfide bonds and a hydrazone moiety in the linkers.

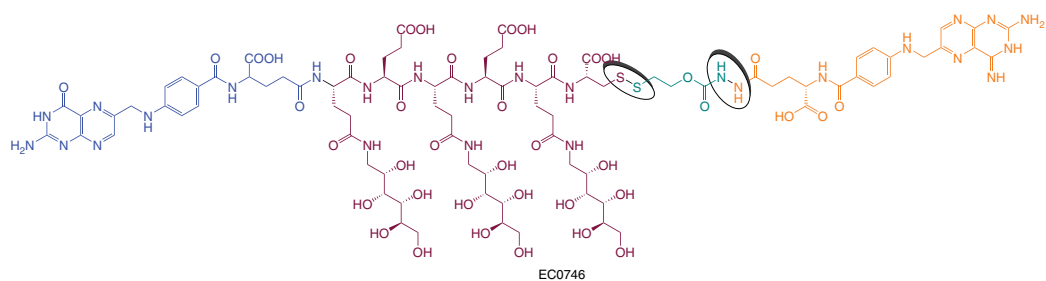


Figure 8.3 The drug conjugate EC0746 is composed of one folate unit (blue) for targeting and one unit aminopterin (orange) as delivered agent. Both are connected by an amino sugar linker (red) that is cleavable in two positions (circles) – at a disulfide bond and at a hydrazine moiety – to release the delivered drug in its free form.

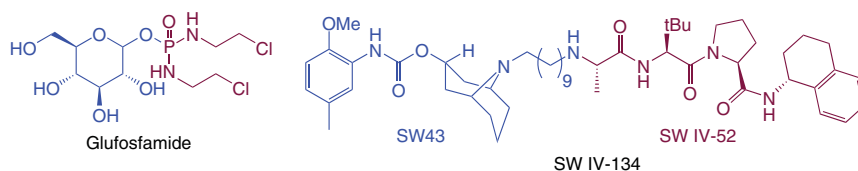


Figure 8.4 Drug conjugates glufosfamide and SW IV-134. Glufosfamide exploits glucose transporters for the targeted delivery of the alkylating agent ifosfamide (red). In SW IV-134, the sigma-2 ligand SW43 (blue) facilitates delivery of the Smac mimetic SWIV-52 (red).

A similar co-development of ^{99m}Tc -based conjugate for imaging and a conjugate delivering the potent cytotoxic agent tubulysin B has been reported, targeting the prostate-specific membrane antigen (PSMA), which is overexpressed on various tumors. However, the molecular structure of the ligand that facilitates the targeting of these agents has not been disclosed yet. Both conjugates are currently investigated in clinical phase I [19]. BIND-014, another PSMA-targeted small molecule conjugate, is undergoing clinical trials [1].

Many tumors overexpress glucose transporters and therefore glucose-conjugated anticancer drugs display another route to targeted delivery to tumors. Glufosfamide (Figure 8.4) is a conjugate of the alkylating agent ifosfamide and glucose and represents the most advanced agent of this class. The clinical data of glufosfamide is very promising and currently the targeted drug is investigated for metastatic pancreatic cancer in phase III [1, 20–22]. In addition, the clinical pipeline of targeted anticancer drugs contains a tripeptide (Asn–Gly–Arg) conjugate of tumor necrosis factor (TNF) α that is directed to the cell surface protein aminopeptidase N [23, 24] and angiopoietin 2-conjugated paclitaxel, which targets low-density lipoprotein receptor-related protein 1 [25, 26].

Also the polyamine transport system has been explored as potential target for specific delivery of drugs to cancer cells. As strongly proliferating cells, cancer cells often display an upregulated polyamine transport system, which might offer at least relative selectivity. To investigate the possibility of targeting the polyamine transport system, a drug conjugate consisting of spermine and podophyllotoxin as cytotoxic agent linked only by an acetamide moiety was evaluated in several cancer cell lines where it exhibited antiproliferative activity against two thirds of the tumor cell lines. *In vivo* in xenograft tumor models, the agent was effective after p.o. administration and induced apoptosis in cancer cells. Using a fluorescent probe with spermine moiety instead of the conjugate showed that by targeting the polyamine transport system, the conjugate accumulated in the sensitive xenograft cancer cells [27, 28].

Very recently, a promising small molecule drug conjugate, SW IV-134 (Figure 8.4), for the treatment of highly therapy-resistant pancreatic adenocarcinoma has been reported. The agent combines a sigma-2 receptor ligand with a mimetic of the proapoptotic protein second mitochondria-derived activator of caspases (Smac). This combination is especially promising for the treatment of pancreatic adenocarcinoma since this cancer overexpresses sigma-2 receptor and circumvents apoptosis by downregulation of X-linked and cellular inhibitors of

apoptosis proteins (XIAP and cIAP), which are inhibited, for example, by Smac. By targeting sigma-2 receptor, the drug conjugate is thought to be taken up especially by sigma-2-positive pancreatic adenocarcinoma cells where it can exhibit proapoptotic activity by mimicking Smac. Sigma-2 receptors are additionally valuable for targeted delivery since they are rapidly internalized upon ligand binding, which helps the uptake of the coupled agent into the targeted cells. Conjugation with the Smac mimetic did not interfere with the binding kinetics of the sigma-2 receptor ligand to its target, and the conjugate robustly activated apoptotic pathways including TNF α - and NF- κ B-dependent cascades. *In vitro*, SW IV-134 killed various pancreatic cancer cell lines with low micromolar IC₅₀ values. *In vivo*, the conjugate was effective against pancreatic tumors in syngenic and xenograft mouse models where it reduced tumor growth and robustly extended survival. In addition, no toxicity or adverse effects were observed related to the agent in the animal studies. The tumors of animals treated with SW IV-134 displayed a twofold increased caspase-3 activity and higher apoptosis rates. With these results, the conjugate offers a promising new experimental treatment option for poorly controllable pancreatic adenocarcinoma [29, 30].

Not especially for targeted delivery but with the intention to increase their half-life and bioavailability, irinotecan and chlorambucil have been linked by a cleavable linker, and the resulting conjugate (Figure 8.5) was investigated *in vitro* and *in vivo*. The linkage of the hydrophilic molecule irinotecan with the lipophilic and insoluble drug chlorambucil led to an amphiphilic drug conjugate that forms nanoparticles in aqueous environment. When taken up by cells, the cleavable ester bond that links the two molecules can easily be hydrolyzed by esterases, enabling the agents to exhibit their cytotoxic effects. A further advantage of the relatively large conjugate molecule is that it avoids to be transported by MDR proteins of tumor cells. *In vivo*, the conjugate showed a significantly longer retention in plasma compared with non-linked irinotecan and chlorambucil, which gives proof to the concept of drug conjugation for longer half-life. Additionally, the nanoparticles were found to accumulate in xenograft tumors that is thought to

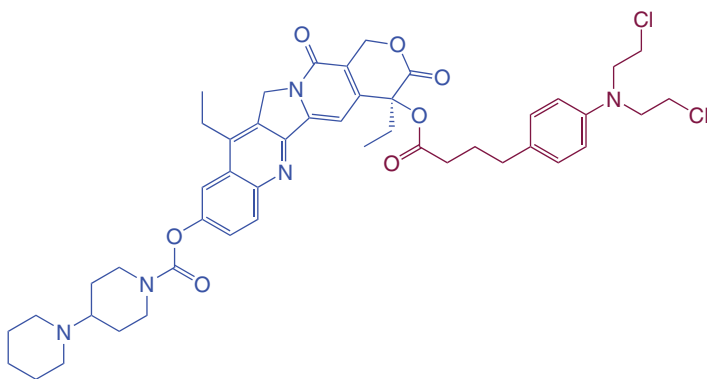


Figure 8.5 Conjugation of the two antitumor agent irinotecan (blue) and chlorambucil (red) led to an amphiphilic drug conjugate that showed enhanced plasma half-life and additionally self-assembled to nano-sized micelles, which can exploit the EPR effect for tumor targeting.

be due to the enhanced permeability and retention (EPR) effect in tumor tissue. All these effects together resulted in a significantly improved therapeutic efficacy of the conjugate *in vivo* compared with the co-administration of non-linked irinotecan and chlorambucil. The improved half-life combined with a passive tumor targeting by the EPR effect and a lower affinity to MDR proteins might hence be a valuable approach to make available antitumor agents more effective and reduce their side effects. However, it is questionable whether the characteristics of the irinotecan–chlorambucil conjugate and especially its self-assembly to nanoparticles will be observed for similar amphiphilic drug conjugates and can be translated into clinically efficacious agents [31].

In addition to drug–drug conjugates, a possible use in cancer therapy has also been described for fluorescence-labeled drugs. Fluorescence-labeled drugs have an important role in *in vitro* pharmacological test systems, but they might also be valuable in cancer diagnostics and early recognition of malignant cells. Since premalignant and malignant cells often display a higher level of cyclooxygenase 2 (COX-2) expression, detection of high COX-2 levels might help to detect tumors at early stage. Based on this hypothesis, a series of fluorescence-labeled COX-2 targeting NSAIDs was investigated, leading to the development of an indomethacin–fluorophore conjugate, which displayed COX-2 selectivity and was effective in detecting tumor xenografts in mice. Eventually, such drug-labeled conjugates can help to detect malignant cells earlier and thereby improve the efficacy of cancer treatment [32].

Although targeting of tumors and tumor cells clearly has the highest significance in drug conjugation and targeted delivery, drug conjugates for other purposes have been developed, as well.

The di- and tripeptide transporter PEPT1 is considered a valuable target for active uptake of prodrugs. With the help of this transport protein, the bioavailability of agents that fail to be absorbed by classical mechanisms might be improvable. The concept was investigated with a library of dipeptides conjugated to azidothymidine as model drug, and the study yielded some promising results, but further experiments are required to support PEPT1 as target and peptide drug conjugates as suitable ligands for active absorption of prodrugs [33].

A valuable role for small molecule drug conjugates might also evolve as antimicrobial agents where a conjugate of two agents might either help microbial uptake or lead to a dual cytotoxic activity. The concept of a dual antimicrobial activity was evaluated with a series of phenothiazinium–fluoroquinolone drug conjugates (Figure 8.6). Phenothiazinium derivatives exhibit antimicrobial

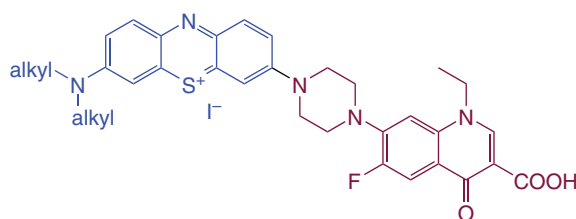


Figure 8.6 Conjugates of photosensitizers (blue) and norfloxacin (red) showed increased antibacterial activity compared with the single agents and lower lipophilicity.

Table 8.1 Properties of exemplary small molecule drug conjugates.

Disease/therapy	Target	Ligand	Delivered agent	References
—	Bile acid transporters	Cholic acid	Chlorambucil	[3, 4]
Cancer	Folate receptor	Folate	Vinca alkaloid	[8–12]
Autoimmune	Folate receptor	Folate	Aminopterin	[12]
Cancer	Folate receptor	Folate	Vinblastine	[14]
Cancer	Folate receptor	Folate	Tubulysin B	
Cancer imaging	Folate receptor	Folate	^{99m} Tc	[15]
Cancer	Polyamine transport system	Spermine	Podophyllotoxin	[27]
Cancer	<i>Passive delivery by the EPR effect</i>	Irinotecan + chlorambucil	[31]	
Cancer (pancreatic adenocarcinoma)	Sigma-2 receptor	SW43	SW IV-52 (Smac mimetic)	[29]
Prostate cancer	PSMA	<i>Not disclosed</i>	Tubulysin B	[19]
Prostate cancer imaging	PSMA	<i>Not disclosed</i>	^{99m} Tc	[19]
Antimicrobial	Topoisomerases + phototoxicity	—	Phenothiazinium + fluoroquinolone	[34]
Cancer	GLUT1	Glucose	Ifosfamide	[20, 22]
Cancer	Aminopeptidase N	Asn–Glu–Arg	TNFα	[23, 24]
Cancer	LRP1	Angiopep 2	Paclitaxel	[25, 26]

activity as photosensitizers, while fluoroquinolones inhibit bacterial topoisomerases and are among the most widely used anti-infective agents. Conjugation of a phenothiazinium dye with norfloxacin produced a series of potent antibacterial agents with strongly improved activity against *Staphylococcus aureus* and *Escherichia coli* combined with lower lipophilicity. The resulting compounds showed lower bactericidal concentrations in light than in darkness, confirming the dual activity of the photosensitizer. Although so far not much research has addressed this topic, small molecule drug conjugates might gain more significance in anti-infective agent development in the future (see Table 8.1) [34].

8.2.2 Antibody–Drug Conjugates/Protein–Drug Conjugates

Conjugation of therapeutic antibodies to small molecule drugs constitutes the most prominent strategy in drug conjugation at present. Several antibody–drug conjugates are investigated in clinical trials, and brentuximab vedotin as well as trastuzumab emtansine, the first representatives of this compound class, have entered the market yet.

8.2.2.1 Chances and Challenges

Monoclonal antibodies loaded with, for example, a cytotoxic agent (Figure 8.7) might generate a combination of several valuable activities. By binding to a specific antigen, a highly specific targeted delivery is generated that helps the conjugate enter the targeted cell type where it is dismantled and the small molecule cargo is released. The small molecule can then exhibit its cytotoxicity (or other effect) inside the targeted cell, but it would not become active in systemic circulation. In addition to facilitating the targeted delivery, the monoclonal antibody can also evoke its typical effects by blocking its antigenic target and, for example, inducing its degradation. On top of this superior selectivity and efficacy, an antibody–drug conjugate has a longer half-life than a small molecule applied alone.

The use of antibody–drug conjugates as targeted agents that predominantly release their cytotoxic load in cancer cells enables the application of drugs that alone have shown too high toxicity to be used as therapeutic agents. Many present antibody–drug conjugates consist of extremely potent antiproliferative agents with nano- to picomolar potency such as microtubule destabilizers and DNA minor groove binders.

To design a potent and efficacious antibody–drug conjugate, the triad of antibody, small molecule agent, and linker has to be adjusted carefully. For cancer treatment that is far the most prominent application of antibody–drug conjugates, an antibody with an antigen suitable for the cancer type intended to treat has to be selected. This antigen should show a high expression rate in tumor cells and at the same time a low density on other cells to retain a maximum selectivity of the antibody–drug conjugate. However, the antigen not only has to be expressed on the surface of the right cells, but it also must be internalized by the cells upon binding of an antibody to enable the release of a cytotoxic load inside the cell.

The linker of antibody–drug conjugates has to be stable enough that the cytotoxic agent is not set free in the circulation. Still, the small molecule agent must be quickly released in the targeted cells. After binding of the antibody–drug

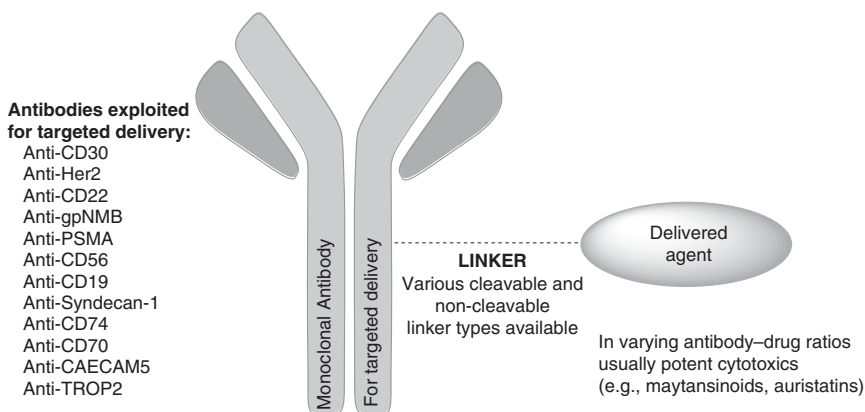


Figure 8.7 Schematic structure of antibody–drug conjugates.

conjugate to its antigen and subsequent internalization of the complex, the conjugate is taken up into lysosomes, which provide acidic environment and contain lysosomal enzymes. Under these conditions, the protein part of the antibody–drug conjugate is metabolized, but the linker design has to ensure that also the small molecule–linker bond is cleaved to set the drug free in its active form.

Naturally, there are also high demands to the antibody of an antibody–drug conjugate. The antibody must not evoke an immune response and therefore is humanized or human in the optimal case. In addition, attachment of a linker and a small molecule agent to the antibody might disturb the binding of the antibody to its antigen or the pharmacokinetic properties of the conjugate [35].

8.2.2.2 Examples

In contrast to small molecule drug conjugates and polymer–drug conjugates whose developmental status is predominantly in an early preclinical state, antibody–drug conjugates are intensively investigated in clinical trials, and two representatives have already entered the market.

Brentuximab vedotin was the first approved agent of this drug class. It consists of a chimeric anti-CD30 antibody and monomethyl auristatin E (Figure 8.8), which are connected through a disulfide bond and a valine–citrulline dipeptide, a linker that is cleavable by cathepsin B. In average, 4 equiv. of the small molecule agent is attached to one antibody. The small molecule part of the conjugate monomethyl auristatin E inhibits the polymerization of tubulin and thereby prevents mitosis. However, the agent is too toxic to be applied alone. The antigen CD30 that is recognized by brentuximab vedotin is a transmembrane protein of the TNF superfamily. Physiologically, CD30 is only expressed on activated B and T cells as well as natural killer cells. Overexpression of CD30 is found

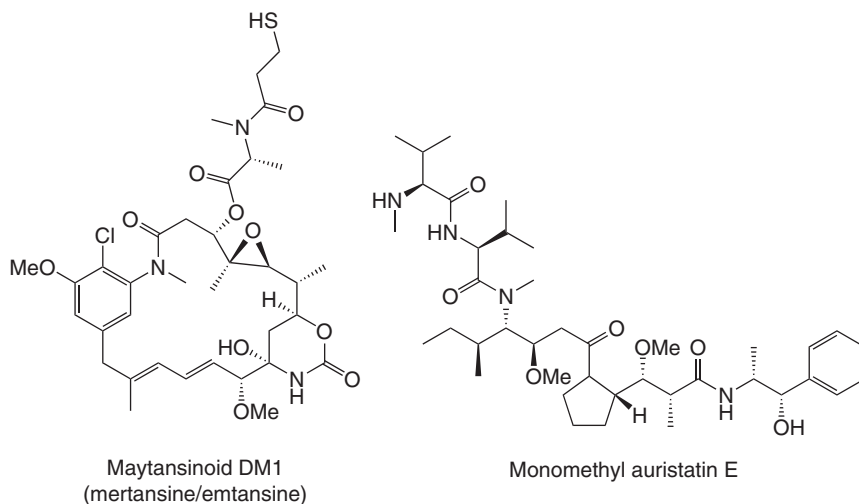


Figure 8.8 Cytotoxic agents that are frequently used as small molecule component of antibody–drug conjugates.

in Hodgkin lymphoma (HL) and anaplastic large cell lymphoma, which makes CD30 a valuable target for the treatment of HL. Hence, anti-CD30 antibodies have been investigated for the treatment of HL, but their efficacy was limited [36]. In contrast, brentuximab vedotin in which the antibody especially facilitates targeted delivery of the cytotoxic agent showed impressive results for the treatment of HL with high response and complete response rates even among strongly pretreated patients. As serious side effects hematological toxicities and peripheral neuropathies were reported. It is also to be noted that HL is considered one of the most curable forms of cancer. Still, based on its great clinical data, brentuximab vedotin received fast track approval for HL. It was an impressive pioneer of antibody–drug conjugates and might further enhance research in this field since it proved that high clinical efficacy can be achieved by attaching a potent cytotoxic agent to an antibody, which alone is only moderately effective [37–40].

After brentuximab vedotin, *trastuzumab emtansine* was the second antibody–drug conjugate to receive approval. It is a conjugate of the monoclonal humanized anti-Her2 antibody and the tubulin inhibitor DM1 (emtansine; Figure 8.8). Structurally, emtansine is a macrocyclic maytansinoid that inhibits mitosis and displays a very potent cytotoxic agent. Her2 belongs to the epidermal growth factor receptor family and constitutes a receptor tyrosine kinase. Her2 activation inhibits apoptosis via mechanistic target of rapamycin (mTor) and stimulates cell proliferation via the mitogen-activated protein kinase (MAPK) pathway. In around 20% of breast cancers and some other cancers, Her2 is overexpressed, which is associated with poor prognosis [37, 41–46].

The two components of trastuzumab emtansine are linked by a non-cleavable *N*-succinimidyl-4-(*N*-maleimidylmethyl)cyclohexane-1-carboxylate linker that is attached to DM1 at its free thiol function and to the antibody at lysine residue side-chain amines. The drug–antibody ratio is 3.5 on average. Since trastuzumab alone already constitutes a potent agent that inhibits tumor growth by blocking the receptor tyrosine kinase Her2, trastuzumab emtansine exhibits a dual pharmacological activity against Her2-positive cancer cells by blocking the receptor tyrosine kinase and additionally delivering a highly potent cytotoxic agent. Experimental data indicated that the conjugate preserves the inhibitory activity of free trastuzumab on Her2 and its Fcγ receptor-mediated activation of antibody-dependent cellular cytotoxicity. *In vitro*, trastuzumab emtansine robustly inhibited growth of breast cancer cells even for trastuzumab-resistant cell lines. Comparable effects were observed in xenograft tumor models in mice. Clinical results from studies with refractory breast cancer patients showed good response rates with trastuzumab emtansine as single agent. Thrombocytopenia and fatigue were reported as the most common adverse events. For first-line treatment, trastuzumab emtansine was evaluated in comparison with trastuzumab plus docetaxel for advanced or metastatic breast cancer. Both therapeutic regimens showed equal efficacy, but trastuzumab emtansine was superior concerning the safety profile. In phase III trastuzumab emtansine was superior to the combination of lapatinib and capecitabine in the treatment of advanced, trastuzumab-resistant, Her2-positive breast cancer and prolonged survival by 5.8 month compared with the combination. In addition,

the antibody–drug conjugate had a preferable toxicity and safety profile. This data led to the approval of trastuzumab emtansine as promising new alternative in Her2-positive breast cancer management [37, 41–46].

Succeeding brentuximab vedotin and trastuzumab emtansine, several other antibody–drug conjugates are in the clinical pipeline targeting various antigens and delivering diverse highly potent cytotoxic agents.

Inotuzumab ozogamicin consists of a monoclonal humanized anti-CD22 antibody linked to calicheamicin via a cleavable acid-labile hydrazone-based linker. CD22 is a sialic acid-binding immunoglobulin-like lectin expressed solely on immature and mature B cells but not on hematopoietic stem cells. The surface glycoprotein is probably important for B-cell activation and homing, but its exact role is not yet clear. Upon binding of a ligand or antibody, CD22 is internalized, which enables the delivery of cytotoxic agents with anti-CD22 antibody–drug conjugates. In several lymphatic neoplasias such as B-cell non-Hodgkin lymphomas (NHL), follicular lymphomas, and B-cell acute lymphoblastic leukemia, CD22 is expressed and offers a target to treat these cancers. However, anti-CD22 antibodies alone are not a sufficiently effective as single treatment option. In inotuzumab ozogamicin the DNA minor groove binder calicheamicin is delivered to such CD22-positive cells, which is a bacterial toxin from *Micromonospora echinospora* and induces DNA double-strand scission. Calicheamicin is an extremely potent cytotoxic and therefore not suitable as single agent. In the conjugate, six equivalents of calicheamicin are attached to the antibody on average. The conjugate is presently investigated in clinical trials. So far, clinical results suggested considerably lower response rates than reported for the approved antibody–drug conjugates, and additionally, severe thrombocytopenia and neutropenia were observed. A study for refractory CD22+ NHL was discontinued since the required doses could not be applied, but the conjugate is currently further investigated for NHL [37, 47–52].

The aforementioned small molecule cytotoxic agents are also used in several other antibody–drug conjugates. The maytansinoids DM1 and DM4 can also be found in the conjugates lorvotuzumab mertansine (IMGN901), coltuximab ravtansine (SAR3419), and indatuximab ravtansine (BT-062), while the conjugates glembatumumab vedotin (CDX-011) and PSMA-vc-MMAE are composed of antibody and monomethyl auristatin E [37].

Glembatumumab vedotin combines a monoclonal human antibody targeted to glycoprotein nonmetastatic melanoma protein B (gpNMB) and the tubulin inhibitor monomethyl auristatin E conjugated via a valine–citrulline dipeptide, which is cleavable by proteases. The antigen of this agent is present on melanoma and breast cancer cell lines. The conjugate has shown good response rates in first clinical trials for unresectable melanoma and metastatic breast cancer with acceptable safety profile. Besides neutropenia most toxic effects were restricted to the skin. At present, two phase II trials for melanoma and breast cancer are ongoing [37, 53–56].

Monomethyl auristatin E was furthermore conjugated with a monoclonal humanized antibody targeting the PSMA, a transmembrane glycoprotein predominantly expressed in prostate epithelium and highly overexpressed in prostate cancer. Antibody and monomethyl auristatin E are connected via a

cleavable citrulline–valine linker. The conjugate entered clinical trials, but so far it could not match the expectations [37, 57–59].

Lorvotuzumab mertansine targets CD56, a neural cell adhesion molecule that is overexpressed in various cancers such as neuroblastoma, multiple myeloma, small cell lung cancer, and ovarian cancers. Physiologically, CD56 is only expressed on natural killer cells, which predicts good tumor selectivity. In lorvotuzumab mertansine, antibody and DM1 are connected via a cleavable disulfide linker that is stabilized by steric hindrance. In clinical trials for different cancers, the conjugate showed a good safety profile and some encouraging complete and partial responses. Currently, a phase II trial for small cell lung cancer is recruiting participants [37, 60, 61].

Coltuximab ravtansine is composed of a monoclonal humanized anti-CD19 antibody and the maytansinoid DM4 that is attached to the protein via a cleavable disulfide-based linker. The conjugate's antigen CD19 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. It is expressed on B cells in all developmental stages as well as in all types of B-cell lymphomas and lymphoblastic leukemia. First clinical results from heavily pretreated NHL patients indicate good response rates to coltuximab ravtansine as well as a favorable safety and toxicity profile. Especially hematological toxicity was low, which might offer the possibility to combine the conjugate with other agents to increase therapeutic efficacy. At present, coltuximab ravtansine is investigated in phase II alone or in combination with rituximab [37, 62–64].

Another antibody–drug conjugate containing the maytansinoid DM4 is *indatuximab ravtansine*. The conjugate targets the transmembrane heparan sulfate proteoglycan syndecan-1 (CD138), which is present in many different tumors. Indatuximab ravtansine contains a chimeric monoclonal anti-CD138 antibody to which DM4 is attached via a cleavable disulfide linker. A first clinical trial with multiple myeloma patients reported some response combined with an acceptable safety profile [37, 61, 65].

Based on the obvious clinical efficacy of the previously described agents, nearly all antibody–drug conjugates that are currently in clinical development contain the same extremely potent cytotoxic compounds, and there are several more antibody–drug conjugates of a maytansinoid or an auristatin derivative in development. They are targeting diverse antigens for the treatment of various cancers, but their fundamental structure is similar to the described agents including the commonly used linkers. However, exceptions are made with conjugates of doxorubicin, SN38, and irinotecan as small molecule agents, which are in earlier stages of development [37].

Doxorubicin was conjugated to the monoclonal humanized anti-CD74 antibody milatuzumab for the potential treatment of lymphoma and myeloma. The antigen CD74 is a membrane protein belonging to the MHC class II chaperones and is highly expressed on malignant B cells, which makes it a valuable target for the treatment of B-cell lymphoma. In addition, CD74 is internalized upon antibody binding and therefore is suitable for targeted delivery of cytotoxic agents to CD74+ cancer cells. Hence, the anti-CD74 antibody milatuzumab was conjugated with several potent small molecule antitumor agents such as doxorubicin,

bortezomib, and SN38, but so far only the doxorubicin conjugate entered clinical development. However, no data has been reported yet [66–68].

MDX-1203 is designed to deliver a derivative of the DNA minor groove binding and alkylating agent duocarmycin to CD70+ cells. CD70 is a co-stimulatory cell surface protein involved in immune response and is found upregulated in several cancers. The conjugate is currently investigated in phase I [69].

Two more promising antibody–drug conjugates in clinical development contain the topoisomerase inhibitor SN38, which is the active metabolite of irinotecan as small molecule agent. In labetuzumab-SN38 (IMMU-130), SN38 is attached to a monoclonal humanized anti-carcinoembryonic cell adhesion molecule 5 (CEACAM5) antibody, while in IMMU-132 the antibody is directed against TROP-2. Both antigens CEACAM5 and TROP-2 (tumor-associated calcium signal transducer 2 (TACSTD2)) are found on many solid tumors. In contrast to most other small molecule agents in antibody–drug conjugates, SN38 is only moderately cytotoxic. Accordingly, the conjugates showed a very favorable safety and toxicity profile in phase I and II. In addition, after binding of the antibody to its antigen, SN38 is slowly released (about 50% in 20 h) from the conjugate and can enter surrounding tumor cells through diffusion. Both agents are investigated in clinical phase II and so far, the results suggested good efficacy [70, 71].

The number of antibody–drug conjugates in clinical development and the fact that many more antibody–drug conjugates are in earlier stages of development point out the high potential of this class of drugs and justify the hope that several new effective therapeutic options might arise with such conjugates in the near future (Table 8.2).

8.2.3 Polymer–Drug Conjugates

Polymer–drug conjugates are usually built up of small molecule drug(s) attached to a water-soluble and often biodegradable polymeric carrier. The resulting conjugates display a high molecular weight (>40 kDa) and size (5–100 nm) and can therefore be considered nanomedicines. The strategy of combining polymers and small molecules is rather old, but so far no polymer–drug conjugate has entered the drug market. However, there are several agents in far stages of clinical development.

In the field of therapeutic proteins, polymer conjugates already have a high significance since the stability of proteins in the circulation can be strongly improved by introduction of a suitable polymer. In addition, the potential of therapeutic proteins to exhibit immune-sensitizing effects can be reduced by conjugation to a polymer.

8.2.3.1 Chances and Challenges

Conjugation of a small molecule agent and a polymer offers many valuable opportunities for drug development. With the help of a water-soluble polymeric carrier, the solubility of a drug can strongly be improved. In addition, a completely different pharmacokinetic profile can be achieved by this strategy.

In targeted delivery, polymer–drug conjugates have a growing significance. Their characteristic as macromolecules enables a specific targeting to solid

Table 8.2 Selected antibody–drug conjugates in clinical development.

Agent	Antibody/antigen	Small molecule drug	Developer	Disease (clinical phase)	References
Brentuximab vedotin	Anti-CD30	Monomethyl auristatin E (tubulin inhibitor)	Seattle Genetics/Takeda	Hodgkin lymphoma (approved)	[37–40]
Trastuzumab emtansine	Trastuzumab/anti-Her2	Emtansine/DM1 (tubulin inhibitor)	Celltech/Pfizer	Her2+ breast cancer (approved)	[37, 41–46]
Inotuzumab ozogamicin	Anti-CD22	Calicheamicin (DNA minor groove binder)	Genentech (Roche)	Lymphatic neoplasias (phase II)	[37, 47–52]
Glembatumumab vedotin	Anti-gpNMB	Monomethyl auristatin E	Celldex Therapeutics	Melanoma, breast cancer (phase II)	[37, 53–56]
—	Anti-PSMA	Monomethyl auristatin E	Progenics	Prostate cancer (phase I)	[37, 57–59]
Lorvotuzumab mertansine	Anti-CD56	DM1	ImmunoGen		[37, 60, 61]
Coltuximab ravtansine	Anti-CD19	DM4	ImmunoGen/Sanofi	Non-Hodgkin lymphoma, leukemia (phase II)	[37, 62–64]
Indatuximab ravtansine	Anti-syndecan-1 (CD138)	DM4	Biotest	Various cancers (phase II)	[37, 61, 65]
Milatuzumab doxorubicin	Anti-CD74	Doxorubicin	Immunomedics	Multiple melanoma (phase II)	[66–68]
MDX-1203	Anti-CD70	Duocarmycin	BMS	Non-Hodgkin lymphoma, renal carcinoma (phase I)	[69]
Labetuzumab-SN38	Anti-CEACAM5	SN-38	Immunomedics	Breast cancer, colorectal cancer, lung cancer (phase II)	[70, 71]
IMMU-132	Anti-TROP2	SN-38	Immunomedics	Solid tumors (phase II)	[70, 71]

gpNMB, Glycoprotein nonmetastatic melanoma protein B; PSMA, prostate-specific membrane antigen; CEACAM5, carcinoembryonic cell adhesion molecule 5; TROP2-TACSTD2, tumor-associated calcium signal transducer 2.

tumors that is based on the so-called EPR effect. The EPR effect leads to increased accumulation of macromolecules in solid tumors and is based on a poor architecture of vessels in solid tumors, an increased production of factors enhancing vascular permeability, and disrupted lymphatic drainage of tumor tissue. This effect offers another principle of delivering agents selectively to solid tumors, thereby reducing side effects and increasing cytotoxicity to tumor cells by application of conjugates of small molecule antitumor agents with biocompatible polymers.

Furthermore, the conjugation of small molecule drugs to a polymer offers the possibility of co-delivering more than one bioactive small molecule by attaching diverse agents to the carrier, and with a specific drug–drug–carrier ratio in this co-delivery, additionally even the proportion of the small molecule agents may be controllable. The polymer–drug conjugate-based combination therapy opens new opportunities in the development of personalized medicines and could strengthen the progress of individualized therapies.

Finally, with the development of modern polymers, the architecture of polymer–drug conjugates can be increasingly governed. This enables, for example, the design of nano-sized agents that self-assemble to micelles further improving the solubility and pharmacokinetics of the resulting conjugates.

Hence, polymer–drug conjugates offer several advantages and chances, but there are also new challenges to be addressed with this class of agents. Intense research focuses on the discovery of new biocompatible polymers with improved properties such as solubility and increased drug load.

Another demanding task is the analytical and *in vitro* characterization of polymer–drug conjugates. Drug-loaded polymers constitute very complex structures with several potentially risk-fraught features. Therefore, many characteristics of these macromolecules need to be determined such as molecular weight and size, drug–polymer ratio and drug impurities, hydrophilic–hydrophobic ratio and solubility, zeta potential, and many others.

The design of the linker attaching the small molecule drug to the polymeric carrier is another challenging topic in polymer–drug conjugate development. In contrast to protein–drug conjugates, many polymers are slower and less easily degraded when taken up by, for example, the targeted tumors. Therefore, the use of a suitable linker seems even more important for this class of conjugates to enable a specific but quick release of the drug payload. For this purpose, diverse linkers have been developed that are cleavable by, for example, lysosomal enzymes or pH sensitive. Especially for polymer carriers that deliver more than one agent, linker chemistry is crucial.

Another crucial demand on a polymer–drug conjugate is its drug load or in other words the polymer–drug ratio, and with a co-delivery of more than one agent, also the drug–drug ratio is highly important. These relationships must carefully be chosen and then realized in the production of the polymer–drug (drug) conjugate, which might be additionally more complicated when the production is increased to large scale.

8.2.3.2 Examples

Besides specifically addressing a receptor or other protein that is overexpressed in tumor cells, the EPR effect as another concept of targeted delivery to tumors is gathering increasing interest. There are several approaches to exploit the EPR effect for cancer treatment, and there are clinical results available for the first representatives of this drug class.

Among the most advanced polymer–drug conjugates is paclitaxel poliglumex (PPX, CT-2103, Opaxio; Figure 8.9), which has already completed several clinical trials for various solid tumor diseases. Paclitaxel is a widely used microtubule inhibitor that is active against a variety of tumors, but its use is restricted by high systemic toxicity, and the fact that the agent is poorly distributed to tumor cells limits its efficacy. In the water-soluble conjugate PPX, the microtubule inhibitor is attached to a poly-L-glutamic acid polymer with an average weight of 38.5 kDa. Small molecule and polymer are linked via an ester moiety, which can easily be cleaved in the acidic environment of a tumor. In average, there is one paclitaxel molecule on about 11 glutamic acid units. Polymer size and ratio of paclitaxel to glutamic acid units vary, however. With the help of the EPR effect, PPX accumulates in solid tumors where it is taken up by tumor cells via endocytosis. The resulting endosomes fuse with lysosomes inside the cells, and an acidic pH for the release of the small molecule agent by ester hydrolysis is generated. For the release of paclitaxel from the conjugate, the lysosomal enzyme cathepsin B is required, which might additionally produce selectivity for tumors with cathepsin B overexpression. *In vivo*, the conjugate produced an about 12-fold increased tumor exposure compared with free paclitaxel. When administered alone or in combination with gemcitabine or vinorelbine, PPX exhibited reduced hematologic toxicity compared with free paclitaxel. Additionally, the combination of PPX and radiation therapy showed promising results and acceptable systemic toxicity. In contrast, the combination with temozolomide led to high toxicity, and there are reports of increased neurotoxicity. Concerning clinical efficacy, PPX produced comparable results as free paclitaxel. Phase III data of PPX was somewhat disappointing due to a lack of (superior) efficacy. However, deeper analysis of these data indicated a gender-dependent activity profile with better efficacy and prolonged

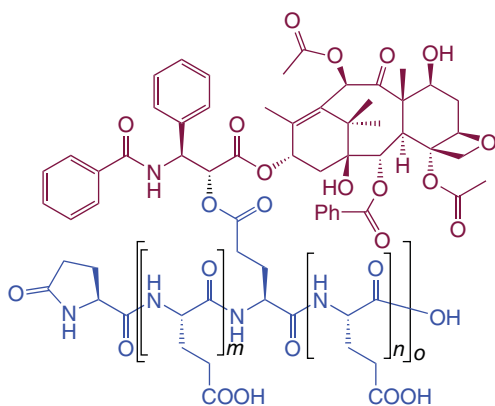


Figure 8.9 In paclitaxel poliglumex, the tubulin inhibitor paclitaxel (red) is attached to poly-L-glutamic acid (blue) via an ester group. The conjugate has an average weight of 38.5 kDa and carries one paclitaxel unit per 11 units of glutamic acid.

survival in women, which is thought to be due to higher cathepsin B activity that increases the release of free paclitaxel [72–75].

Poly-L-glutamate camptothecin (PGA-CPT, CT-2106), a very similar drug conjugate as PPX, has been applied to clinical studies as well. The conjugate improves stability and solubility of camptothecin and additionally ought to improve tumor accumulation with the help of the EPR effect. In phase I, PGA-CPT displayed a more controllable toxicity compared with free camptothecin, and the conjugate produced a prolonged release of free camptothecin [76]. A phase I/II study was completed but no results were reported.

With *N*-(2-hydroxypropyl)methacrylamide copolymer-linked pirarubicin and *N*-acetylglucosamine polyethylene glycol (PEG)-linked doxorubicin, two very similar approaches as PPX have been described. Both cytotoxic antibiotics suffer from comparable limitations of high systemic toxicity, and tumor targeting might improve their clinical applicability.

In the water-soluble *N*-(2-hydroxypropyl)methacrylamide conjugate of pirarubicin, small molecule and polymer are linked via a hydrazone bond that is similarly cleavable in acidic environment of a tumor but might be more stable against enzymatic hydrolysis than an ester, which could eventually further reduce systemic exposure to the free drug. The conjugate led to significant accumulation in tumors *in vivo* and produced robust antitumor activity in mice with strongly reduced toxicity compared with free pirarubicin. However, clinical studies have to prove the actual clinical value of the pirarubicin polymer conjugate [77–79].

N-acetylglucosamine PEG-linked doxorubicin combines two principles of tumor targeting. The macromolecule again exploits the EPR effect to accumulate in tumors and additionally contains an *N*-acetylglucosamine moiety that as saccharide is recognized on cellular surfaces and is thought to enhance cellular uptake. The conjugate showed reduced cytotoxicity *in vitro*, but no data is available on the agent's anticancer activity *in vivo* [80].

Additionally, very promising preclinical and clinical data is available for a PEG-conjugated fragment SN38 of the topoisomerase inhibitor irinotecan. The PEGylated active moiety of irinotecan EZN-2208 (Figure 8.10) constitutes a water-soluble 40 kDa macromolecule in which the small molecule is attached to the polymer via a glycine linker. The first advantage of the conjugate is its strongly improved solubility compared with the fragment SN38, which is not suitable for clinical development due to insolubility. The conjugate displayed a long circulation time and accumulation in tumors with the help of the EPR effect and was very effective in several *in vivo* tumor models. Especially for xenograft neuroblastoma, the conjugate showed very promising activity in mice [81]. Several clinical trials have been conducted with etirinotecan (NKTR-102, EZN-2208), and the agent showed acceptable tolerability. For several advanced solid tumors including pediatric neuroblastoma and metastatic breast cancer, beneficial clinical efficacy was observed, but there are also results indicating that NKTR-102 alone may not be sufficient to treat some forms of cancer. Currently, there are several active clinical trials with etirinotecan of which a phase III study for metastatic breast cancer is most sophisticated [82–87]. NKTR-105 comprises a very similar PEG conjugate of docetaxel that has successfully completed a clinical phase I trial [88, 89].

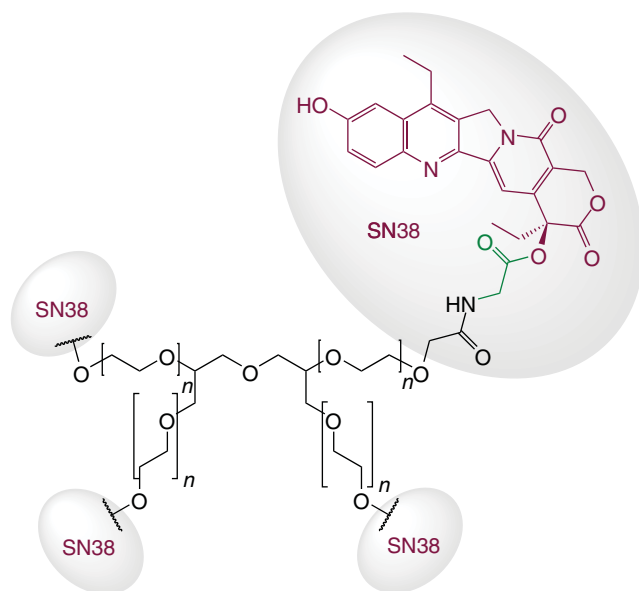


Figure 8.10 EZN-2208 is a PEG conjugate of SN38 (red) that is the active moiety of irinotecan. The approximately 40 kDa polymeric carrier is branched and holds 4 units of the active agent. With the help of the hydrophilic carrier system, the conjugate is highly soluble (180 mg/mL).

Also platinum-based anticancer agents are currently investigated as potential candidates for polymer–drug conjugates. AP5346 (ProLindac) is a hydroxypropylmethacrylamide-linked 1,2-diaminocyclohexane platinum derivative. The platinum derivative is attached to the polymeric carrier by a pH-sensitive chelator instead of a linker that releases the cytotoxic agent in acidic environment, which is usually present in tumors. AP5346 showed promising data in clinical phase I and II for ovarian cancer. In the trials AP5346 was equally effective or superior to oxaliplatin but displayed lower systemic toxicity [90].

There are several more polymer–drug conjugates in clinical trials at present (Table 8.2), and there is justified hope that advanced candidates might receive approval in the near future.

Conjugation of small molecule drugs with a polymer also offers the opportunity to deliver more than one agent to tumors that could generate synergies. An exemplary polymer–drug–drug conjugate is a mPEG-*b*-P(LA-*co*-MCC/OH) polymer connected with dimethylcantharidin and oxaliplatin. In the agent, the serine/threonine phosphatase 2A (PP2A) inhibitor dimethylcantharidin is attached to the carrier via an ester bond. Platinum is present as Pt(IV), which has to be reduced to Pt(II) for antitumor activity. Hence, reduction and acidic hydrolysis are required to release the small molecule anticancer drugs. This might offer a dual selectivity for tumor cells in which the concentration of redox-active molecules such as glutathione should be higher than in normal (resting) cells and the pH ought to be lower. Combined with the EPR effect, this might lead to good tumor selectivity and low systemic toxicity. In addition, both small agents are thought to generate a synergistic antitumor effect since

oxaliplatin induces DNA damage and inhibition of PP2A prevents DNA repair mechanisms. *In vitro*, the release of the small molecules from the carrier was more pronounced in the presence of higher concentrations of redox-active agents (5 mM sodium ascorbate) and under acidic conditions (pH 5.0). Furthermore, it was rapidly internalized by ovarian cancer cells (SKOV-3) *in vitro* and exhibited higher cytotoxicity than either of the small molecule agents or their combination [91]. So far, no *in vivo* data has been reported to support these promising *in vitro* results.

In addition to their most prominent role as experimental cancer therapeutics, small molecule polymer–drug conjugates have also been investigated for other purposes. For example, an ibuprofen–PEG conjugate has been reported for use as transdermal prodrug. For transdermal delivery the PEG carrier and the small molecule were linked via a 2-mercaptoethyl residue according to observations that sulfur-containing amides and esters enhance transdermal transport and increase stability against oxidative processes in the skin. *In vitro*, the conjugate displayed good stability at a variety of pH values but quickly released the small molecule in human plasma due to its esterase activity. However, not ibuprofen was released as drug but its 2-mercaptoethyl derivative since the thioester between PEG carrier and mercaptoethyl ibuprofenate is less stable against hydrolysis. Unfortunately, nothing is reported about the pharmacological activity of the released mercaptoethyl derivative of ibuprofen (Table 8.3) [99].

8.3 Linker Chemistry

8.3.1 Demands on a Linker or How to Link Drugs

Drug conjugates are characterized not only by their pharmacologically active agents but also by the way they are attached to each other. The linkage influences the stability of the conjugate in the circulation, the compartment where it is activated by cleavage, and the speed of its activation. In addition, the linker can influence the conjugates' solubility and might even alter the pharmacological potency of the agents it connects. Considering this high influence, the selection and design of a suitable linker for the desired drug conjugate is crucial.

The regiochemistry of the linkage can have significant impact on the efficacy and behavior of the conjugate. Especially, the linker may not affect the affinity of the ligand part of the conjugate that is supposed to facilitate targeted delivery since a compromised affinity of the targeting ligand to its target structure could disrupt the desired selectivity. Such influences on target binding not only include covalent bonds but may also result from intramolecular interactions between targeting ligand and linker or delivered cargo. The discovery of suitable positions for linker attachment might therefore require intensive studies on the structure–activity relationships [1].

Often, the use of a linker that increases the conjugates' polarity and hydrophilicity is advantageous. On the one hand, this improves the solubility of the conjugate, and on the other hand, a polar linker can prevent nonselective uptake of the conjugate into cells by diffusion, which would enhance selectivity [1].

Table 8.3 Advanced polymer–drug conjugates and their properties.

Agent	Polymer	Small molecule drug	Developer	Disease (clinical phase)	References
Mureletcan MAG-CPT (PNU166148)	HPMA	Camptothecin	—	Cancer (phase I, terminated)	[92–95]
PNU166945	HPMA	Paclitaxel	—	Cancer (phase I, terminated)	[96]
FCE28068	HPMA	Doxorubicin	—	Breast cancer, non-small cell lung cancer (phase II, terminated)	[97]
Paclitaxel poliglumex (Opaxio)	Poly-L-glutamic acid	Paclitaxel	—	Solid tumors (phase III, terminated)	[72–75]
PGA-CPT (CT-2106)	Poly-L-glutamic acid	Camptothecin	Cell Ther.	Solid tumors (phase II)	[76]
AP5346 (ProLindac)	HPMA	1,2-Diaminocyclohexane-platinum	Access Pharma	Cancer (phase I/II)	[90]
—	HPMA	Pirarubicin	—	Cancer (preclinical)	[77–79]
—	N-Acetylglucosamine PEG copolymer	Doxorubicin	—	Cancer (preclinical)	[80]
NKTR-102 (etirinotecan pegol)	PEG	SN38 (active moiety of irinotecan)	Nektar Therapeutics	Solid tumors (phase II/III)	[82]
NKTR-105	PEG	Docetaxel	Nektar Therapeutics	Solid tumors (phase I)	[98]
—	mPEG- <i>b</i> -P (LA- <i>co</i> -MCC/OH)	Oxaliplatin + dimethylcantharidin	—	Cancer (preclinical)	[91]
—	PEG	Ibuprofen	—	Inflammatory (preclinical)	[99]

HPMA, (2-hydroxypropyl)methacrylamide.

For polymer–drug conjugates and antibody–drug conjugates, also the carrier–drug ratio is very important since it determines the amount of the small molecule drug that is delivered to the targeted tissue. In antibody–drug conjugates, the linker is usually attached to the side chains of lysine or cysteine residues. The process of conjugation determines the amount of drug attached to the antibody and generates a heterogeneous mixture of conjugates with different antibody–drug ratios and varying sites of attachment.

8.3.2 Linker Types

Several types of linkers (Figure 8.11) have been developed for the high requirements on this part that unites all kinds of drug conjugates. Linkers can generally be divided into cleavable and non-cleavable. Cleavable linkers are labile against acid, reducing agents, or degradation by enzymes. The choice of the right linker strongly depends on the application of the desired drug conjugate.

Among usual acid-cleavable linkers such as acetals, hydrazones, imines, and esters, the hydrazone-based moieties are most prominent. At the neutral pH value of the blood, hydrazones are quite stable, but as soon as the pH value is changed to acidic environment, the hydrazone is quickly hydrolyzed. Hydrazone-based linkers are hence suitable for drug conjugates that are

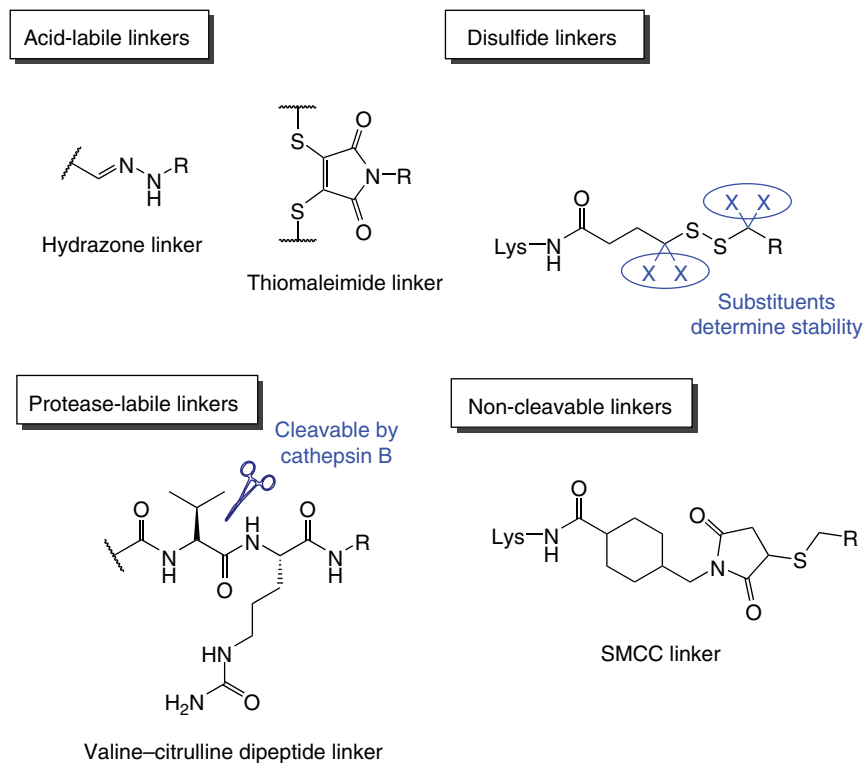


Figure 8.11 Frequently used linkers.

supposed to be stable in circulation but cleaved in acidic environment such as lysosomes (pH 4.5–5.0), tumors, or inflamed tissues. *In vivo*, hydrazone linkers are associated with a significant cleavage and release of the small molecule agent in the circulation, which limits their use. Alternative acid-labile linkers are, for example, based on thiomaleamic acid [1, 100–102].

Disulfide bonds represent the most prevalent reductive cleavable linkers. They are less easily degraded and therefore generate higher plasma stability. Disulfide-linked drug conjugates are cleaved in lysosomes with the help of reductive agents such as glutathione. Their stability can to a certain extent be controlled by substituents next to the disulfide bond where higher substitution causes steric hindrance that makes the disulfide more stable [1, 101, 102].

Probably the most selectivity concerning cleavage in a specific compartment can be generated by linkers that are labile to hydrolysis by a certain enzyme. For this purpose a linker composed of a valine–citrulline dipeptide has been developed that is a substrate of the lysosomal enzyme cathepsin B. Drug conjugates with this type of spacer can – at least theoretically – only be hydrolyzed after they have entered the cell that would strongly reduce the systemic toxicity of their single components. Alternative dipeptides that are relatively stable in circulation but easily cleaved by proteases are phenylalanine–lysine and valine–lysine. Similar as cathepsin B, also the lysosomal enzyme β -glucuronidase can be exploited as linker-cleaving enzyme when β -glucuronic acid-based linkers are used [101–104].

Another interesting approach to enzymatically cleavable linkers is based on imide bonds. Through reaction of a thiol and an azidoformate, such imide moieties can easily be introduced between two components of a drug conjugate with good to excellent yields, and no complex multistep reaction is required to link the two drugs. The resulting imide residue is cleavable by endopeptidases, which enables a cyclization process to release the drug from the linker [105].

Although enzyme-cleavable linkers display the best selective cleavage and stability in circulation, it has to be considered that the small molecules can leave the cell after cleavage of the linker. This can be desirable, for example, when the released cytotoxic agent kills surrounding cancer cells, but it might also cause side effects when the agent reenters the circulation.

In addition to the cleavage site, the linkers of modern drug conjugates contain a spacer that upon cleavage of the linker by acid, reducing agent, or enzyme undergoes an intramolecular reaction to release the free drug that shall be delivered without any remainder of the linker. Such self-cleavable bridges can, for example, be realized by the introduction of an elimination cascade that guarantees that no atom of the linker remains attached to the active moiety of the conjugate [1, 106, 107].

Although cleavable linkers might seem to be superior on first glance, also non-cleavable linkers have their advantages. A non-cleavable linker reduces the amount of the small molecule agent that is released in the circulation to a minimum. Additionally, a small molecule cytotoxic agent that is attached to an antibody via a non-cleavable linker will after the lysosomal degradation of the antibody be released as attached to a lysin or cysteine via the spacer. The

resulting metabolite then carries a charge, which hinders its diffusion out of the targeted cell.

The non-cleavable linkage of two pharmacologically active agents – be it small molecule or macromolecule – must ensure that the resulting agent still exhibits the desired pharmacological effect and that the non-cleavable linker does not interfere with binding of the agent to its target by blocking important parts of the pharmacophore. Therefore, the position for attachment of the linker has to be chosen even more carefully for non-cleavable conjugates.

Theoretically, many moieties could serve as non-cleavable linkers. So far, thioether-based linkers such as in the thiol maleimide system [108] are most prevalent among non-cleavable linkers probably due to their easily controllable synthesis. In non-cleavable antibody–drug conjugates such as trastuzumab emtansine, the SMCC linker (*N*-succinimidyl-4-(*N*-maleimidylmethyl) cyclohexane-1-carboxylate linker) is used. Furthermore, PEG spacers and simple alkyl chains have been reported [101]. In addition to these most widely used linker types, there are many more systems with different selectivity and complexity that might find use in future drug conjugates [109].

8.4 Conclusion and Future Perspective

In the last two decades much research has focused on various ways of conjugation of two (or seldom more) pharmacologically active agents. Reasonable conjugation offers many possibilities and therapeutic benefit.

Predominantly, drug conjugates tend to exploit a targeting effect generated by one active component to deliver the second part in tissues or compartments where it shall be active. Such targeted delivery reduces side effects and systemic toxicity on the one hand and increases the amount of drug at the site of action on the other hand, which enhances therapeutic efficacy.

For small molecule drug conjugates, targeted delivery is facilitated by the high affinity of one small molecule to a target structure that in the best case is only present at the site of action. For small molecule conjugates, also physiologic transport systems can be exploited to generate a high drug concentration at the desired site of action. Several such targets have successfully been utilized for targeted delivery *in vitro*, and especially folate polyamine transporters seem valuable to dispose drug conjugates in specific compartments of the body. As most drug conjugates, also small molecule agents of this class are predominantly evaluated for cancer therapy, but they might also have a future role in the development of anti-infective agents especially since bacteria offer target structures that are not present in human, which would help to generate a very selective delivery. In addition to a targeted delivery, the physicochemical properties of drugs can be improved by conjugation as well and a controlled release from conjugates is also thinkable.

In addition to cancer, which presently is the most prominent indication for drug conjugates by far, other fields and diseases might similarly profit from targeted conjugates, and correspondent research is conducted. As interesting

examples, there are small molecule drug conjugates, polymer–drug conjugates, and antibody–drug conjugates as experimental therapeutics for several inflammatory diseases in early development. Furthermore, preclinical studies [110] have reported a potential role of drug conjugates as antiparasitic agents, and very recently, a drug conjugate [111] that combines antiresorptive and osteoanabolic effects showed very promising preclinical data for the potential treatment of osteoporosis.

Small molecule drug conjugates might have several advantages over macromolecular conjugates. In contrast to drug conjugates that include a macromolecule such as an antibody or a polymeric carrier, preparation as well as characterization of small molecule drug conjugates is easier and less expensive. In addition, the threat of immune reactions to the conjugate is considerably lower for small molecule agents [1].

Antibody–drug conjugates are designed for targeted delivery of small molecule agents as well. Through their high affinity to a specific antigen, a very selective deposition at the site of action is possible, which reduces systemic side effects and even enables the use of highly potent agents that would not be applicable alone. As therapeutic antibodies that are most widely used in cancer treatment also for antibody–drug conjugates, cancer therapy is the dominant indication. For this purpose, highly toxic small molecules are attached to the antibodies and kill cancer cells after the delivery facilitated by the antibody. In some cases, the antitumor activity of the small molecule cytotoxic agent is further assisted by an antitumor activity of the antibody as it is the case for trastuzumab emtansine, for example. With modern linkers, the release of the cytotoxic agent is highly controllable, which strongly reduces systemic toxicity and increases therapeutic efficacy. With a suitable antibody–drug conjugate virtually every kind of tumor would be addressable given that the right antibody for the right antigen is developed. Therefore the class of antibody–drug conjugates could have therapeutic value for all kinds of cancers – solid tumors or hematopoietic malignancies – even for cancers where the antibody alone is not sufficiently effective but can facilitate targeted delivery of a cytotoxic agent. With these valuable properties, antibody–drug conjugates might significantly improve therapeutic management of cancers and play an important future role.

The third promising approach of drug conjugation is based on polymer–drug conjugates. The attachment of a small molecule to a biocompatible polymer again offers new possibilities. Polymer–drug conjugates are also suitable for targeted delivery, but in this case the selectivity of targeting is limited and the targeting is merely of passive nature. As macromolecules, polymer–drug conjugates can target solid tumors using the EPR effect by accumulating in tumor tissue due to a higher vascular permeability and a poor microvasculature. In addition, biocompatible polymers as drug carriers can strongly improve the physicochemical properties of drugs such as solubility and stability and can aid managing pharmacokinetic behavior. With the help of modern linkers, the release of small molecules from the macromolecular carriers can be controlled in various ways including release in a certain compartment of the body or a slow time-dependent release. And finally, polymer–drug conjugates offer the possibility of co-delivering more than one pharmacologically active

agent. With a polymeric carrier several agents can be applied in a defined ratio and set free at a defined site of action, which in times of expanding polypharmacology is a very attractive possibility. Furthermore, research on polymers yields more and more biocompatible carriers with highly controllable properties and behavior. Therefore, the development of personalized polymer–drug conjugates as next step in individualized medicine is conceivable. Still, most of the polymer–drug conjugates are currently in an experimental stage of development. Their potential seems not yet exhausted by far.

For all types of conjugates – small molecule drug conjugates, polymer–drug conjugates, and antibody–drug conjugates, intense preclinical research has been conducted and has shown that all approaches hold a lot of promise. However, for drug conjugates the available *in vitro* test systems cannot simulate all relevant aspects of a potential *in vivo* activity. Typical test systems, for example, fail to represent a full picture of circulation and uptake of such drug conjugates and therefore do not seem suitable to predict their *in vivo* accumulation in tumor cells. Eventually, more sophisticated assays could further improve the preclinical development and evaluation of future drug conjugates.

So far, robust clinical experience is only available for antibody–drug conjugates. Some of the investigated agents show significant advantages over unconjugated agents – be it antibody or small molecule cytotoxic agent – concerning efficacy and safety profile. Additionally, the clinical success of antibody–drug conjugates proves that the concept of attaching two or more pharmacologically active agents to each other is effective and that the advanced linkers provide stability in systemic circulation and specific cleavage at the site of action. Currently, several antibody–drug conjugates are in clinical development succeeding the first approved agents and promise much therapeutic progress. However, stringent head-to-head clinical trials of these agents are required to judge their clinical value especially in light of their high therapeutic costs. At present, the variety of small molecule agents used in antibody–drug conjugates is limited, but with the obvious clinical success of currently investigated and used antibody–drug conjugates, their number might increase in the future. In addition, antibody–drug conjugates might also access the field of severe autoimmune and inflammatory disorders.

Although the conjugation of small molecules also offers several therapeutic opportunities and potential advantages, small molecule drug conjugates have not yet reached clinical attention but are mostly in a preclinical, experimental stage of development. Based on the conceivable success of antibody–drug conjugates, this might change in the future since the advantages of antibody–drug conjugates might also be translatable to small molecule conjugates and the development of small molecule drug conjugates can profit from the experiences with antibody conjugates, for example, concerning linker chemistry.

Polymer–drug conjugates hold therapeutic potential especially as carriers for co-delivery of personalized combination therapies. However, for that purpose, a lot of further research is necessary to resolve the long-term compatibility of the polymeric carriers with the human body and to develop robust processes to produce polymer–drug conjugates with defined polymer–drug–drug ratio for

various agents. In case these open questions can be answered, polymer–drug conjugates could become a very valuable tool for personalized medicine.

Every class of drug conjugates composed of small molecules or macromolecules is a valuable therapeutic approach and offers its own possibilities and advantages. Antibody–drug conjugates already have proven their clinical value, but to exploit the full potential of drug conjugates will require more research. With increasing knowledge on the behavior of drug conjugates *in vivo* and improving technologies of preparation and characterization of this exciting drug class, many new fields in therapy and diagnostics seem accessible with drug conjugates.

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9

Merged Multiple Ligands

Hongming Chen¹, Udo Bauer², and Ola Engkvist¹

¹Discovery Sciences, Innovative Medicines and Early Development, AstraZeneca, Pepparedsleden 1, 431 83 Mölndal, Sweden

²Cardiovascular and Metabolic Diseases, Innovative Medicines and Early Development, AstraZeneca, Pepparedsleden 1, 431 83 Mölndal, Sweden

9.1 Introduction

As is comprehensively described in this book, there are several ways of designing a molecule that modulates more than one target. This chapter will focus on what is defined as merged multiple ligands (MMLs). In MMLs the molecular frameworks, which contain the underlying pharmacophore elements for each target, are “merged” by taking advantage of the commonalities in the structures of the starting compounds [1]. However, it should be stressed that there is a continuum of examples of designed ligands from merged to fused and further to linked multiple ligands. Rational design of MMLs is per definition more complex than optimizing a compound for a single target since an extra optimization constraint has been added to the lead optimization process [2]. As has already been discussed in the literature, there is a tendency that MMLs are both larger and more lipophilic than molecules that have been designed to modulate only one target [1]. This is not surprising since an additional optimization constraint has been added to the design process, making it inherently more difficult to achieve good physicochemical properties. It is therefore advisable before starting an MML drug discovery program to carefully evaluate the likelihood of success of designing an MML with a good physicochemical profile.

Additionally, it is also important to take into account the probability of success for designed MMLs from a target validation perspective. The likelihood of success for MML hypothesis can be assessed by taking into account relevant literature data through text mining and pathway analysis to estimate the strength of the underlying target combination hypothesis in comparison with modulating only one target. In recent years it has become popular to view each protein as a node in a network and many diseases as perturbation of the network of proteins. An influential review that in detail describes network pharmacology and its implications for drug discovery was published a few years ago [3]. Network pharmacology provides a rationale for the empirical fact that for many potential diseases, it is necessary to modulate more than one protein. In this chapter

we will first briefly describe examples of using computational methods that can be used to identify MMLs; thereafter the focus will be on successful examples, mainly from a medicinal chemistry perspective, of designed MML lead optimization projects.

9.2 Computational Methods Utilized in Designing MMLs

Numerous efforts have been done to develop computational methods that could be applied for rationally designing MMLs, and it would be impossible to fully cover all the computational approaches that can be used in designing MMLs; instead we are focusing on representative examples where computational methods have been a key factor in identification or design of an MML. A useful review of representative computational methods that is applicable for designing MMLs has also recently been published [4].

9.2.1 Bioactivity Data Sources

To be able to make a fair judgment of an opportunity to rationally design MMLs, it is very important to have access to high-quality biological activity data for a large set of molecules. Schematically, there exist three types of sources for biological activity data: proprietary in-house data, data licensed from commercial vendors, and publicly available data. With the increase of high-quality structure/bioactivity data, computational knowledge-based methods will become more and more powerful tools for data mining. A key event during the last years has been the larger accessibility of bioactivity data in the public domain. PDB is the main source for crystal structures for small molecules binding to proteins [5]. PubChem BioAssay has established itself as the main repository for *in vitro* screening data [6]. ChEMBL is the main public repository of bioactivity data curated from the scientific literature [7]. It is important that bioactivity data from different sources are compatible. This has recently been largely facilitated with the development of the BioAssay Ontology (BAO), which has been used to annotate the assays in PubChem and ChEMBL [8]. An important project to standardize different sources of bioactivity data is the Open PHACTS project [9].

9.2.2 Utilizing Known Polypharmacology to Identify MMLs

The fastest way to initiate an MML project is to start from a molecule with known activity on the targets of interest. Identification of such a molecule is a clear indication that the binding cavities of the targets of interest might be similar enough to bind the same type of molecules. However, it is important to also check the general promiscuity of the identified molecules. If the identified molecule shows a high degree of general promiscuity, the scaffold might not be suitable for an MML drug discovery project. Chen *et al.* has used PubChem as a data source to explore polypharmacology and build drug–target network [10]. Drug–target network is a powerful way to display and analyze the bioactivity

data for identifying opportunities for MML. Ligand similarity-based methods have been widely used for predicting molecular polypharmacology. Several different ways of identifying similar molecules exist, which all have their pros and cons. The most common way to assess if two molecules are similar to each other is through comparing their structures with a molecular fingerprint technique. There exist a plethora of molecular fingerprints and methods to compare them to assess the similarity [11]. They can be based on substructures or more generalized descriptions like pharmacophore patterns. Structural fingerprints have been used frequently in computational drug repurposing of existing drugs as well as predicting off-targets for drugs and clinical candidates. An interesting approach is the similarity ensemble approach (SEA), which has been successfully been used to predict so far unknown targets for drugs [12]. Circular fingerprint has been popular in predicting bioactivities for molecules. Bornot *et al.* used circular fingerprint to calculate the similarity of known actives for a target pair, and a heat map was generated based on the similarity metrics to highlight the opportunities existed for a target pair. It was noted that many of the identified dual-acting molecules were not published as such; instead the activities of the different targets were reported in different sources [13].

9.2.3 Applying QSAR Models to Identifying and Optimizing MMLs

Another option to estimate the MML potential of a molecule is to build quantitative structure–activity relationship (QSAR) models to predict the activity for the targets of interest to assess the potential for the molecule to modulate these targets. The prediction can either be based on the three-dimensional protein structures, which will be described later or be based on activity data. Based on the known active and inactive molecules for a target, a predictive model is built for each target. There are several machine learning methods that can be used to build QSAR models. Examples of machine learning methods are artificial neural networks (ANN), decision trees (DT), and support vector machines (SVM) [14]. Bayesian modeling is a popular method for building bioactivity prediction models because of its simplicity. The models utilize compounds that are active and inactive on each target. A set of compounds are then scored against each model, and if the molecule obtains a high score for a model, the compound is likely to be active on that target. Thus, compounds can be identified, which have a high probability to bind to the targets desired for the MML. Recently, it has been suggested that SVM might slightly outperform Bayesian modeling for target prediction [15].

An automated method to design ligands to a polypharmacological profile has recently been proposed [16]. An evolutionary process of optimizing potencies and physicochemical property profiles simultaneously in iterative cycles was developed. This method was exemplified by the evolution of the known acetylcholinesterase inhibitor drug donepezil to a dual dopamine type 4 (D4) antagonist and dopamine type 2 (D2) antagonist via several rounds of chemical transformations derived from medicinal chemistry knowledge. After several rounds of optimization, a molecule with high D4 and D2 potencies were obtained. Multitarget QSAR models for identification of MMLs [17] and active agents against multiple bacterial and viral [18] and fungal [19, 20] species have

been developed based on multitarget spectral moment descriptors in which the binding site features are integrated from a stochastic Markov drug–target binding process model. These multitarget QSAR models achieve high retrieval rates of 72–85% and moderately low false-hit rates of 15–28%. Ma *et al.* used SVM algorithm to predict dual inhibitors for specific kinase pairs from compounds in PubChem database [21]. SVM models for six individual kinase targets – EGFR, FGFR, VEGFR, PDGFR, Src, and Lck – were built individually by using known non-dual inhibitors of each kinase, while the dual inhibitors of related kinase pairs were explicitly excluded from the training set. SVM models for individual kinase target were then combined to predict dual inhibitors for specific target pairs. Altogether four kinase pairs – EGFR-FGFR, VEGFR-Lck, PDGFR-Src, and Src-Lck – were investigated, and the retrieval rate for dual inhibitors in the test set was 38–53% for these specific target pairs.

9.2.4 MMLs Developed Based on Fragments

As has been proposed in the literature, fragment-based drug design might be a suitable lead generation tool for identifying starting points for an MML drug discovery program [1]. An example was recently published where dual-acting fragments were prioritized through the use of self-organizing maps (SOM) [22]. Known modulators of 5-lipoxygenase (5-LO) and soluble epoxide hydrolase (sEH) were collected from the literature. The molecules were fragmentized, and the fragments appearing much more frequent among the active molecules for the two targets in comparison to the baseline frequency were identified. Through applying SOM, commercially available fragments were identified that were similar to the fragments identified for the two targets. The commercially available fragments were validated by an NMR binding assay to bind to the targets of interest.

9.2.5 Utilizing Protein Crystal Structures in Identifying MMLs

If crystal structures exist for the targets of interest, it is possible to exploit them in the MML design. An example is the identification of dual modulators of LTA₄ and hnp-PLA₂ [23]. An algorithm was developed with the following steps:

- 1) Pharmacophore generation for each binding pocket individually followed by overlaying the pharmacophores to identify common pharmacophores.
- 2) Identifying molecules fulfilling the pharmacophore constraints.
- 3) A more accurate and time-consuming docking method was then applied to dock the compounds to select the most promising MMLs.
- 4) Validating the top scoring compounds experimentally.

After running the protocol, nine compounds were tested experimentally, and three of them were found to inhibit both targets in the double-digit micrometer range. A somewhat similar strategy for searching to identify dual inhibitors for human thymidylate synthase (hTS) and human dihydrofolate reductase (hDHFR) has been utilized [24]. A dual-target pharmacophore model was built based on known dual inhibitors from literature; compounds were thereafter docked independently into individual protein structures to find common hits.

These common hits were then filtered by the dual pharmacophore model to remove any compounds that didn't match the pharmacophore model. Zhou *et al.* [25] investigated the possibility of using docking program to predict dual kinase inhibitors. In their study, docking-based virtual screening was first carried out on each individual kinase structure, and the common docking hits for a pair of kinases were then predicted as the dual kinase inhibitor. It was found that in some test cases dual inhibitors can be predicted very well; however, the enrichment is in general still limited due to a high rate of false positives.

It is also possible to compare binding pockets for the whole proteome that has been crystallized to identify MML opportunities [26]. The advantage of this method is that it is possible to investigate all target combinations and to identify the target combination that has the highest probability of success from a chemistry perspective. One common method for cavity comparison is to calculate the cavity similarity based on pharmacophore patterns of the cavity. In a recent study, hydrogen bond donors/acceptors, hydrophobic, positive and negative ionizable, and aromatic ring were used as pharmacophoric features [27]. Pharmacophore patterns were generated for the 8166 protein–ligand complexes in the sc-PDB database, and a set of ligands were thereafter profiled against the set of pharmacophore patterns to identify potential binders to each crystal structure. The performance of the protein structure-derived pharmacophore patterns was compared with docking and pure ligand-based similarity methods. Rather surprisingly the ligand-based methods outperformed on average both the structure-based pharmacophore patterns and the docking methods. Another example of a cavity based method is CavBase, where five physicochemical descriptors are used to characterize the binding pocket. A clique algorithm is then used to identify similar pockets. The method has been successfully applied to identify dual angiotensin-converting enzyme (ACE) and COX inhibitors [28]. Another recently developed structure-based method is the Drug Profile Matching where docking scores are used to predict novel targets [29].

9.3 Examples of Medicinal Chemistry Efforts of Designing MMLs in Drug Discovery Projects

9.3.1 MMLs in Oncology

The majority of postmenopausal breast tumors are hormone dependent and estrogen receptor (ER) positive. A common therapeutic intervention for this type of cancer is either by diminishing the availability or by inhibiting the binding of estrogens to the ER. Aromatase catalyzes the conversion of androgens to estrogens in the final step of the biosynthesis of estrogens and is therefore an attractive therapeutic target for inhibition. The steroid sulfatase (STS) enzyme catalyzes the hydrolysis of steroid sulfates, including estrone 3-sulfate, which is the main source of estrogens in tumors [30]. Dual inhibition of aromatase and STS leads to enhanced estrogen deprivation for hormone-dependent breast cancer. Woo *et al.* reported the dual aromatase–STS inhibitor by introducing the STS inhibitory pharmacophore element of compound **2** into a potent aromatase

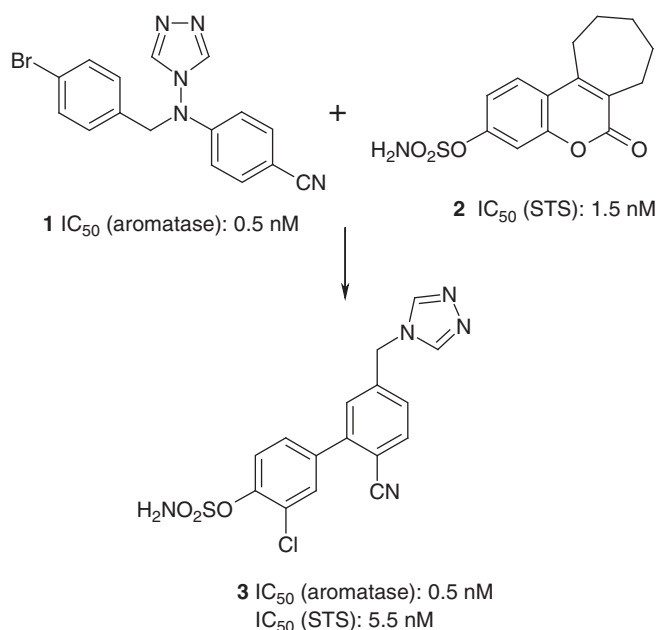


Figure 9.1 Design of a dual aromatase–STS inhibitor.

inhibitor 1 [31]. After including the phenyl sulfamate group, several compounds demonstrated both strong aromatase and STS inhibition, and particularly compound 3 shows strong dual potency in cellular systems (Figure 9.1).

One promising pathway for tumor growth inhibition is the epigenetic and protein acetylation state modulation by histone deacetylases (HDACs). HDAC inhibitors have been shown to cause growth arrest, differentiation, and apoptosis in cancer cells. Two HDAC inhibitors, SAHA (compound 4 in Figure 9.2) and romidepsin, have been approved by the FDA for the treatment of cutaneous T-cell lymphoma. Topoisomerase I (TopoI) is a well-validated anticancer target. It relieves the torsional strain on DNA during DNA replication by cutting one strand of the DNA double helix and passing one strand over the other [32]. Due to the inherent need for rapid replication in cancer, TopoI inhibitors result in a DNA strand break, cell cycle arrest, and apoptosis. Guerrant *et al.* designed and synthesized a series of dual HDAC and TopoI inhibitors [33]. The design strategy was to merge the fused ring system of the TopoI inhibitor camptothecin with a similar linker to that of SAHA (Figure 9.2). The hydroxamic acid in SAHA is a zinc-binding motif and crucial for the HDAC inhibition. The synthesized compounds retain inhibition activities against both target enzymes and inhibit the proliferation of selected cancer cell lines.

Inosine monophosphate dehydrogenase (IMPDH) inhibitors show a significant ability to trigger differentiation and apoptosis and were found to block tumor-induced angiogenesis *in vivo*. Mycophenolic acid (MPA) is an IMPDH inhibitor and was used as a starting point to design a dual HDAC and IMPDH inhibitor by including the HDAC pharmacophore element hydroxamic acid

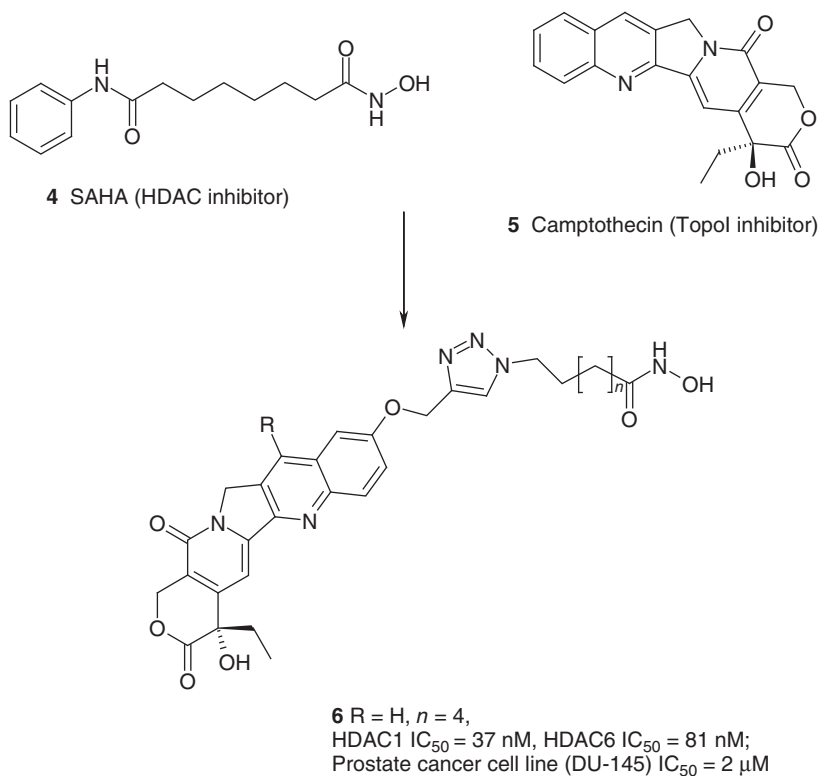


Figure 9.2 Design of a dual HDAC and Topol inhibitor.

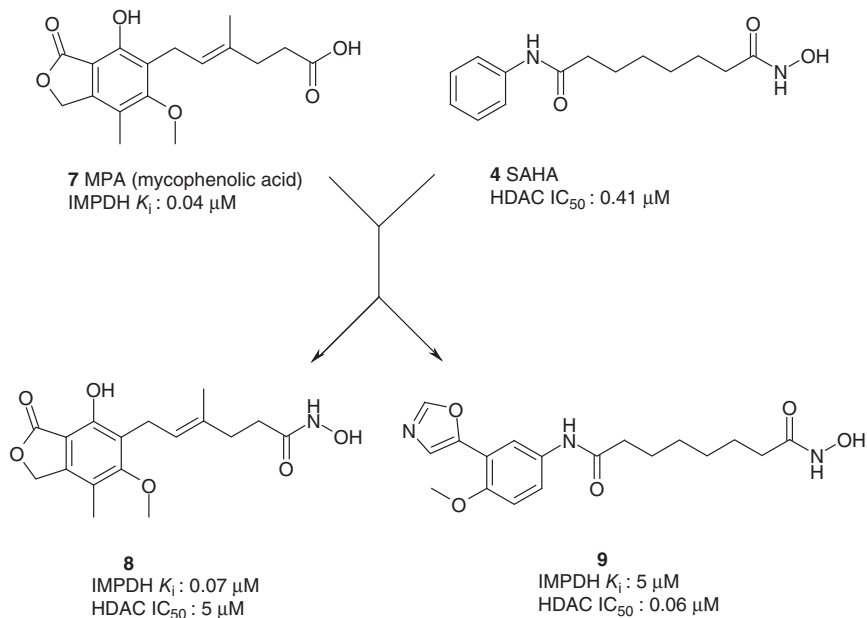


Figure 9.3 Design of dual HDAC and IMPDH inhibitors.

from SAHA (Figure 9.3) [34]. For compound **8**, the carboxylic acid group of MPA was replaced with a hydroxamic acid moiety. The design of compound **9** is based on an opposite strategy, that is, SAHA was modified by addition of groups known to interact with IMPDH. Both compounds **8** and **9** were found to act as MML for IMPDH and HDAC inhibition. The activity against both targets was not perfectly balanced for either compound, and further optimization of these series might be necessary, but the principle that dual inhibition is possible was established.

Matrix metalloproteases (MMPs) are a class of zinc-dependent endopeptidases capable of degrading all components of the extracellular matrix. MMPs are involved in several steps of cancer progression including tumor growth, angiogenesis, and apoptosis and have long been considered as a target for cancer chemotherapy. Several MMLs targeting MMPs and other targets were reported in literature. MMP inhibitor **10** (Figure 9.4) has hydroxamic acid as a functional group chelating the zinc ion in the binding site. It has a benzyl group in the P_2' position, while the cathepsin L (CatL) inhibitor **11** has a benzyl group at the P_2 position and an aldehyde group, which is generally used as a functional group of CatL inhibitor interacting with thiol group at the active site. Compound **12**

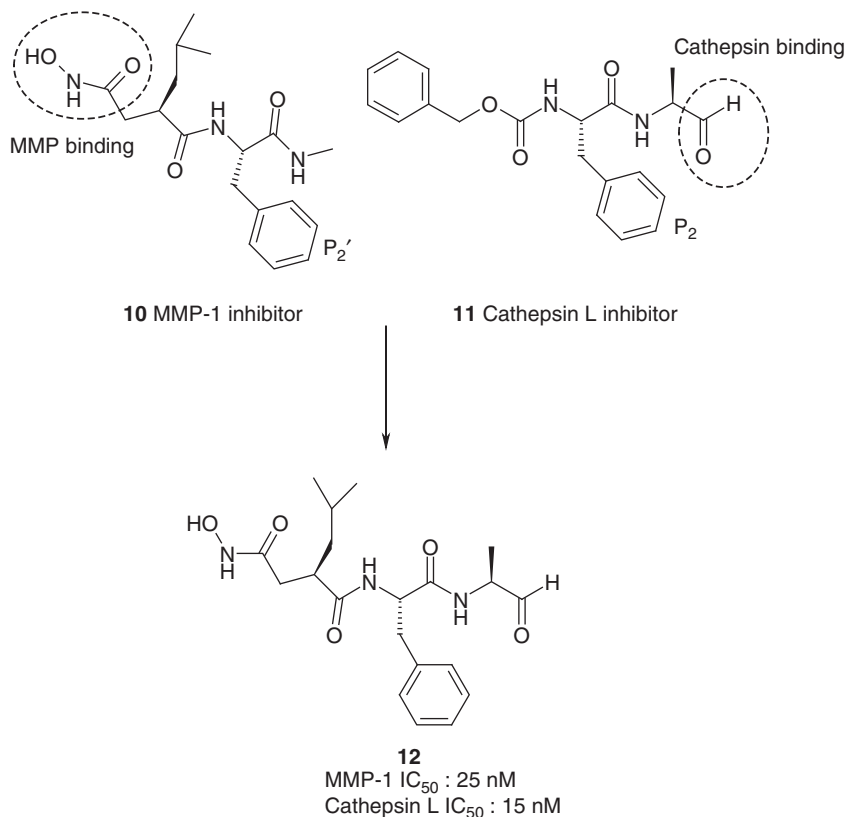


Figure 9.4 Design of dual MMP-1/CatL inhibitors.

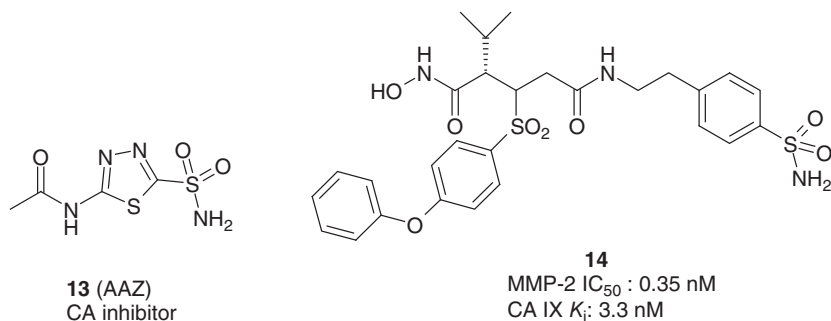


Figure 9.5 Design of dual MMP-2/CA IX inhibitors.

was designed by combining the desired hydroxamic acid and aldehyde groups at the opposite end of a peptide fragment [35]. This compound demonstrated dual inhibition of both targets (IC_{50} value is 25 nM for MMP-1 and 15 nM for CatL).

Like MMPs, α -carbonic anhydrase (CA) is a class of widespread zinc-containing enzymes that catalyze the CO_2/HCO_3^- interconversion. The expression of the CA IX transmembrane isoform is increased in many types of hypoxic tumors, and there is strong interest in selective inhibition of CA IX over other isoforms for cancer. Known CA inhibitors mostly belong to the class of primary sulfonamides, sulfamates, and sulfamides. One example is acetazolamide (AAZ, compound **13**; Figure 9.5) in clinical use for more than 50 years as an antiglaucoma and antiepileptic drug. Although both MMPs and CAs possess a catalytic zinc ion, the major differences between their catalytic sites make it difficult for the common zinc-binding motifs like hydroxamic acid to be suitable for both classes of enzymes. By combining the hydroxamic acid for MMP inhibition and a primary arylsulfonamide for CA inhibition, it was possible to develop inhibitors for both targets simultaneously [36]. Following this strategy, a series of compounds possessing the iminodiacetic acid as the main backbone were synthesized. These compounds were demonstrated to strongly inhibit both MMPs and CAs, some of them in the nanomolar to subnanomolar range. Compound **14** (Figure 9.5) was identified as the most potent MML for both targets. A docking study based on the MMP-2 X-ray structure showed that the arylsulfonamide group of compound **14** is well accommodated in the catalytic site and extends toward the $S_2'-S_3'$ region to establish several binding interactions with the enzyme.

9.3.2 MML Targeting for Neurodegenerative Disease

Neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD), and Huntington's diseases, have long been regarded as among the most enigmatic and complex issues in biomedicine. It has become increasingly clear that the major basic processes involved are multifactorial in nature, caused by genetic, environmental, and endogenous factors. Although each of these diseases has its own molecular mechanisms and clinical manifestations, some general pathways might be recognized in different pathogenic cascades. They include protein misfolding and aggregation, oxidative stress and free radical formation, metal

dyshomeostasis, mitochondrial dysfunction, and phosphorylation impairment, all occurring concurrently. Potential drugs modulating a single target may be inadequate for the treatment of such complex diseases. Thus, there is a growing interest in MMLs to provide disease-modifying drugs for neurodegenerative diseases.

9.3.2.1 MMLs for the Treatment of Alzheimer's Disease

AD is the most common form of dementia associated with progressive loss of memory, speech, and recognition, occurring most frequently in elderly people. The etiology of AD is not fully known, but some hallmarks, such as low levels of acetylcholine, β -amyloid ($A\beta$) deposits, τ -protein entangling, oxidative stress, and dyshomeostasis of metals, are considered to play important roles in the pathogenesis of the disease [37]. Acetylcholinesterase inhibitors (AChEI), namely tacrine, donepezil, rivastigmine, and galantamine, are currently the primary therapeutic options approved by the FDA for the treatment of AD. However, these drugs can only reverse the symptoms for a short period of time. Current MML efforts focus on modifying AChEI structures to add up additional biological responses useful for treating AD.

Recently it has been reported that AChE might act as a “pathological chaperone” in inducing $A\beta$ aggregation through direct interaction of its peripheral anionic site (PAS). Some research efforts have been devoted to the development of the so-called “dual binding site” AChEIs, that is, simultaneously interacting with AChE catalytic and PSA site. Compound **15** (Figure 9.6) was designed by linking two moieties, a benzylamino group and coumarin heterocycles, which were selected from previously developed AChEIs [38]. Besides having an AChE inhibitory activity similar to donepezil, compound **15** demonstrated an enhanced anti- $A\beta$ aggregation compared with other known AChEIs. This gave direct evidence of the $A\beta$ antiaggregation action of dual binding site-based AChEI. An improved ACE-induced $A\beta$ aggregation inhibition was obtained by a series of compounds that combine an isosteric group of the indanone moiety of donepezil and the fused ring system of tacrine (Figure 9.6) through a spacer of variable chain length. The most potent compound in this series (compound **18**; Figure 9.6) displays AChE inhibition at 20 pM and AChE-induced $A\beta$ aggregation inhibition with an IC_{50} value of 2 μ M [39]. Efforts of combining AChEI with monoamine oxidase (MAO) inhibitors have been published [40, 41]. A series of AChE/MAO dual inhibitors were rationally designed by introduction of the carbamate functional group of the AChEI rivastigmine in either rasagiline or selegiline, both of which are MAO-B inhibitors with neuroprotective activity *in vitro* and *in vivo* (Figure 9.7). Compounds **22** and **23** were identified as interesting leads having a well-balanced dual MAO/AChE inhibition profile [42]. Further development of compound **22** resulted in the more potent compound, ladostigil, which has entered into clinical trials [43].

In addition to MAO inhibitors, investigations have also been done to combine serotonin transporter (SERT) and AChEI to provide additional antidepressant benefit for AD patients [44, 45]. Based on the X-ray complex structure of AChE with donepezil and molecular modeling, an MML strategy was proposed by merging the AChEI rivastigmine and the SERT inhibitor fluoxetine (Figure 9.8)

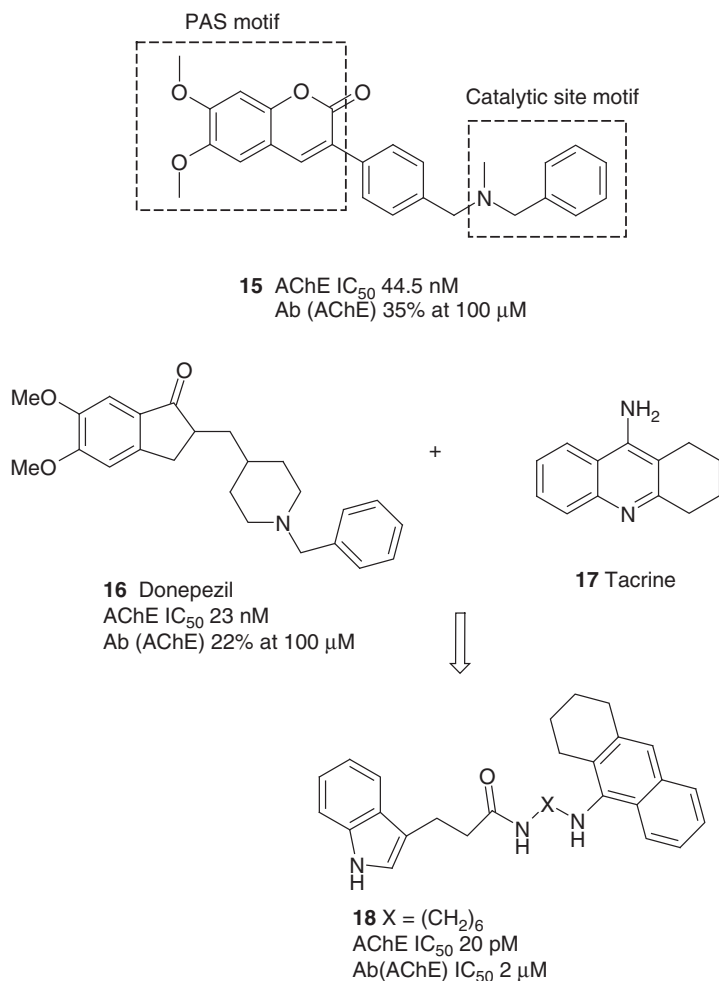


Figure 9.6 Design of dual AChEI and AChEI-induced β -amyloid aggregation.

[46]. Compounds of series A were designed by linking the methylenedioxyphenyl moiety of compound **26** to the ethylamine part [47], while series B compounds were designed as ring closure compounds to explore the restrained conformation [48]. The best compound (**26**) exhibited excellent inhibitory activities against AChE and SERT.

9.3.2.2 MML for the Treatment of Parkinson's Disease

PD is the second most common neurodegenerative disorder in the industrialized countries. It is characterized by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* and other subcortical nuclei. So far little is known about the molecular pathogenesis of PD. Current PD therapies are based mainly on exogenous replacement of dopamine (DA) within the striatum.

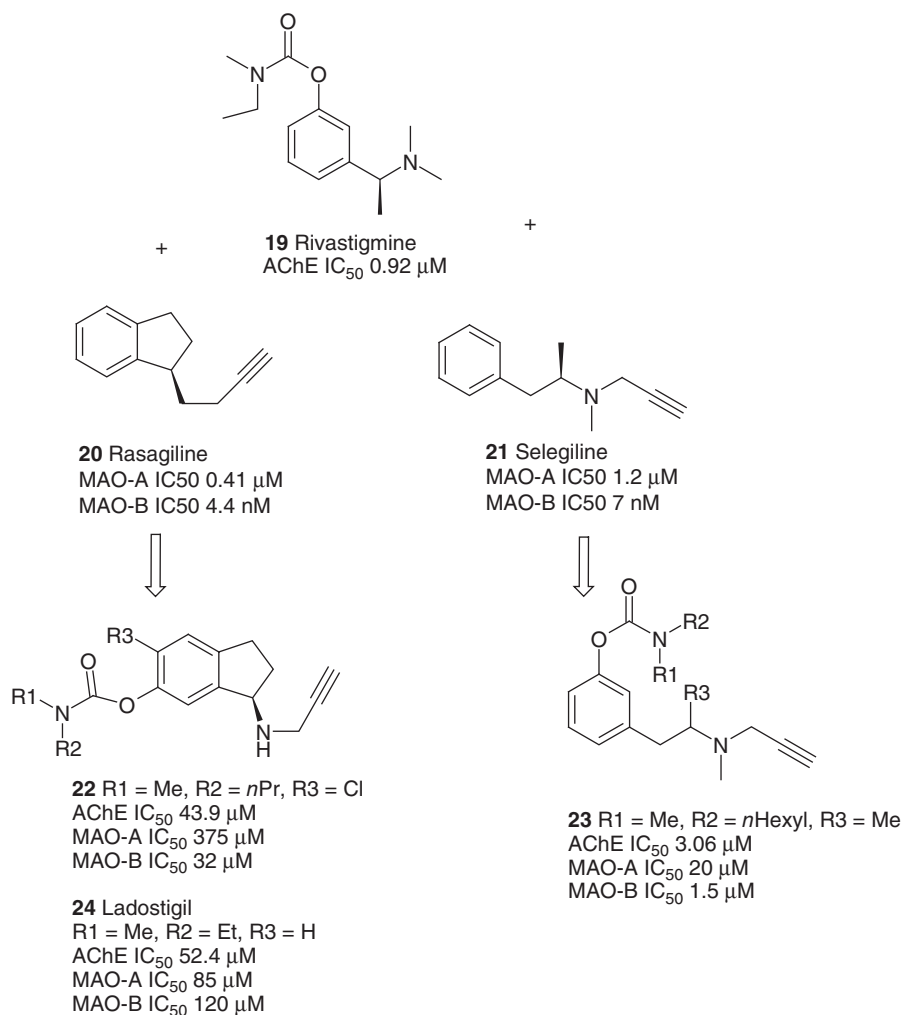


Figure 9.7 Design of dual AChE and MAO-B inhibitors.

This can improve the symptoms but without halting or reversing the progression of the neurodegenerative process. The major anti-PD drugs include L-dopa, dopaminergic receptor agonists, and DA-modifying agents such as (i) peripheral dopa decarboxylase inhibitors, (ii) catechol-*O*-methyltransferase (COMT) inhibitors, and (iii) selective MAO-B inhibitors.

MML discovery efforts in PD area have been actively pursued. The first series of dual functional compounds was rationally designed to combine the ion-chelating compound **32** and the propargylamine group of MAO-B inhibitor rasagiline (Figure 9.9) [49]. The propargyl group of compound **33** was proposed to be responsible for its MAO-B inhibition and also for a wide neuroprotective profile unrelated to MAO inhibition. Compounds **33** and **34** were found to be potent lipid peroxidation inhibitors, and this may be due to their

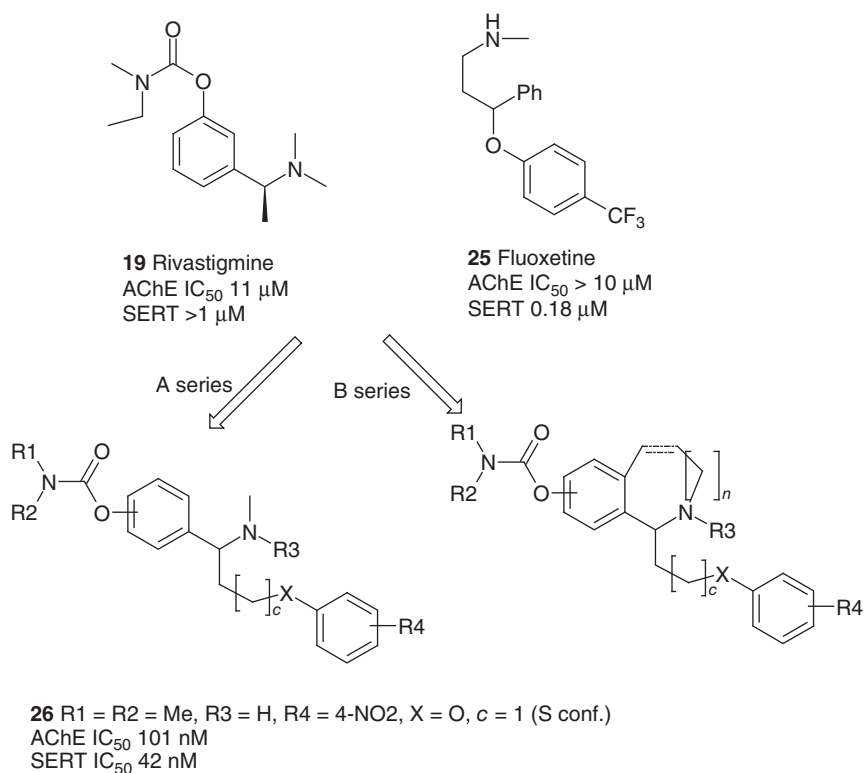


Figure 9.8 Design of dual AChE and SERT inhibitors.

metal chelating capability. Both compounds showed a neuroprotective activity comparable with that of parent compound **33** in a cellular assay [50]. Compound **34** demonstrated superior MAO-B inhibition compared with compound **33** and was further tested *in vivo*. It turned out that compound **34** preferentially inhibits MAO-A and MAO-B in the CNS, while it has lower potency in the liver and intestines (Figure 9.9). This avoids potentiation of the tyramine-induced cardiovascular activity, which is a major side effect among other nonselective MAO inhibitors.

Compound **35** (8-(3-chlorostyryl) caffeine) was originally developed as a potent adenosine A_{2A} receptor antagonist [51]. It was found out later that compound **35** also possess MAO-B inhibitory activity [52]. This finding has raised the possibility of designing dual-target-directed drugs that block both the A_{2A} receptor and MAO-B. The bifunctional profile of **37** was rationalized as a combination of caffeine, an adenosine A_{2A} antagonist, and 1,4-diphenyl-2-butene, a reversible MAO-B inhibitor (Figure 9.10). SAR data for compound **37** showed that the saturation of the styryl double bond has a negative effect on inhibition potency [53]. This supports the observation that many MAO-B inhibitors contain planar conjugated heterocyclic systems. Molecular modeling study also shows that the two terminal rings of compound **37** are at a comparable distance with that found in compound **36** [54].

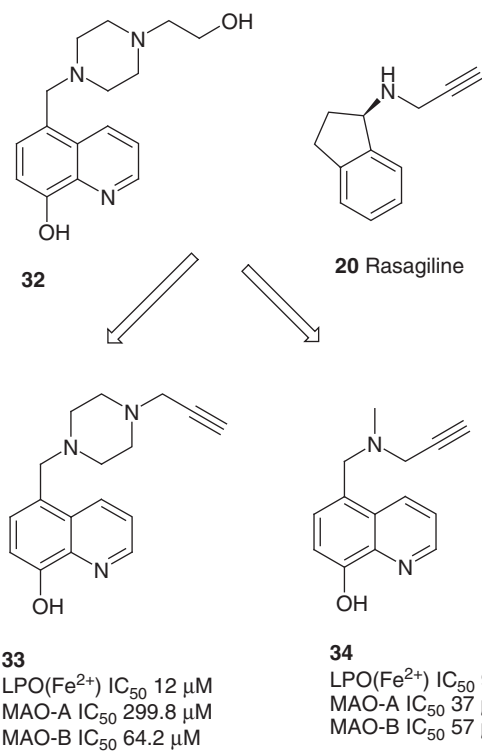


Figure 9.9 MML of an MAO-B inhibitor with an ion chelator.

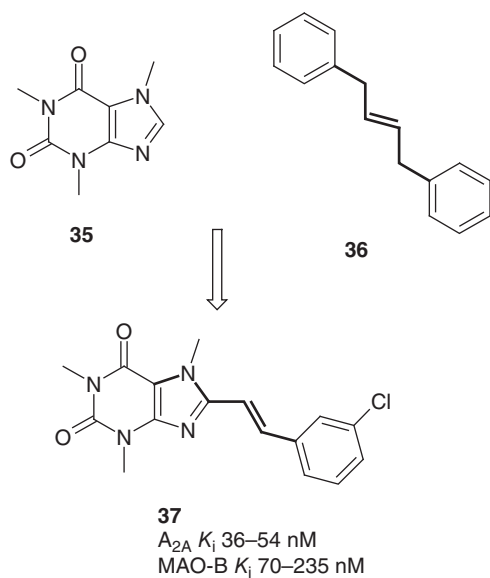


Figure 9.10 Design of a dual $\text{A}_{2\text{A}}$ and MAO-B inhibitor.

9.3.3 MML for the Treatment of Depression

Depression is associated with reduced levels of serotonin (5-HT) in the brain. Drugs such as fluoxetine that increase 5-HT levels by blocking selectively the SERT have revolutionized the treatment of depressive patients. In order to address some of the deficiencies of this class of antidepressants, industry and academia have paid much attention to the design of SERT inhibitors with additional activity at a secondary monoamine target, such as serotonin 5-HT_{1A} and 5-HT_{1D}, or adrenaline alpha2 receptors, or norepinephrine transporters (NET) and dopamine transporters (DAT).

For example, robalzotan (38, NAD-299) has been reported to be a potent 5-HT_{1A} antagonist (Figure 9.11). Preliminary studies found that the combination of chroman with the straight chain of **39**, the prototypic serotonin uptake pharmacophore, generates dual-acting molecules with affinity for the 5-HT_{1A} and serotonin uptake site. Compound **40**, as an example of the designed MMLs, demonstrated excellent 5-HT_{1A} and SERT binding affinities and full antagonism at the 5-HT_{1A} receptor. Furthermore, this compound also showed greater than 100-fold selectivity for the 5-HT_{1A} receptor against a number of other monoamine receptors as well as *in vivo* activity [55, 56].

Substantial preclinical data suggest that neurokinin (NK-1) receptor antagonists have the utility in the treatment of depression. Therefore, selective NK-1 receptor ligands, such as MK-869, have been advanced into clinical development for the treatment of depression; however the results were disappointing. Since NK₁ receptor antagonists are believed to modulate 5-HT neurotransmission via input into noradrenergic pathways, their combination with SERT inhibition was envisaged to deliver an improved antidepressant profile with faster onset of action and improved efficacy. In this context, screening of a compound library provided a hit (compound **41**) with modest activity at both the NK-1 receptor

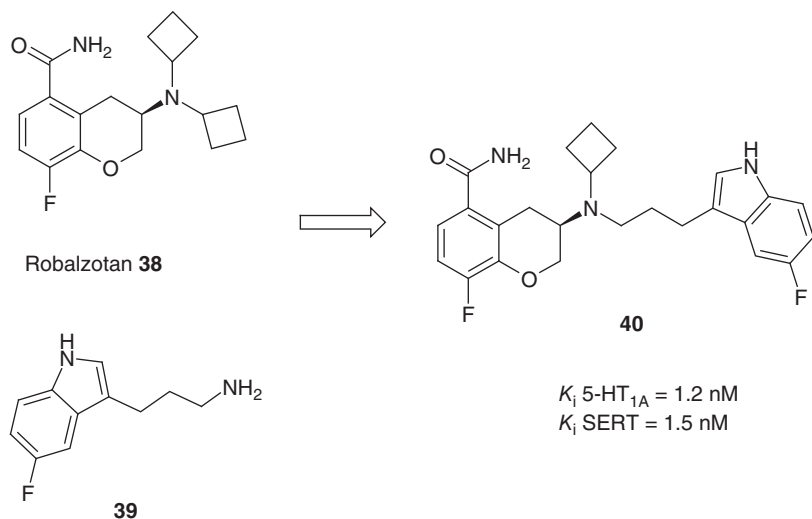


Figure 9.11 Design of a dual SERT and 5-HT_{1A} modulator.

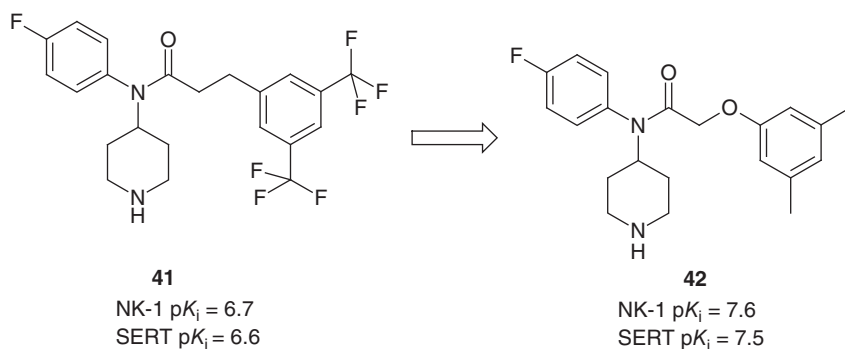


Figure 9.12 Dual NK_1 receptor antagonist and SERT inhibitor identified by HTS.

and SERT. Systematic optimization provided a more potent compound with a balanced activity at both targets (compound **42**) (Figure 9.12). An aryl ether moiety was introduced to reduce lipophilicity, providing physicochemical properties predictive of CNS penetration following oral administration [57]. Compound **42** is one of the few examples of dual ligands with a merged pharmacophore identified by HTS.

Histamine H_3 receptor antagonists have been reported to be able to improve cognition and increase wakefulness in a variety of animal models without showing nonspecific stimulant effects such as increased locomotor activity. Thus a combined drug aiming at H_3 and SERT activity could serve as a new treatment for depression and related disorders. 4-(Aminoalkoxy)benzylamines have been reported to possess high binding affinity at the human histamine H_3 receptor, for example, compound **43** [58]. Keith *et al.* used **43** as a template to which SERT activity could be added. Indeed, attachment of a piperidinylpropyloxy side chain from compound **43** to the pyrrolidino-tetrahydroisoquinoline scaffold in compound **44**, a potent SERT inhibitor, resulted in MMLs (**45**; Figure 9.13) with dual activity as H_3 antagonists and serotonin reuptake inhibitors [59].

9.3.4 MMLs for the Treatment of Cardiovascular Diseases

Cardiovascular diseases (CVDs), including coronary heart disease, ischemic heart disease, arrhythmias, and hypertension, among others, currently represent the main cause of worldwide mortality, with the latter affecting billions of people around the world, despite the numerous drugs available able to target the different mechanisms leading to the disease.

ACE inhibitor such as captopril (**46**) gained wide acceptance for the treatment of hypertension and congestive heart failure (Figure 9.14). Neutral endopeptidase (NEP) is another metalloproteinase that is responsible for the degradation of atrial natriuretic peptide (ANP), a peptide hormone with opposing actions to those of angiotensin II, which is released by the heart in response to atrial distension and causes vasodilation. Consequently, it has been postulated that dual ACE/NEP inhibition may produce beneficial synergistic effect in the management of hypertension and congestive heart failure. Furthermore, inadequate responses of some hypertensive patients to selective ACE or NEP

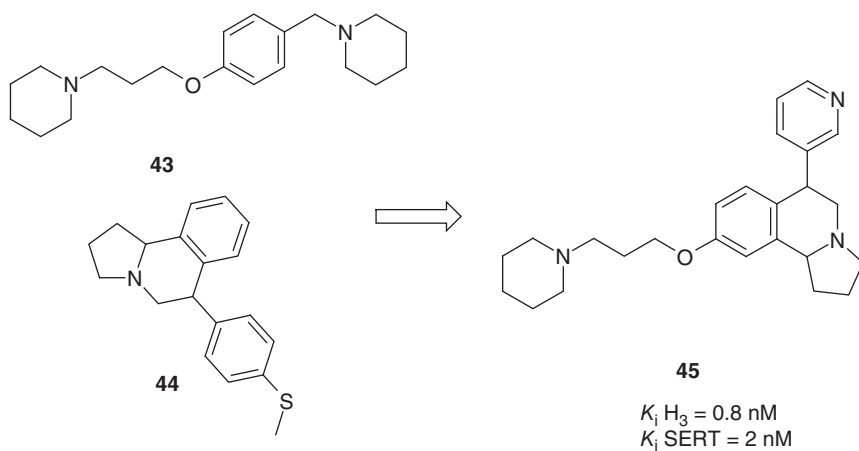


Figure 9.13 Design of a dual H_3 and SERT modulator.

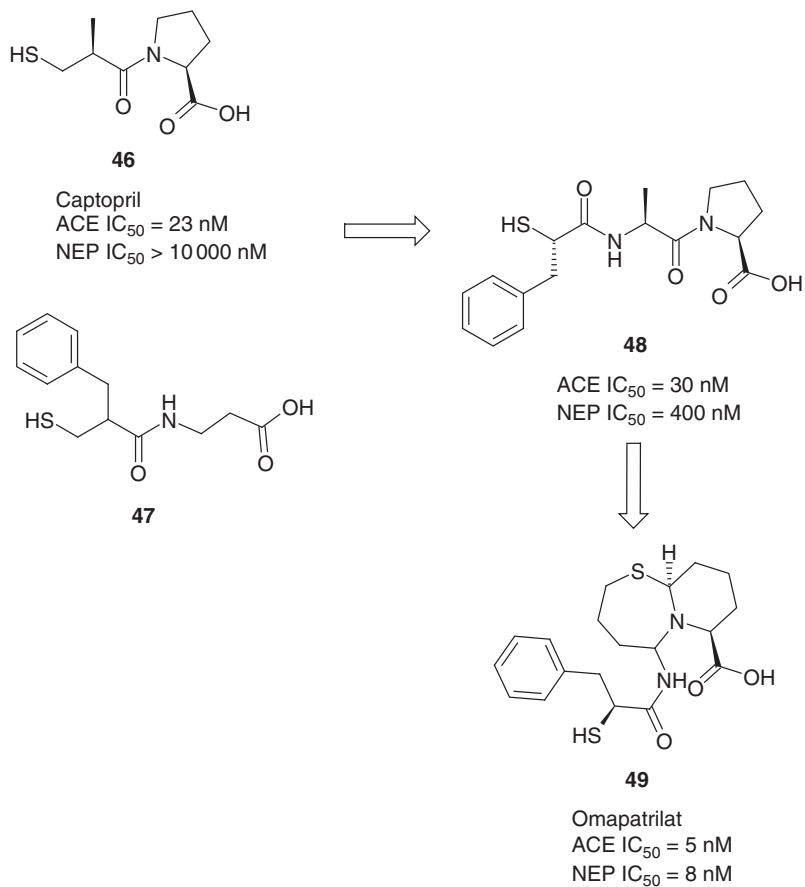


Figure 9.14 Design of omapatrilat, a dual ACE and NEP modulator.

inhibitors and demonstration that coadministration of NEP inhibitors with ACE inhibitors reduces blood pressure more effectively than either drug alone led to the development of molecules with dual action [60].

One of the first dual ACE/NEP inhibitors, dipeptide **48**, was designed using the knowledge of binding requirements for both enzymes. NEP favors a hydrophobic substituent at S_1' , preferably a benzyl group that is present in the selective NEP inhibitor **47**, whereas ACE is more tolerant in this region but strongly favors a proline at P_2' . Further optimization of **48** based on the relatively tight SAR for NEP inhibitors counterbalanced by flexible SAR for ACE inhibitors led to the discovery of conformationally restricted dipeptide mimetics, among which the 7,6-fused bicyclic thiazepine omapatrilat **49** was advanced into clinical development for the treatment of hypertension, but later failed in phase III due to angioedema side effects [61].

In another elegant example of the knowledge-based approach to MMLs, Murugesan *et al.* reported the design of dual AT_1 and endothelin-A receptor (ET_A) antagonists [62]. Again, the interest in this particular profile was generated by results of clinical studies suggesting that a combination of an AT_1 selective antagonist, losartan, and an ET_A/ET_B selective antagonist, SB-290670, produced an additive reduction in blood pressure compared with either drug alone. Taking the framework combination approach, the authors selected as a starting point selective AT_1 and ET_A antagonists with common structural features, compounds **50** and **51** (Figure 9.15). Both ligands contain a biaryl core. The heterocycle in the 4'-position of the biaryl, required for AT_1 activity, was well tolerated by ET_A , and on the other hand, a pyrrolidine moiety in the C_2' position of the merged MML was found to be optimal for the balanced activity at both receptors. Further optimization of compound **52** focused on improving pharmacokinetic profile, especially oral bioavailability, as well as on improving binding to both receptors resulted in the discovery of BMS-346567, an MML with superiority over irbesartan [63].

9.3.5 MML for the Treatment of Diabetes and Related Metabolic Diseases

Type 2 diabetes mellitus (T2DM) is considered a pathophysiologically complex disease resulting from the interaction of environmental (lifestyle) factors that act on a polygenic background. The disease is characterized by high blood glucose in the context of insulin resistance. Risk factors include poor diet, lack of physical activity, and obesity. No cure has yet been found for the disease; however a number of treatment modalities available today include lifestyle modifications, metformin, GLP-1 agonists, DPP-IV inhibitors, SGLT2 blockers, and insulin. For most patients, lifestyle interventions alone are insufficient to maintain blood glucose within a target range over the long term, and pharmacotherapy is required. Current international guidelines for the treatment of T2DM recommend the use of combination therapy when oral monotherapy fails to achieve or maintain sufficient blood glucose control, and combination therapy should be considered at the time of diagnosis for patients with poor blood glucose control. Given that T2DM is a multifactorial disease, linked to both environmental and

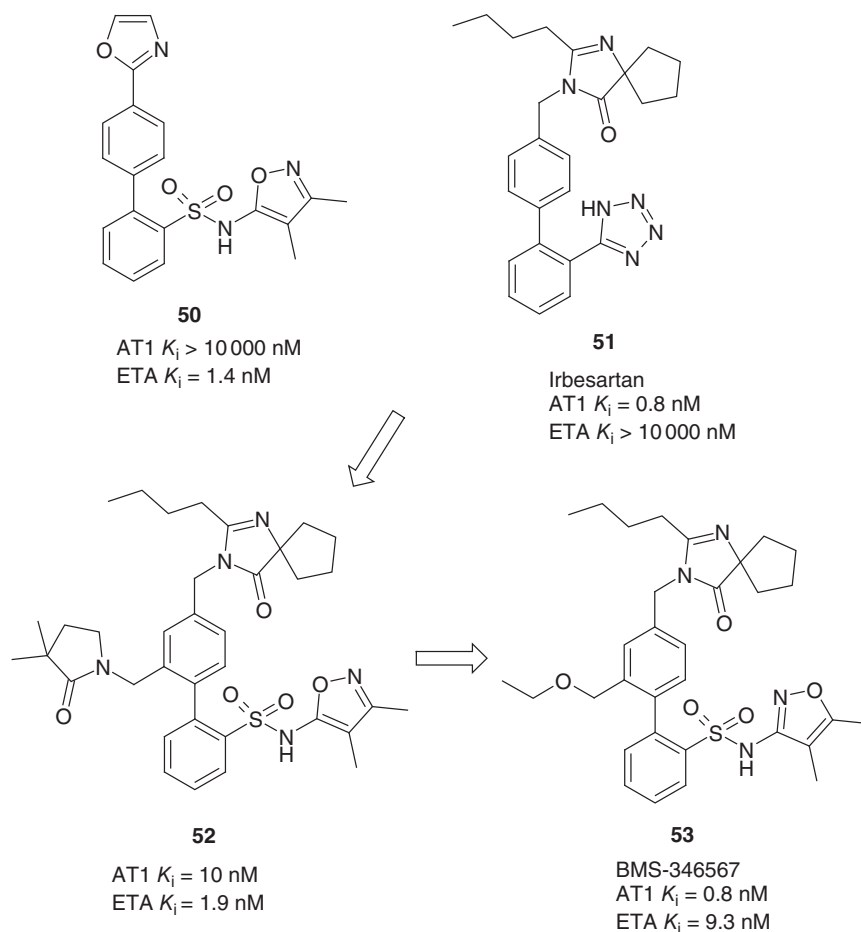


Figure 9.15 Design of a dual AT₁ and ET_A receptor antagonist.

genetic factors, the MML concepts represent an attractive avenue to novel and innovative treatment options.

DPP-IV inhibitors have been on the market for over 5 years and have been shown to be well tolerated and weight neutral. On the other hand, MCH-1 receptor antagonists have been explored as anti-obesity drug opportunities, and oral dosing of these antagonists is well documented to give significant weight loss in rodent models. To date only a few MCH-1 programs have been advanced into the clinic, and validation in man remains elusive. The combination of a DPP-IV inhibitor and a MCH-1 receptor antagonist appears to be appealing, providing an oral antidiabetic agent that gives significant weight loss. Gattrell *et al.* used a ligand-based approach to identify starting points for a medicinal chemistry program aiming at the discovery of dual-acting DPP-IV and MCH1R modulators [64]. Although ligands known to interact with both targets show structural diversity, X-ray crystallography has shown that DPP-IV possess a large solvent-exposed cavity proximal to the active site, suggesting a potential

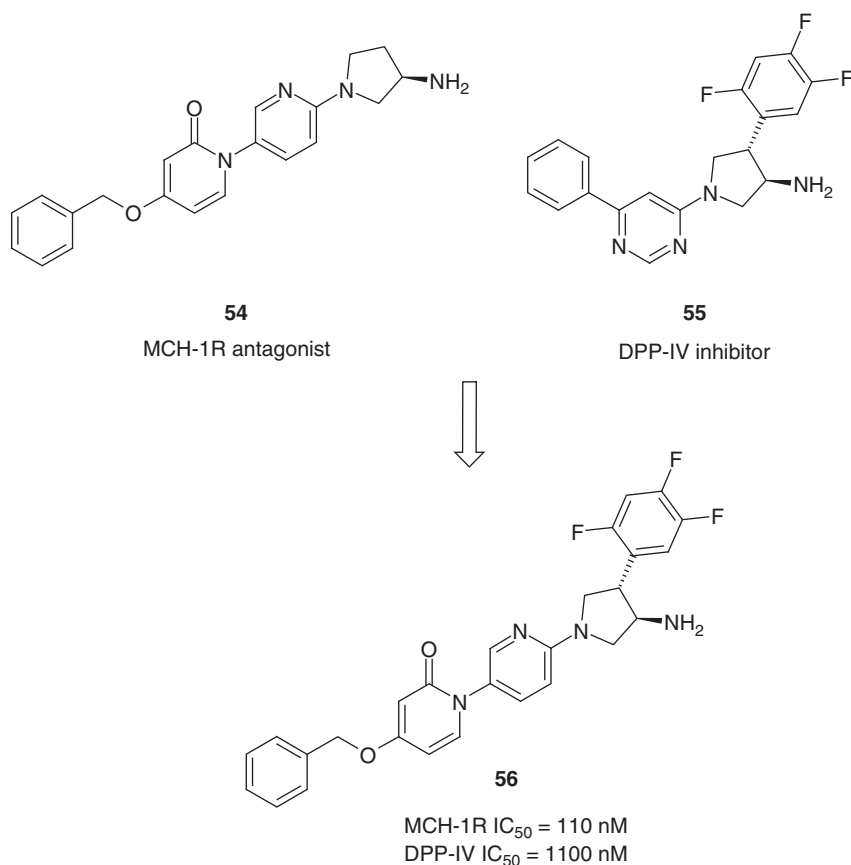


Figure 9.16 MML example for MCH-1 and DPP-IV.

to accommodate a significant part of the pharmacophore of a MCH-1 receptor antagonist. Following this theme, MCH-1R ligands such as compound **54** were cross-hybridized with a DPP-IV inhibitor (compound **55**) as shown in Figure 9.16 to provide dual MCH-1R antagonists and DPP-IV inhibitors with compound **56** as a typical example.

Analogously, Gattrell *et al.* used a ligand-based approach in the design of MMLs combining inhibition of DPP-IV and agonism of GPR119. The crystal structure of the DPP-IV enzyme active site revealed an extensive cavity in the enzyme well positioned to allow the incorporation of the GPR119 pharmacophore. Subsequent chemical design led to the identification of potent dual modulators with compound **57** as an example (Figure 9.17). In fact, this program culminated with the nomination of a clinical candidate, PSN491 [65].

A final and elegant example of an MML is PSN602 (Figure 9.18), a potent inhibitor of both noradrenaline (NE) and serotonin (5-HT) reuptake transporters and a full agonist at the 5-HT_{1A} receptor. Its design is based on the dual NE/5-HT reuptake inhibitor sibutramine, which was approved for the treatment of obesity. Sibutramine is associated with elevations in blood pressure and heart

Figure 9.17 Design of a dual GPR119 and DPP-IV MML.

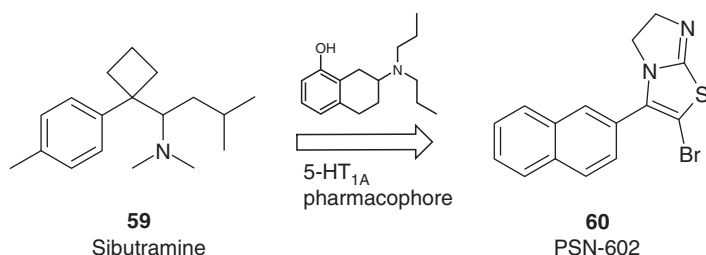
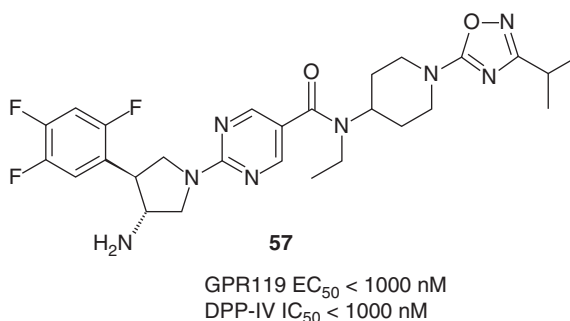


Figure 9.18 MML design of PSN-602.

rate in some patients, raising safety concerns and limiting the ability to dose titrate upward to achieve better efficacy. It was hypothesized that the addition of 5-HT_{1A} agonism to monoamine reuptake inhibition would reduce the risk of unwanted cardiovascular activity. In preclinical models PSN602 was effective as a high dose of sibutramine at reducing body weight but exhibited a more favorable cardiovascular profile. The compound has reached clinical development.

9.3.6 MML for the Treatment of Inflammation and Pain

Inflammation and pain are complex biological processes that involve a large number of mediators. For over a century, the arachidonic acid (ARA) pathway has been the target of numerous drugs. In particular, nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) selective inhibitors (coxibs) are widely used in the treatment of inflammation and pain. However, gastrointestinal and cardiovascular adverse effects of NSAIDs and coxibs, and recent findings demonstrating that there are significant risks from the disruption of oxylipin levels when inhibiting a single ARA cascade pathway, have led to studies involving the simultaneous modulation of multiple pathways. These studies suggest that a multitarget approach represents a new and valuable option to enhance efficacy or reduce side effects in the treatment of inflammation and pain [66].

Recently, Chen *et al.* reported dual inhibitors that target COX and the downstream leukotriene-metabolizing enzyme LTA₄H, an epoxide hydrolase that catalyzes the conversion of LTA₄ into LTB₄ (Figure 9.19). A dual inhibitor, compound **63**, was designed by merging the COX-2 selective inhibitor nimesulide with an LTA₄H inhibitor, 1-(2-(4-phenoxy)ethyl)pyrrolidine [67].

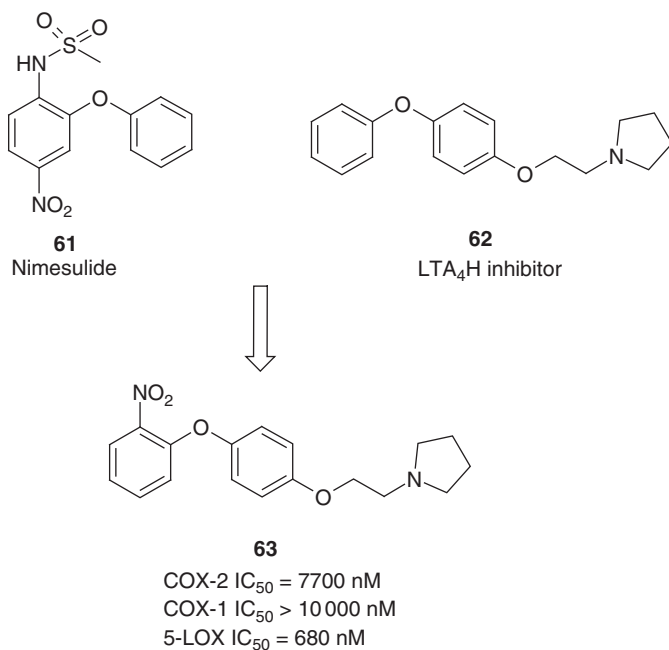
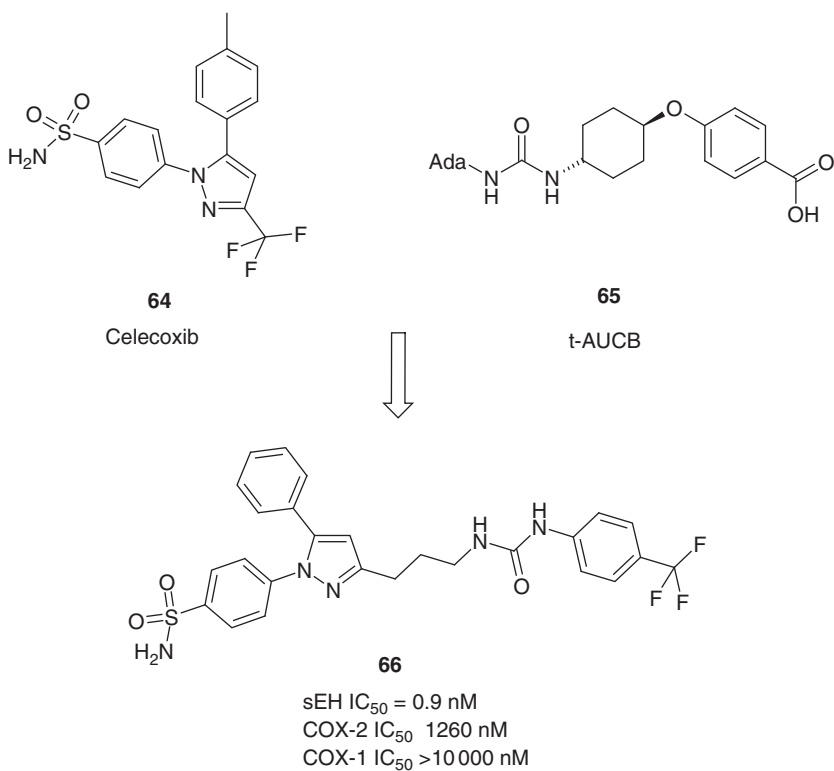
Figure 9.19 Design of a dual COX-2/LTA₄H inhibitor.

Figure 9.20 Design of a dual COX-2 and sEH inhibitor.

A further example is the development of the COX-2/sEH dual inhibitors (Figure 9.20) [68]. Combining a COX-2 inhibitor (compound **64**) with t-AUCB, an sEH inhibitor, led to the dual inhibitor compound **66**, which exhibited in an LPS-induced rat pain model by s.c. injection an anti-allodynic activity. Compound **66** was more efficacious than the same dose of either a celecoxib or a combination of an sEH inhibitor and celecoxib in this model.

9.4 Conclusions and Future Outlook

Designed MMLs have been established as an opportunity to reach sufficient efficacy when a one-molecule–one-target hypothesis hasn't provided the desired outcome. Many impressive examples in the literature from a variety of disease areas have been described in this chapter to show the feasibility of MMLs. As has been shown in several examples, computational methods can provide essential support in an MML project. There are several reasons to be optimistic for the future of MMLs. The available bioactivity data to identify starting points for MML projects will only increase. The increased understanding of disease networks will facilitate the identification of suitable target combinations, and the learnings from more and more successful examples of designing MML will further guide future MML projects.

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10

Pharmacophore Generation for Multiple Ligands

Norbert Handler

RD&C Research, Development & Consulting GmbH, Neuwaldegger Strasse 35/2/3, Vienna, 1170, Austria

10.1 Introduction

The concept of pharmacophore is known for a long time and was first introduced by Ehrlich [1], when he defined the pharmacophore as “a molecular framework that carries (phoros) the essential features responsible for a drug’s (pharmacon) biological activity.” This basic concept still remains unchanged, although, after a century’s development, its intentional meaning and application range have been expanded considerably. A relevant definition was given by IUPAC [2], who stated that a pharmacophore model is “an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response.” These interaction patterns of bioactive molecules and their targets are represented by a three-dimensional (3D) arrangement of abstract features that define interaction types rather than specific functional groups including hydrogen bonds, charged interaction, metal interactions, or hydrophobic and aromatic contacts. Additionally, steric constraints, so-called exclusion volumes mimicking the geometry of the binding pocket, may be introduced in pharmacophore models to avoid prediction of inactive molecules clashing with the protein surface. Further details and definitions as well as the development and history of the pharmacophore concept through the past century may be found in literature. [3–6].

In principle, for the development of pharmacophore models, two different approaches are known, the ligand-based and the structure-based approaches. In the ligand-based method, a set of active molecules is aligned and common chemical features essential for bioactivity are extracted, whereas in the structure-based method, the possible interaction patterns between macromolecular targets and their ligands are explored. Both methods can be used in pharmaceutical research for a broad range of tasks like virtual screening, *de novo* ligand design, lead identification and optimization, structure–activity relationship studies (SAR), and so on for selective as well as multiple ligands. The rapid development and improvement of computational chemistry played an essential role in the development and improvement of pharmacophore modeling tools. Additionally, the increasing availability of computing power, velocity, and

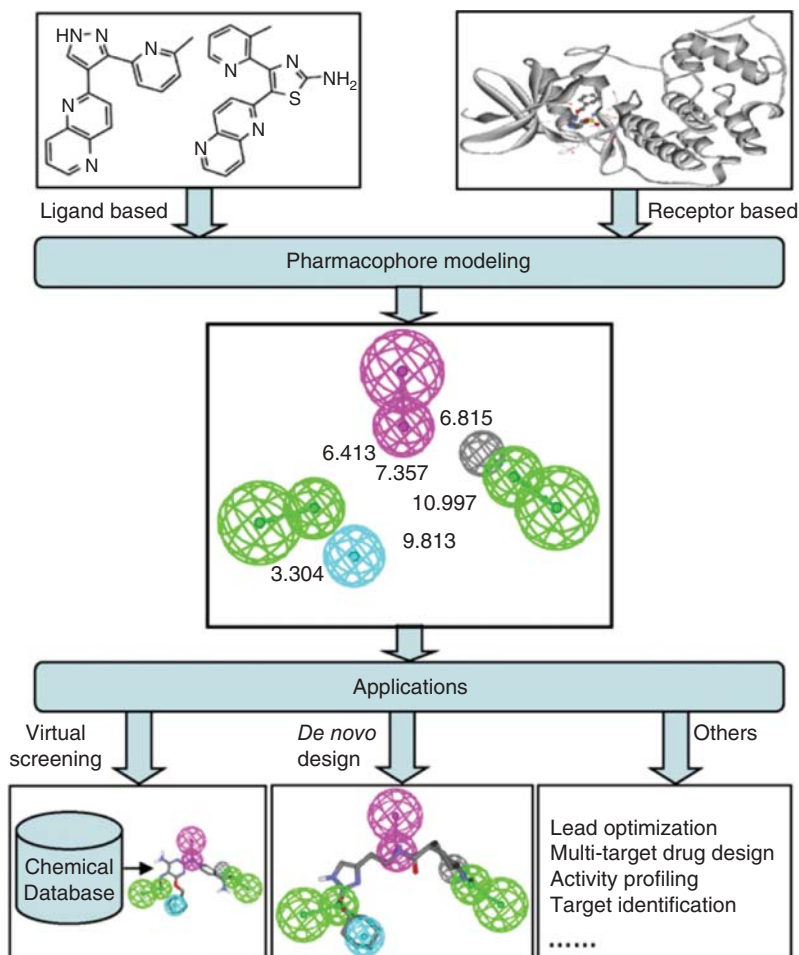


Figure 10.1 The basic framework of pharmacophore architecture. (Yang 2010 [7]. Reproduced with permission of Elsevier.)

storage media supported the proceedings in the last 20 years. In recent years several success stories have been documented emphasizing the importance and potential of these *in silico* methods; still, many challenges and limits have to be overcome to exploit the full potential for the application in pharmaceutical routine research and development. Figure 10.1 shows the basic framework and scope of pharmacophore modeling [7].

10.2 Ligand-Based Pharmacophore Modeling

Ligand-based pharmacophore modeling is an important tool for facilitating drug discovery in the absence of a macromolecular target structure. Therefore, a set of known ligands is compared, and common chemical features from their

3D structures are extracted representing the essential interaction between the ligands and the macromolecular target. This approach may be used to generate a pharmacophore model from numerous ligands (the training set compounds) and usually involves two main steps: (i) creation of conformational space for each ligand in the training set to represent conformational flexibility of ligands and (ii) alignment of multiple ligands in the training set and determining the essential common chemical features to construct pharmacophore models. Although several automated programs have been developed, both essential steps remain a major challenge and are prone to errors and misinterpretations. Some programs for pharmacophore modeling are presented and reviewed in literature and mainly use different algorithms for handling the flexibility of ligands and alignment of the molecules. Further details may be found in review articles and is beyond the scope of this book chapter [8, 9].

Despite the current proceedings pharmacophore modeling remains a challenging and sophisticated work especially for designing multiple ligands. A main challenge is the modeling of ligand flexibility, where two strategies have been developed to overcome this issue. In the pre-enumerating method multiple conformations for each molecule are pre-computed and saved in a database [8], having not only the advantage of lower computing cost for the molecular alignment but also the need for high mass storage capacity. Alternatively, the on-the-fly method could be used, where the conformation analysis is carried out in the entire pharmacophore modeling process with the need for higher CPU time for the subsequent rigorous optimization step [10].

Currently, a substantial number of advanced algorithms have been established to sample the conformational spaces of small molecules [7]; some of these algorithms, such as poling restraints [11], systematic torsional grids [12], directed tweak [13], genetic algorithms [14], and Monte Carlo [15], have been implemented in various commercial and academic pharmacophore modeling programs. However, at least the following requirements should be satisfied with a good conformation generator: (i) all putative bound conformations of small molecules during interaction with the macromolecules should be generated efficiently, (ii) the list of low-energy conformations should be kept as short as possible to avoid combinational explosion problems, and (iii) the conformational calculations should be time-efficient. Recently developed algorithms like MED-3DMC and Cyndi of CAESAR seem to be very efficient and fast and suitable for calculation of conformations.

As described above, the molecular alignment is the second major challenge in ligand-based pharmacophore modeling. Basically, there are two fundamentally different possibilities for molecular alignment: point-based and property-based approaches [10], whereas the points in a point-based method can further be differentiated in atoms, fragments, or chemical features [5]. There, pairs of atoms, fragments, or chemical feature points are usually superimposed using a least-squares fitting. Since anchor points need to be predefined, the method has its limitations especially in the case of dissimilar ligands. On the other hand, property-based algorithms use molecular field descriptors, usually represented by sets of Gaussian functions, which generate alignments. The optimization is based on some variants of similarity measure of the intermolecular overlap

of the Gaussians as the objective function. Conventional molecular alignment algorithms have been extensively reviewed elsewhere [10]; however, new alignment methods like stochastic proximity embedding [16], atomic property fields [17], fuzzy pattern recognition [18], and grid-based interaction energies [19] are actively developed. Another essential step in pharmacophore modeling is the proper selection of training set compounds, which highly influences the final pharmacophore model through the type of ligand molecules, the size of the dataset, and its chemical diversity [8]. Subsequently, highly different pharmacophore models of ligands interacting with the same macromolecular target could be generated from the same algorithm and program by using different training sets. For example, three different pharmacophore models of cyclin-dependent kinase 2 (CDK2) inhibitors were generated by three different training sets using the same modeling program [20–22].

10.3 Structure-Based Pharmacophore Modeling

Structure-based pharmacophore modeling is based on the 3D structure of a macromolecule–ligand complex or a macromolecule without a ligand. In principle, complementary chemical features of the active site and their spatial relationships and the subsequent pharmacophore model assembly with the selected features are determined. The macromolecule–ligand approach is used for locating the ligand binding site of the macromolecular target and determining the key interaction points between ligands and macromolecule. LigandScout [23] and Discovery Studio [24] are very good representations that incorporate the macromolecule–ligand complex-based scheme; however, they will only work when the 3D structure of the macromolecule–ligand complex and the target in the binding site are known. In case no information on the complex is available, a macromolecule-based approach can be used instead, again LigandScout [23] and Discovery Studio [24] offer such kind of modeling. There, LUDI [25] interaction maps within the protein binding site are converted into Catalyst features like H-bond acceptor, H-bond donor, and hydrophobes; however, these interaction maps consist of a large number of unprioritized Catalyst features, which complicate its application in 3D database searches. To overcome this drawback, hot spot-guided receptor-based pharmacophores [26] may be applied to enable prioritization of cavity atoms from training machine learning algorithms with atom-based fingerprints of known ligand-binding pockets. Also, an apoprotein-based approach was reported [27], where the GRID [28] molecular interaction fields (MIFs) are first calculated by using different probes for the binding site of interest, followed by the selection and subsequent conversion of the points of minimum of MIFs into pharmacophoric features.

A general problem of structure-based pharmacophore models may be the identification of a very large number of chemicals usually not prioritized features, since more than seven chemical features may not be suitable for practical applications like 3D database screenings. Typically, three to maximum seven chemical features have to be selected to construct a practical pharmacophore hypothesis; however, it cannot reflect the SAR, as the model is derived just by a single

macromolecule–ligand complex or a single macromolecule. A way to overcome this problem may be the use of a multicomplex-based comprehensive map as described by Zou *et al.* [29].

Certainly, any preliminary model generated with any approach will need further refinement steps like deletion or addition of pharmacophore features and adaptations concerning the feature weight and size; more sophisticated changes may comprise the modification of feature definitions, for example, the functional groups covered by a pharmacophore feature.

A critical review on pharmacophore models, computational programs, and differences in algorithms and analyses of results for Catalyst, Unity, LigandScout, Phase, Pharao, Molecular Operating Environment (MOE), Pharmer, and POT may be found in literature [7, 30, 31].

10.4 Pharmacophore-Based Virtual Screening

After generating a pharmacophore model, it can basically be used for querying the 3D chemical database to search for potential ligands doing a “pharmacophore-based virtual screening,” which is together with the docking-based virtual screening, a main tool in drug discovery. The pharmacophore-based screening reduces the problems arising from inadequate consideration of protein flexibility or the use of insufficiently designed or optimized scoring functions by introducing a tolerance radius for each pharmacophoric feature. Accordingly, the pharmacophore hypothesis is taken as a template to find molecules (hits), which possess chemical features similar to the template. A variety of compounds may be obtained, some with scaffolds similar to known active structures, while others with entirely novel structures, whereas the search for compounds with different scaffolds is generally known as “scaffold hopping” [22]. In any case, the aim of a pharmacophore-based virtual screening is to enrich active molecules in the virtual hit list by improving the quality of the model by applying multiple quality metrics like the enrichment factor (the enrichment of active molecules compared to random selection), yield of actives (the percentage of active compounds in the virtual hit list), specificity (the ability to exclude inactive compound), sensitivity (the ability to identify active molecules), and the area under the curve of the receiver operating characteristic area under the curve plot (ROC-AUC) [32]. Details concerning applied quality parameters may be found in literature [33–36]. However, some difficulties have to be solved during the screening process, like the handling of the conformational flexibility of small molecules and the identification of the pharmacophore pattern. For handling the conformational flexibility, the same strategies as for pharmacophore modeling, namely, pre-enumerating multiple conformations for each molecule in the database or conformational sampling at search time, could be applied. The “substructure searching,” that is, the identification of a pharmacophore pattern, may check whether a query pharmacophore is present in a given conformer of a molecule. This searching is usually based on graph theory including Ullmann [37], the backtracking algorithm [38], and the generic match algorithm (GMA) [39]. Despite the increases in computing power, the screening of large chemical

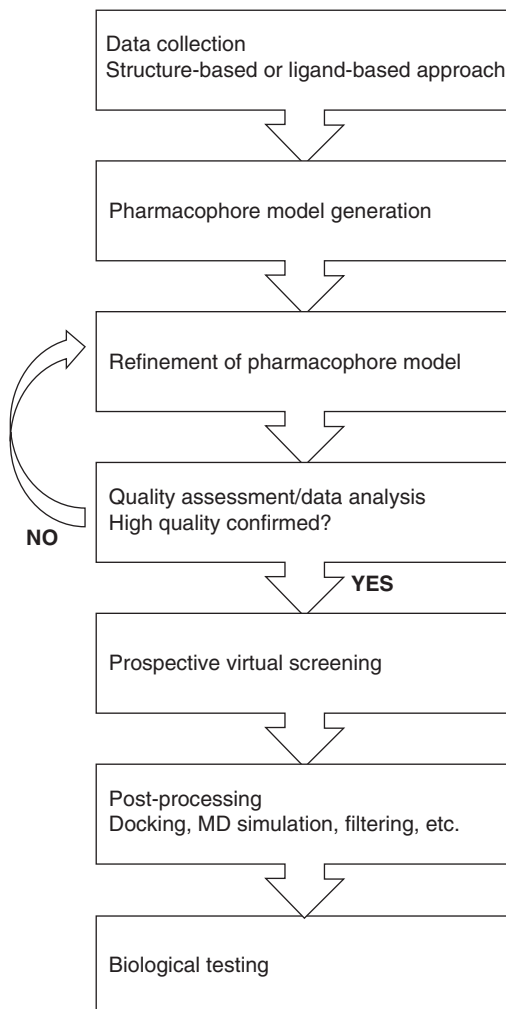
databases with flexible molecules may still be time-consuming. Accordingly, a multilevel searching approach may make sense [5], where a series of screening filters is applied to the molecules in an increasing order of complexity, so that the first filters are fast and simple, whereas successive ones are more time-consuming but are applied only to a small subset of the entire database. The filters may be selected individually, especially filtering according to Lipinski's rule of 5 (≤ 5 H-proton donors, ≤ 10 H-proton acceptors, molecular weight ≤ 500 , $c \log P \leq 5$ [40]); certain substructures or physicochemical properties like solubility may make sense.

In any case the low number of really bioactive virtual hits with high rates of false positive and/or high rates of false negatives may be a major drawback. Again, the quality and composition of the pharmacophore model and the macromolecular target information and the lack of a sound pharmacophore hypothesis will play an essential role. Accordingly, a comprehensive validation and optimization of the pharmacophore model by, for example, cross-validation and test set method is required [41]. The validation and optimization process can also be automated by adjusting the position and tolerance radius of each pharmacophoric feature as described in literature [42]. Additionally, the 3D pharmacophore model used for query is generally one of the subgraphs of the full pharmacophore map; screening with this pharmacophore query might not retrieve molecules that match other subgraphs except for the selected one, which is probably an important reason for the higher false negative rate in some studies. Also the flexibility of target macromolecule in pharmacophore approaches is handled by introducing a tolerance radius for each pharmacophoric feature, which is unlikely to fully account for macromolecular flexibility in some cases. There, the incorporation of molecular dynamics simulations could be a good solution, which should show considerably a better representation of the flexibility of the pharmacophore. Finally, the steric restriction by the macromolecular target may not be considered sufficiently in the models, which may lead to high false positive rate. Additionally, most interactions between ligand and protein are distance sensitive, particularly the short-range interactions, such as the electrostatic interaction, of which a pharmacophore model is difficult to account for. Therefore, the synergistic combination of pharmacophore-based and docking-based virtual screening may be used in a hybrid protocol to mutually compensate the limitations. Reviews on combined strategies and their validity can be found in literature [43–48] (see Figure 10.2).

10.5 Pharmacophore-Based *De Novo* Design

The pharmacophore model may also be applied for a *de novo* design of ligands to create completely novel candidate structures that conform to the requirements of a given pharmacophore. An older program is NEWLEAD [49], where a set of disconnected molecular fragments that are consistent with the pharmacophore model and the selected sets of disconnected pharmacophore fragments are connected by using linkers (like atoms, chains, or ring moieties). To name the drawbacks of NEWLEAD, only concrete functional groups can be handled; the

Figure 10.2 Basic steps for pharmacophore model generation, refinement, and application. (Adapted from Kaserer *et al.* 2015 [30], <http://www.mdpi.com/1420-3049/20/12/19880>. Used under CC BY 4.0, <https://creativecommons.org/licenses/by/4.0/>.)



sterically forbidden region of a binding site is not considered, and some obtained compounds may be difficult to synthesize. However, also the old program LUDI [50] combines identification of structure-based pharmacophore and can be used for *de novo* design; in any case the knowledge of macromolecular target 3D structure is required. To overcome these drawbacks further programs for *de novo* pharmacophore-based design were developed, for example, PhDD [51], which can automatically generate drug-like molecules, which satisfy the requirements of an input pharmacophore hypothesis. Also abstract chemical features and sterically forbidden regions of the binding site can be included to conform with the requirements of the given pharmacophore model. Additional programs for *de novo* drug design like SMOG, LigBuilder, SYNOPSIS, FLUX, DOGS, and so on were developed; a very recent publication gives more details on the advantages and disadvantages for multitarget drug design [52].

10.6 Limitations for Pharmacophore Modeling

Although pharmacophore modeling is an important and versatile method in drug discovery and development, the limitations clearly have to be addressed. Different programs follow different screening algorithms. Accordingly, highly different hit list may be derived. A recent study compared two pharmacophore modeling programs, LigandScout and Discovery Studio, on the identification of novel cyclooxygenase inhibitors [53]. Both programs succeeded in finding novel bioactive molecules; however, the generated hit lists turned out to be completely different without a single overlap. It seems that neither program covered the active space comprehensively and models from different programs need to be combined to obtain a complementary and full hit list.

As outlined in another study [54], the role and treatment of halogens in medicinal chemistry is not considered satisfactorily in the modeling programs. Some consider halogen only a hydrophobic moiety [24, 55, 56], and some additionally match fluorine to H-bond acceptors. However, in many ligand–target complexes, halogens participate in strong halogen bond formations, for example, with aromatic rings contributing to the interaction of ligand and target considerably. These types of interactions are usually not yet implemented in common pharmacophore-based virtual screening tools [54].

As every virtual screening tool, also pharmacophore modeling very much depends on the data employed for model generation, refinement, and theoretical validation. Although many repositories exist with a plethora of data and information, many of them are erroneous, and up to 10% of the original structures have to be removed as described in literature [57]. Accordingly, manual inspection steps are recommended to check not only the structures but also biological data, which might be incorrect or useless. Thus, the exclusion criteria for inappropriate testing system (e.g., data from intact cell assays or from animal tissue preparations are of limited use for human enzyme models) and the application of suitable activity cutoffs and critical comparison with the original literature are crucial steps to clear up the data basis and ensure high quality of the pharmacophore model.

Another limitation is the lack of information on confirmed inactive compounds for a specific target, since these data are usually not published, though this information would be important for the balancing between the selectivity and sensitivity of a model during the validation step.

Additionally, due care has to be taken for defining detailed interaction pattern of active compounds in the dataset. Although the data may be of high quality, the determination of an exact binding mode at the respective binding site may be difficult. Despite high similarity, some molecules may occupy a slightly different part of the binding pocket, and the interaction patterns may therefore differ. This factor is even more pronounced in ligands binding to allosteric sites, disrupting conformational changes, or interfering with posttranslational modifications. Similar concerns also apply for the experimental validation of the *in silico* predictions, as it is often not known whether the newly identified compounds indeed exert the predicted binding mode. Though an X-ray crystal

may be desirable and delivers more information, the 3D structure resolution of, for example, transmembrane proteins by crystallization remains difficult. In cases where homology modeling is not possible, a ligand-based pharmacophore modeling approach may be feasible.

10.7 Practical Strategy for Pharmacophore-Based Discovery of Multiple Ligands

In 2004, Morphy *et al.* [58] described the principle of designed multiple ligands, where a balanced modulation of several targets may provide a superior therapeutic effect and side effect profile compared with the action of a selective ligand. The major challenge is to gain a balanced activity at each target of interest while simultaneously achieving a wider selectivity and a suitable pharmacokinetic profile. Additionally, the basic pharmacophoric attributes of multiple ligands ranging from conjugates to overlapping pharmacophores were described. In a simple approach pharmacophores from two selective ligands may be linked together via cleavable or non-cleavable chemical linkers. On the other hand, pharmacophores may show structural commonalities with various degrees of overlapping structural features, forming a continuum from linked multiple ligands with high molecular weight to structures with highly integrated pharmacophores and lower molecular weight (see Figure 10.3).

As mentioned above, a simple technique to design multiple target ligands is to integrate structural elements from various single target ligands [58, 59], but many of them are quite inefficient in terms of their binding energy per unit of molecular weight, since they bear essential groups for binding one of the targets, but might be merely tolerated by the other targets [60, 61]. Multitarget compounds were examined for their mean molecular weight and the total number of known targets with at least minimal activity ($<10\ \mu\text{M}$), and they tended to show

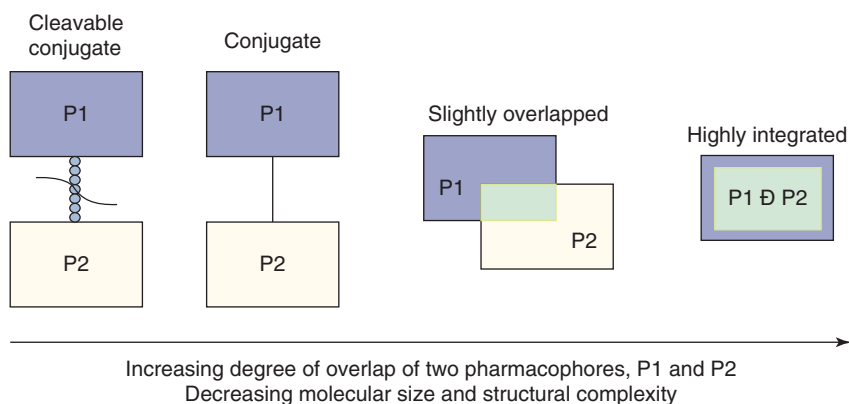


Figure 10.3 The degree of pharmacophore overlap varies significantly among designed multiple ligands. There is a continuum from conjugates where the pharmacophores are well separated by a linker group to ligand where the pharmacophores are highly intermingled. (Morphy *et al.* 2004 [58]. Reproduced with permission of Elsevier.)

lower molecular weight than highly target-specific ones [60, 62]. Subsequently, a fragment-based approach for screening multiple ligands was proposed by Morphy and Rankovic due to the lower size and the binding energy per heavy atom. Screening of fragment libraries (fragments are organic molecules in the range of 100–300 Da) may lead to some hits; however, significant workload at high costs and low overall hit rates may limit the success of this method. The success rate may easily be increased by including protein structural information of the targets. Subsequently, a set of molecular structural features that is recognized at the binding site and responsible for the biological activity of molecules is integrated together with the protein data. This approach can be executed in several steps: (i) generating the pharmacophore model for each protein with a common computer program, (ii) identifying the common pharmacophores by comparing the models (if any), (iii) executing a fast docking approach to predict the binding conformations of small molecules in one of the target proteins and selecting of those molecules whose binding conformations accommodate the common pharmacophores, (iv) executing a more rigorous docking approach to analyze the binding conformations of the selected compounds in the multiple target proteins and choosing of candidates whose binding conformations accommodate the common pharmacophores in all expected proteins, and (v) experimental activity testing of candidates on target proteins. Figure 10.4 shows the schematic procedure.

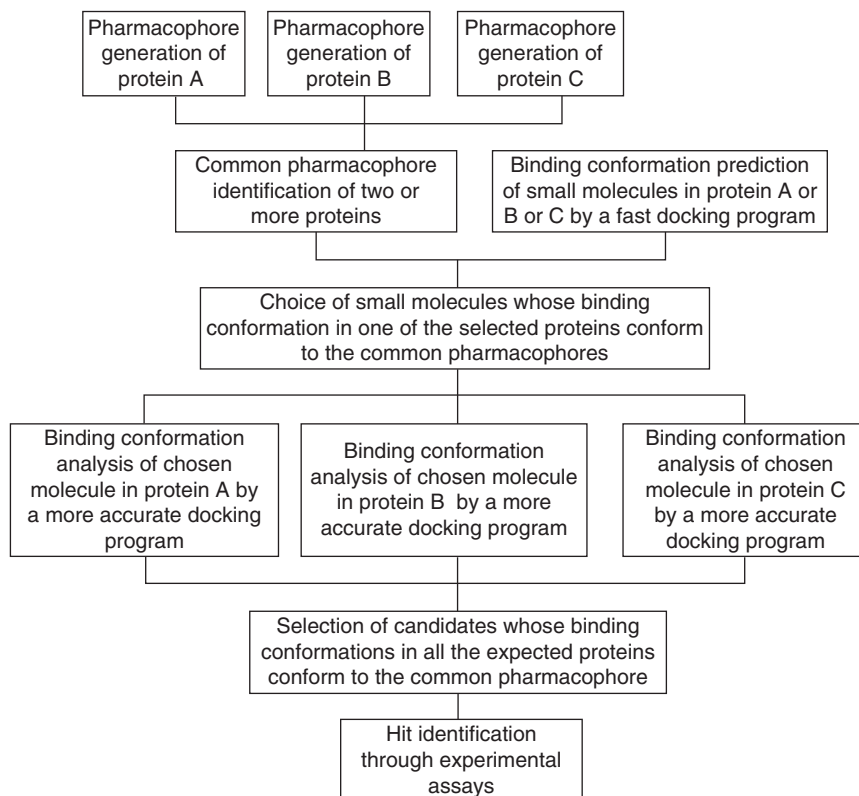


Figure 10.4 Proposed strategy for developing multitarget compounds. (From Ref. [63], with permission, © 2008 American Chemical Society.)

This strategy is a rational and versatile general approach for the design and development of multiple ligands; however, several alternatives have been explored for a broad range of targets and molecules. Generally speaking, a plethora of studies on pharmacophores for different targets and multiple acting molecules was published; however, the majority lacks the essential final step, that is, the hit identification through the experimental assays, and thus confirmation of the computer model by biological results. The following section describes the entire multiple ligand continuum from linked pharmacophore patterns to highly integrated examples including biological results to illustrate the broad spectrum of successful pathways for design and development of multiple ligands.

10.8 Linked Fluoroquinolone-Flavonoid Hybrids as Potent Antibiotics against Drug-Resistant Microorganisms

To overcome the evolution of bacterial drug resistance against common topoisomerase II inhibitors, a novel concept for dual acting molecules was designed [64]. Common quinolone pharmacophore was combined with the flavonoid pharmacophore due to the unique biological properties in the modulation of multidrug resistance. For example, modulation of bacterial efflux pump NorA was inhibited by the flavonoids teramethylscutellarein (II) and sarothrin. Subsequently, essential pharmacophoric features of the antibiotic component floxacine were combined with the essential pharmacophoric features of the flavonoids via linker (see Figure 10.5).

Various molecular entities were designed and synthesized. Finally, compound **1** could be identified with excellent antibacterial properties against four different resistant bacterial strains; the dual mode of action was confirmed by drug accumulation studies (see Figure 10.6 and Table 10.1).

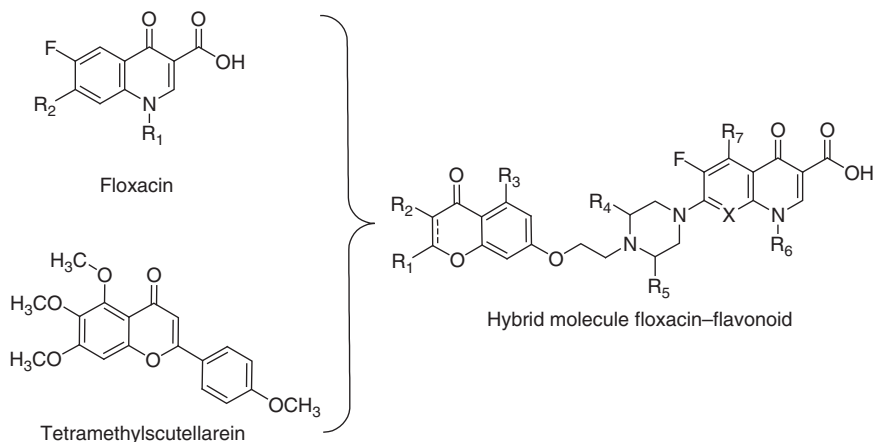
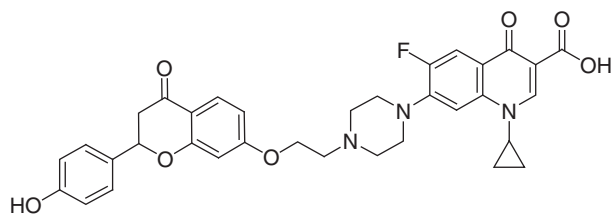


Figure 10.5 Molecular structure of floxacines. Tetramethylscutellarein and a dual-acting hybrid molecule.



Compound 1

Figure 10.6 Molecular structure of compound 1.**Table 10.1** Inhibitory activity (MIC_{50}) of the synthetic compound 1 against microbes.

Compound	MIC_{50} ($\mu\text{g/mL}$)			
	A	B	C	D
Compound 1	0.71	0.062	0.29	0.14
Ciprofloxacin	5.65	2.70	6.82	32.4

A, multiple drug-resistant *Escherichia coli* ATCC 35218; B, tetracycline-resistant *Bacillus subtilis* ATCC 6633; C, methicillin-resistant *Staphylococcus aureus* ATCC 25912; D, amphotericin B-resistant *Candida albicans* ATCC 90873.

10.9 N-Phenylquinazolin-4-Amine Hybrids as Dual Inhibitors of VEGFR-2 and HDAC

To enrich the broad range of cancer therapeutics and offer innovative treatment strategies, novel molecules showing inhibition of multiple targets and pathways of cancer promotion were designed and tested for their *in vitro* inhibitory potency [65]. A useful synergistic effect in cancer therapy may be derived by dual inhibition of vascular endothelial growth factor/histone deacetylase (VEGFR-2/HDAC) with a single molecule. Vascular endothelial growth factor and its receptor tyrosine kinase VEGFR-2 or kinase insert domain receptor (KDR) are the most important regulator of tumor angiogenesis, and inhibition of the VEGF/VEGFR-2 signaling pathway has become a valuable approach in the treatment of cancers. Although several VEGFR-2 inhibitors have been approved already, a significant number of patients do not respond to this therapy. Additionally, drug resistance is occurring following treatment. Histone deacetylases (HDACs) are a class of enzymes catalyzing the removal of acetyl groups from the ϵ -amino groups of lysine residues in histones, leading to chromatin condensation and transcriptional repression. Several HDACs have been identified in humans, 10 of them being zinc-containing enzymes; the inhibition of them has widely been recognized as promising targets for therapeutic intervention. Hence, the pharmacophoric features of selective VEGFR-2 inhibitor vandetanib and HDAC inhibitor vorinostat were analyzed, and two major pharmacophoric properties were combined in a single molecule by linking both parts with a linker chain. Several molecules were synthesized and tested for their inhibitory effect against VEGFR-2 and HDAC *in vitro*, where the most potent molecule showed IC_{50} of

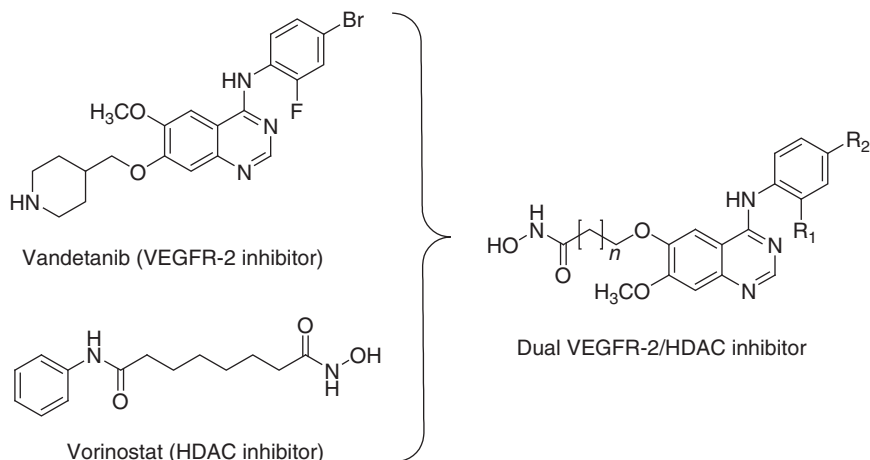


Figure 10.7 Design of dual VEGFR-2/HDAC inhibitors.

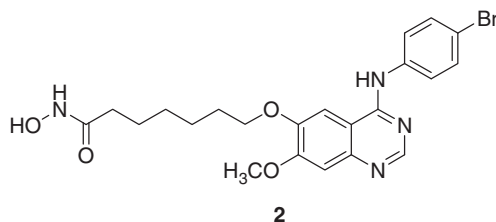


Figure 10.8 Chemical structure of most effective dual VEGFR-2/HDAC inhibitor.

Table 10.2 IC_{50} values of vandetanib, vorinostat, and the dual ligand **2** against VEGFR-2 and HDAC and MCF-7 cell line.

Compound	VEGFR-2 (nM)	HDAC (nM)	MCF-7 (nM)
2	$IC_{50} = 74$	$IC_{50} = 2.2$	$IC_{50} = 0.85$
Vandetanib	$IC_{50} = 54$	$IC_{50} > 10.000$	$IC_{50} = 18.5$
Vorinostat	$IC_{50} > 10.000$	$IC_{50} = 15$	$IC_{50} = 4.2$

2.2 nM against HDAC and IC_{50} of 74 nM against VEGFR-2. Additionally, the antiproliferative effect could be proven against solid tumor cell line MCF-7 with IC_{50} of 0.85 μ M. The initial pharmacophoric hypothesis could be confirmed by docking simulations; a follow-up research program may be justified to look for further potent dual VEGFR-2/HDAC inhibitors (Figures 10.7 and 10.8, Table 10.2).

10.10 Dual Inhibitors of Phospholipase A2 and Human Leukotriene A4 Hydrolase as Anti-Inflammatory Drugs

Wei *et al.* developed a computer-assisted strategy to screen for multitarget inhibitors using a combination of molecular docking and common

pharmacophore matching to obtain dual inhibitors of human leukotriene A4 hydrolase (LTA4H-h) and human nonpancreatic secretory phospholipase A2 (hnps-PLA2) [65]. Both enzymes play a major role in the arachidonic acid metabolic network depleting the sources of arachidonic acid and controlling the generation of downstream metabolites and amplifying many inflammatory disease states, respectively. A dual inhibition of both LTA4H-h and PLA2 may provide relief to patients suffering from inflammatory disease. The pharmacophore model was developed from the 3D structure of a target protein, revealing the key elements of protein–ligand binding. Therefore, the structure of LTA4H-h with 2-(3-amino-2-hydroxy-4-phenylbutyrylamino)-4-methyl-pentanoid acid (bestatin) and the structure of hnps-PLA2 with [3-(1-benzyl-3-carbamoylmethyl-2methyl-1*H*-indol-5-yloxy)propyl]phosphonic acid were used to derive pharmacophores for these two proteins by Pocket v.2. Accordingly, three hydrophobic pharmacophoric features, one for coordination of the metal zinc and one H-bond acceptor, could be identified for LTA4H-h. For hnps-PLA2, three hydrophobic centers and two for coordination with a calcium ion could be derived. These two sets of pharmacophores were compared, and finally two common hydrophobic pharmacophores and one that coordinated with a metal could be defined. In the next step a fast docking approach for LTA4H-h was executed by Dock 4.0, and the binding conformations were predicted; the results were ranked by docking energy score and checked to determine whether their binding conformation accommodated the common pharmacophores. 163 compounds were selected, and the binding conformations with LTA4H-h and hnps-PLA2 were further refined using AutoDock 3.05. Subsequently, 9 compounds matched the common pharmacophore, and they were tested for their inhibition against both enzymes resulting in micromolar inhibition of both enzymes. Additionally, this result could be used for designing a combinatorial library in which different fragments matching single pharmacophore can be combined to construct active compounds or identify existing single target inhibitors with possible multiple functions. Finally, 350 LTA4H-h inhibitors were analyzed yielding some structures, which matched the common pharmacophores for the inhibition of both enzymes. Thus, a compound with low micromolar inhibition on hnps-PLA2 and high-to-medium nanomolar inhibition on LTA4H-h could be identified in alignment with the defined common pharmacophores (compound 4) (see Figures 10.9–10.11 and Table 10.3).

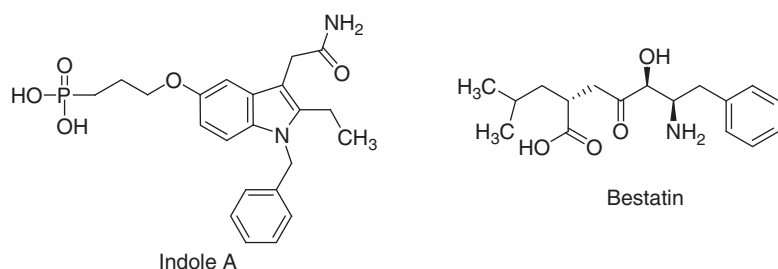


Figure 10.9 Chemical structures of selective hnps-PLA2 inhibitor (LY311727) and LTA4H-h inhibitor (bestatin).

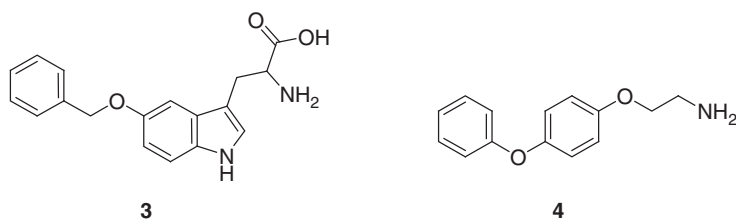


Figure 10.10 Chemical structures of dual LTA4H-h/hnps-PLA2 ligands.

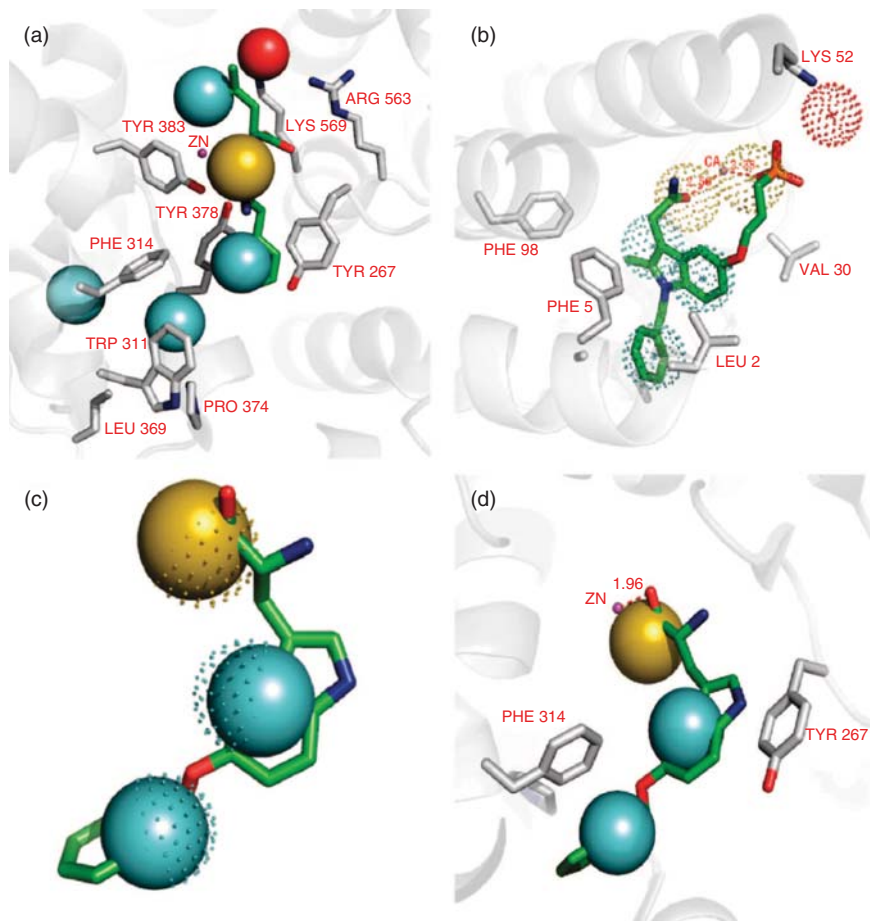


Figure 10.11 Pharmacophores and inhibitors of LTA4H-h and hnps-PLA2. Solid spheres represent the pharmacophore of LRA4H-h and dotted spheres the pharmacophores of hnps-PLA2. Cyan spheres stand for hydrophobic centers; red spheres represent the H-bond acceptor center; yellow spheres stand for the feature that coordinates with a metal. To clearly show the interactions between the metals and other atoms, the radii of metals are not shown in realistic ratios. (a) Pharmacophore model of LTA4H-h; the inhibitor bestatin is shown. (b) Pharmacophore model of hnps-PLA2; the inhibitor indole A is shown. (c) Alignment of common pharmacophores of LTA4H-h and hnps-PLA2; the inhibitor compound 3 is shown; (d) interaction of compound 3 with LTA4H-h; (e) interaction model of compound 4 with hnps-PLA2.

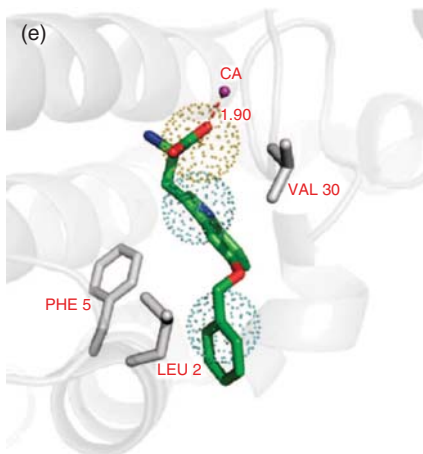


Figure 10.11 (Continued)

Table 10.3 IC_{50} values of dual-acting compounds against LTA4H-h and hnpS-PLA2.

Compound	IC_{50} (μM)		
	LTA4H-h		hnpS-PLA2
	Aminopeptidase	Epoxide hydrolase	
3	17.3 ± 0.2	231 ± 4	42.1 ± 1.9
4	0.228 ± 0.014	0.035 ± 0.002	7.3 ± 1.0

Data are the average values of three separate experiments $\pm$ standard error of the mean from at least three independent experiments.

10.11 Dual Antagonists of the Bradykinin B_1 and B_2 Receptors Based on a Postulated Common Pharmacophore from Existing Non-Peptide Antagonists

Bradykinins are blood-derived peptides exerting inflammatory effects in several *in vivo* models; hence bradykinin antagonists may be useful in the treatment of inflammatory pain and inflammation [66]. Since both receptor subtypes, the preformed and widely expressed bradykinin B_2 and the strongly regulated B_1 subtype, play a major role in the inflammatory cascade, a dual antagonist may make sense. Several B_1 inhibitors were reported so far, where a core motif phenyl-SO₂-NR-(spacer₂₋₄)-CO-NRR is present in all compounds. Merck's compound A was derived from homology modeling, and several interactions in the common region of the drug were predicted, notably the interaction of Gln²⁹⁵ in the TM7 domain with the sulfonic acid function of the drug. Additionally, specific B_2 receptor antagonists also possess core motifs remarkably similar to the postulated B_1 antagonists (phenyl-SO₂-NR-CRR-CO-NRR). This structural element was defined as common pharmacophore, and related

compounds were selected and measured in a competition assay. The results confirm the essential role of the common pharmacophore for inhibition of both bradykinin receptors and may be used for further optimization of dual inhibitors. Figure 10.12 (Table 10.4) shows the broad variety of possible molecules ranging from highly selective to balanced bradykinin antagonists possessing the same phenylsulfonamide pharmacophoric core motif.

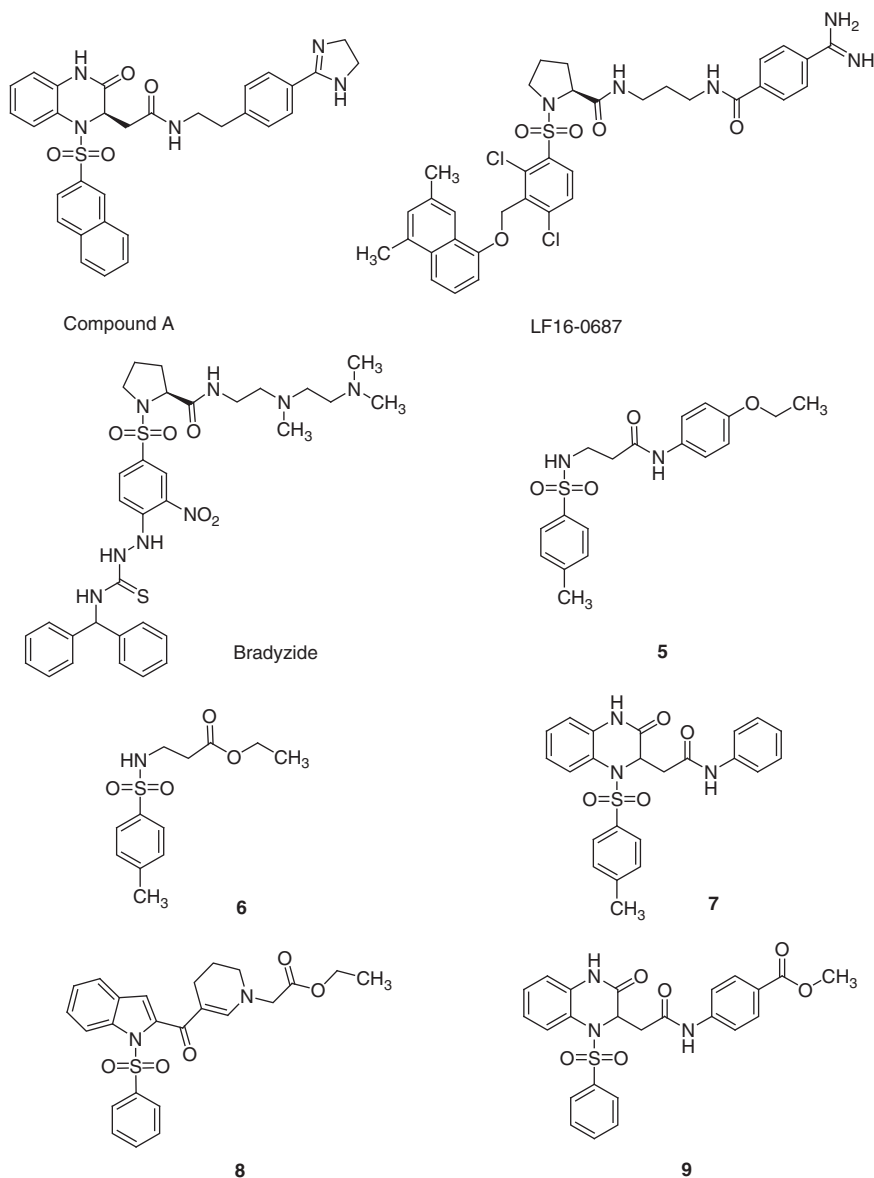


Figure 10.12 Chemical structures used as bradykinin B₁/B₂ inhibitors.

Table 10.4 Ligands at the kinin B₁ and B₂ receptors.

Compound	Selectivity
Compound A	B ₁ ; 13,708-fold
LF 16-0687	B ₂ ; 56.3-fold
Bradyzide	B ₂ ; 244.5-fold
5	B ₂ ; 19.3-fold
6	—
7	B ₁ ; 59.2-fold
8	B ₂ ; 9.7-fold
9	B ₁ ; 3.9-fold

The competition assay for radioligand binding is based on rabbit aortic smooth muscle ([³H]Lys.-des-Arg⁹-BK) and mesenteric artery smooth muscle cells ([³H]BK).

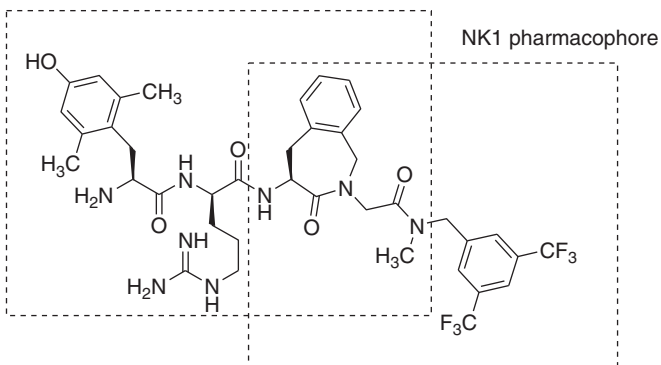
10.12 Dual-Acting Peptidomimetics with Opioid Agonist–Neurokinin-1 Antagonist Effect

Despite major drawbacks, morphine and other opioid ligands are still the prime analgesic for the treatment of severe and moderate pain [67]. Opioids, when administered over a longer period of time, show severe side effects like constipation, physical dependence, and analgesic tolerance. Additionally, they have less analgesic efficacy against neuropathic pain, which is caused by damaged nerves in the nervous system coming from trauma, infection, or diabetes. On the other hand, antagonism at neurokinin-1 (NK1) receptors may block the signals induced by substance P, an endogenous neurotransmitter and pronociceptive peptide involved in pain signaling, and lead to prolonged antinociceptive efficacy. Additionally, the side effect profile may be improved compared with morphine treatment alone. Hence the design and effect of multiple ligands with dual opioid agonistic and NK1 antagonistic profile were studied. The different pharmacophores were identified and finally merged to yield several hybrid molecules (Figure 10.13 and Table 10.5). Two compounds were selected and investigated *in vitro* and *in vivo* in acute and neuropathic pain models showing promising activity profiles. Both showed superior effect especially in the neuropathic pain model compared with morphine.

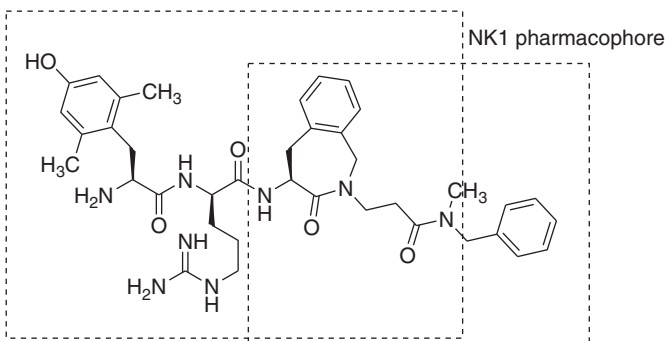
10.13 Novel Dual-Acting Compounds Targeting the Adenosine A_{2A} Receptor and Adenosine Transporter for Neuroprotection

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG trinucleotide expansion in the huntingtin (Htt) gene, which

Opioid pharmacophore

**10**

Opioid pharmacophore

**11****Figure 10.13** Example for a designed dual ligand as opioid agonist-NK1 antagonist.**Table 10.5** Calculated ED₅₀ values for the effect of hybrid molecules **10** and **11** compared with morphine (M) in acute pain (tail-flick test) and neuropathic pain (von Frey and cold plate test) as a measure on naive rats (acute pain) and neuropathic pain model at day 7 after chronic constriction injury (CCI).

Drugs	Response (ED ₅₀) with 95% confidence limits (nmol i. t.)		
	Acute pain	Neuropathic pain	
	Tail flick	Von Frey	Cold plate
M	0.49 (0.27–0.89)	6.25 (2.0–19.44)	377 (130–1093)
10	1.66 (1.11–2.51)	1.05 (0.65–1.69)	1.14 (0.74–1.77)
11	2.78 (1.80–4.30)	0.24 (0.18–0.32)	0.34 (0.25–0.46)

shows chorea, dementia, and psychiatric symptoms [68]. In a transgenic mouse model, it could be demonstrated that the selective A_{2A} adenosine receptor ($A_{2A}R$) agonist could attenuate HD symptoms. Besides $A_{2A}R$, the adenosine transporters, like equilibrative nucleoside transporter (ENT1), are localized in the striatum, where mutant Htt aggregates. The combination of an $A_{2A}R$ agonist and inhibitor of the ENT1 transporter may increase favorable adenosine concentration and be a valuable therapeutic option. Compound **12**, an adenosine analog and $A_{2A}R$ agonist, also exhibited ENT1 inhibition. Subsequently, analogs with dual ligand activity were rationally designed using a pharmacophoric approach, since no high-resolution X-ray or nuclear magnetic resonance (NMR) structures were published for the target biomolecules. First, two pharmacophoric models were constructed, one for $A_{2A}R$ agonists and another for ENT1 inhibitors, and after pharmacophore mapping, several designed compounds with acceptable predicted activities were synthesized. To construct the pharmacophore models, the training set compounds were collected from published sources, and suitable structure were selected and calculated with HypoGen module of Catalyst (Accelrys). The 3D pharmacophores for both targets can be found in the publication (Figure 10.14).

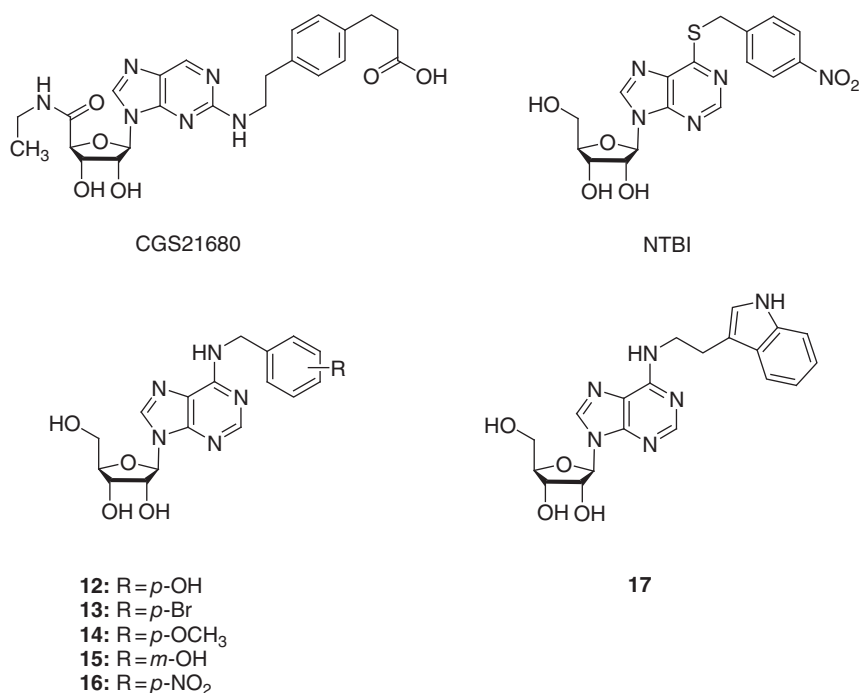


Figure 10.14 Chemical structures of selective $A_{2A}R$ agonist CGS21680, the selective ENT1 inhibitor NTBI, and developed dual $A_{2A}R$ /ENT1 ligands.

Table 10.6 Binding activity of selective and developed dual ligands at A_{2A} receptor and ENT1, including their effect on cell viability.

Compound	K _i A _{2A} R (μM)	K _i ENT1 (μM)	Cell viability (%)
CGS21680	7.77e-2	—	88.6 ± 9.6
NBT1	>10	2.9e-4	29.1 ± 2.1
12	2.62	0.538	81.5 ± 1.8
13	14.4	1.44e-2	63.7 ± 2.9
14	30.1	0.318	42.3 ± 1.8
15	3.21	3.72	83.9 ± 4.5
16	27.7	6.51e-3	48.2 ± 1.3
17	4.39	3.47	118.8 ± 3.9

Several molecules, which were aligned with the pharmacophoric model, were synthesized and tested for their binding affinities at A_{2A}R and ENT1. Additionally the cell viability was measured in serum-deprived PC12 cells, which are a crucial indication for neuroprotection and their potential for treating neurodegenerative diseases. The results showed that finally a moderate affinity toward both targets yielded the highest prevention of apoptosis in serum-deprived PC12 cells, a crucial indication for neuroprotection and potential for treatment of neurodegenerative diseases (Table 10.6).

10.14 Aminobenzimidazoles as Dual-Acting Butyrylcholinesterase Inhibitors and hCB₂R Ligands to Combat Neurodegenerative Disorders

Many pathophysiological processes do not run consecutively, but in parallel and independently, especially in diseases like neurodegenerative disorders, such as Parkinson's disease or Alzheimer's disease (AD) [69]. In AD, the interaction of β-amyloid (βA) plaque formation and τ-protein hyperphosphorylation and the formation and subsequent reactions of reactive oxygen species and cell changes, leading to the irreversible loss of neurons, especially cholinergic neurons, are yet unsolved. A promising solution may be the development of dual- or multiple-acting compounds addressing different processes simultaneously. Thus, a pharmacophore model for butyrylcholinesterase (BChE) inhibitors and human cannabinoid subtype 2 receptor (hCB₂R) agonists was established, and various derivatives were synthesized yielding dual-acting compounds with balanced affinities for potential further optimization as therapeutics in neurodegenerative disorders.

The pharmacophore model is based on previous knowledge on the development of both reversible and irreversible BChE inhibitors, and therefore two essential features could be formulated: first, the condensation of a (substituted) aromatic system to a basic (N-containing) heterocycle and, second, the connection to a sterically demanding hydrophobic moiety. These requirements could also be observed for two other structures, a benzimidazole-based CB₂R agonist described by AstraZeneca (compound C = **18**) and a bivalent CB₂R ligand published by Gonzalez-Naranjo *et al.* (Figure 10.15). Then the inhibition of BChE was examined and verified, and dual SAR studies toward ChE and CB₂R were conducted including molecular docking and molecular dynamics simulations. Although further evaluation and optimization of synthesized compounds may be necessary (exact mode of action, structure optimization, *in vivo* testing), structures representing sub-micromolar BChE inhibitors and *h*CB₂R ligands with selectivity over AChE and *h*CB₁R could be obtained (Figure 10.16, Table 10.7).

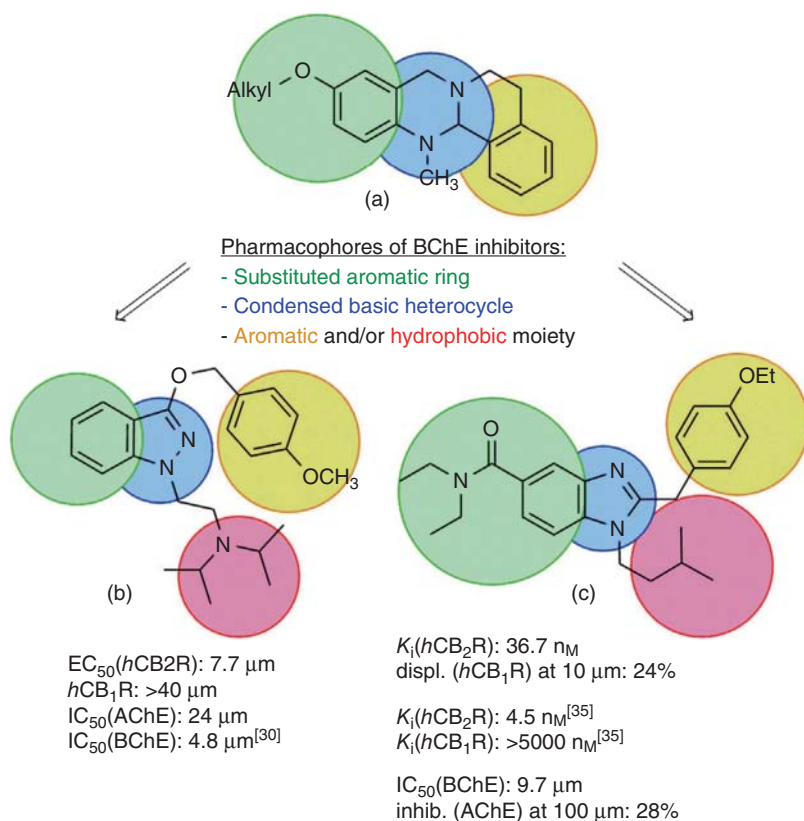


Figure 10.15 Simplified pharmacophore model (pharmacophores circled) of a prototypical competitive BChE inhibitor A [70, 71] and application of this model on a recently described dual-acting compound with an indazole ether scaffold (B) [72] and on a CB₂R-selective ligand described by AstraZeneca (C = **18**). (Pagé *et al.* 2008 [73]. Reproduced with permission of Elsevier.)

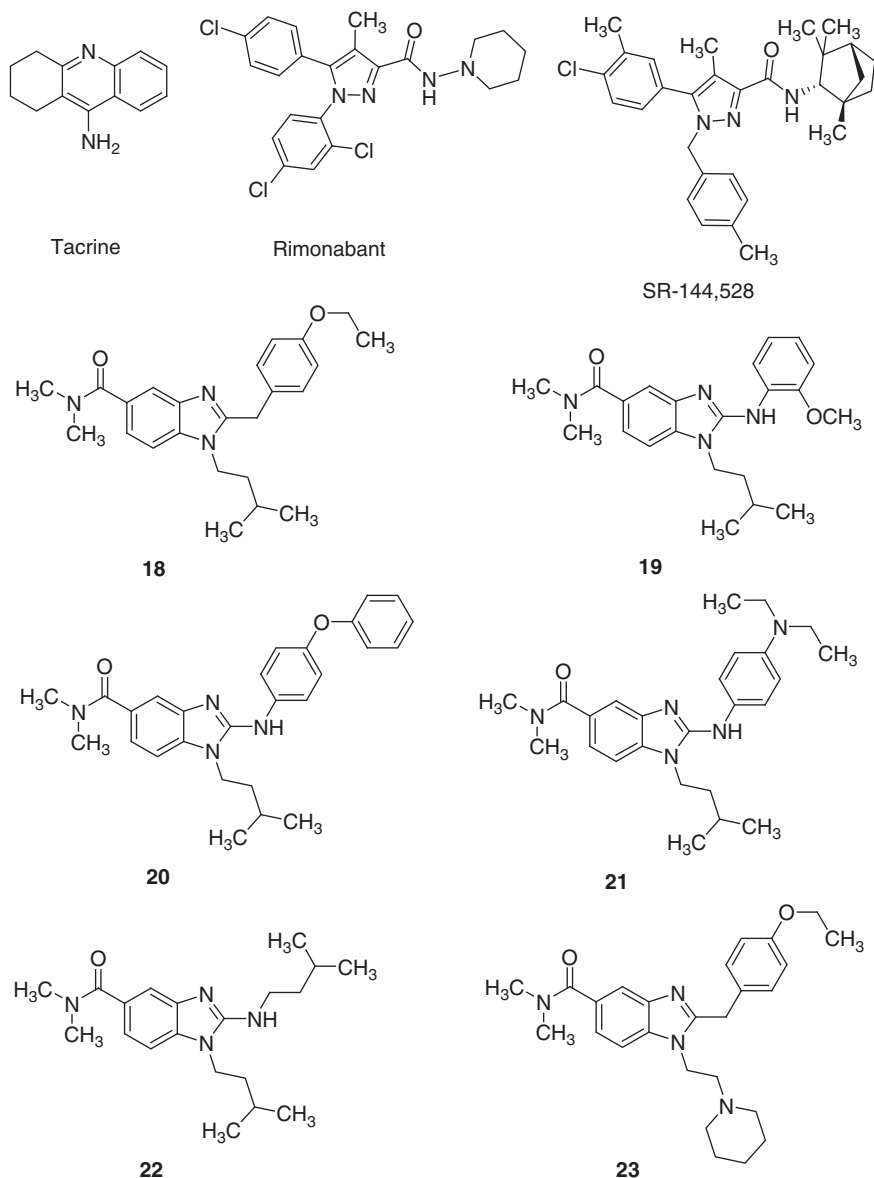


Figure 10.16 Chemical structures to investigate SARs at AChE, BChE, and hCB₁R, hCB₂R, respectively.

10.15 Dual Acetylcholinesterase Inhibitors–Histamine H₃ Receptor Antagonists for Treating Alzheimer's Disease

A previous publication dealing with the effective treatment of AD suggested to combine the effect of AChE inhibitors with histamine H₃ antagonists, since both components should improve and augment cholinergic neurotransmission

Table 10.7 Affinities of selective and dual ligands at BChE, AChE, and *hCB₂R*, *hCB₁R*, respectively.

Compound	IC ₅₀ or inhibition (%)		K _i or [³ H] CP55,940 displ. at 10 μ M	
	BChE	AChE	<i>hCB₂R</i>	<i>hCB₁R</i>
Tacrine	3.3 nN	15.6 nN	ND	ND
Rimonabant	ND	ND	4.0%	143.0 nM
SR-144,528	ND	ND	19.7 nM	687.0 nM
18	9.7 μ M	28% at 100 μ M	36.7 nM	>5 μ M
19	8.9 μ M	10% at 10 μ M	6.7 μ M	4%
20	3.7 μ M	2% at 10 μ M	426.0 nM	45%
21	0.2 μ M	10% at 10 μ M	8.1 μ M	4%
22	5.1 μ M	18% at 100 μ M	7.0 μ M	12%
23	2.3 μ M	48% at 100 μ M	188.0 nM	14%

in the cortex [74]. The major advantage would be the central effect of the H₃ component in the brain without peripheral side effects. Additionally the peripheral anionic site (PAS) of AChE interacts with the β A peptide; thus an inhibition may have a significant impact on the production or the aggregation of β A. To design dual-acting molecules, the available crystal structure information, pharmacophore modeling, and docking (automated, manual, classical, and QM/MM) were used. First, the crystal structure of decamethonium in AChE enzyme was analyzed, and the key interactions were determined (both quaternary ammonium functions were important). On the other hand, a 3D pharmacophore model for histamine H₃ antagonism was developed before and could be used for modeling a joint pharmacophore hypothesis with Catalyst using the Hip Hop option. Accordingly, common features of both models were identified and overlaid in space to obtain a pharmacophore model for dual inhibition with two positive ionic centers and an aromatic ring in the center of the molecule (Figure 10.17).

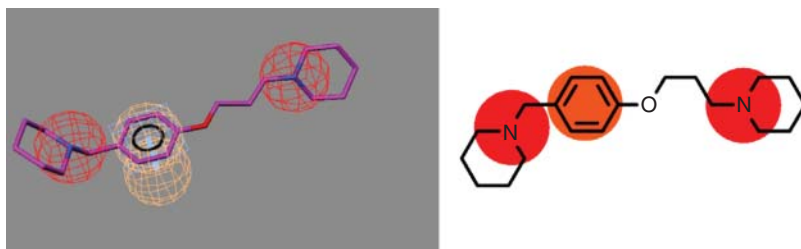
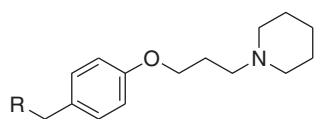
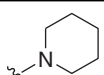
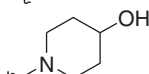
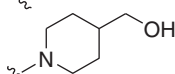
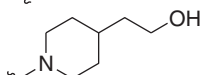
**Figure 10.17** The proposed three-dimensional pharmacophore for histamine H₃ with 1. The main features are two positive ionic centers and an aromatic ring forming the center of the molecule.

Table 10.8 Data for hydroxyl linkers of various lengths in the four position of the piperidine ring.


Compound	R	AChE IC ₅₀ (nM)	H ₃ IC ₅₀ (nM)
24		3,200 ± 400	0.53 ± 0.49
25		23,000 ± 100	0.72 ± 0.19
26		1,100	1.30 ± 0.60
27		350 ± 10	0.98 ± 0.21
	Decamethonium	5,900 ± 1,400	820 ± 170
	Donepezil	160 ± 30	350 ± 70

Following this pharmacophore model, several compounds were synthesized and tested for their AChE and H₃ inhibitory activity yielding dual-acting compound **27** with IC₅₀ of 350 nM for AChE and 0.98 nM for H₃ receptor, a potential drug for treatment and deceleration of AD (Table 10.8).

10.16 Identification of Potential Dual Agonists of FXR and TGR5 Using E-Pharmacophore-Based Virtual Screening

Bile acid receptors play a major role for insulin sensitivity and glucose control, lipids, and bile acid homeostasis. Accordingly, farnesoid X receptor (FXR) and Takeda G-protein coupled receptor-5 (TGR-5) are promising targets for treatment of diabetes [75]. In the current study, novel dual agonists for FXR and TGR5 were explored starting from a well-known dual-acting molecule INT-767. The compound was docked into the binding sites of both target molecules to generate an energy-optimized pharmacophore hypothesis. Additionally a ligand-based virtual screening using the ChemBridge database and a structure-based virtual screening was conducted to identify four best agonists. Furthermore, the compounds were ranked after calculating and predicting molecular dynamics, absorption, distribution, metabolism, and excretion (ADME) toxicity, density functional theory, and binding free energy calculations.

The most potent compound was selected for further *in vitro* studies, and the desired activity as dual FXR/TGR5 agonist could be confirmed (Figure 10.18).

Figure 10.18 shows the e-pharmacophore sites of FXR_{INT-767} (a) and TGR5_{INT-767} (b), along with the intersite distances. The docking results of the two different complexes were used to generate the energy-optimized pharmacophore hypothesis using the e-pharmacophore script available in the Schrödinger script center. The docking poses of the two protein–ligand complexes served as the input to generate six different pharmacophore sites. The pharmacophore hypothesis was selected based on the ranking and quantification process, and finally two constructed e-pharmacophore models (AAN and ADHN) were used as a query to search chemical databases for novel and desired chemical features. The compounds derived from ChemBridge database had to match at a minimum of three or four sites on each generated hypothesis; the final hits were ranked in order of Phase fitness score, volume terms, and root mean square deviation (RMSD) site matching. Figure 10.19 shows the scheme for the overall work flow applied for lead identification with e-pharmacophore-based virtual screening.

Finally, the following molecule was chosen for further *in vitro* testing (Figures 10.20–10.23).

Overall, the e-pharmacophore-based virtual screening supported the identification of potential novel potential drugs for the treatment of diabetes type II. The pharmacological activity could be confirmed by *in vitro* assays; further studies and optimization were considered.

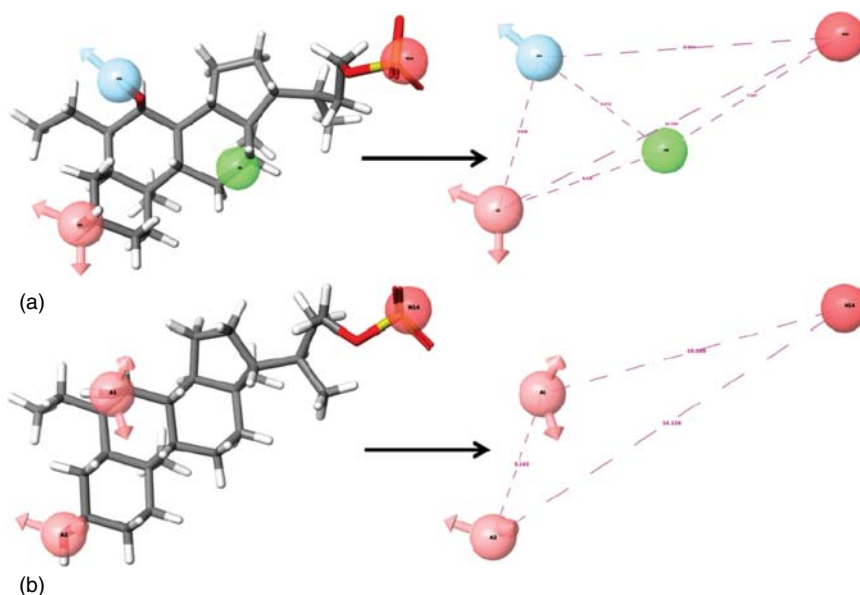


Figure 10.18 The e-pharmacophore sites of FXR_{INT-767} (a) and TGR5_{INT-767} (b) along with the intersite distances.

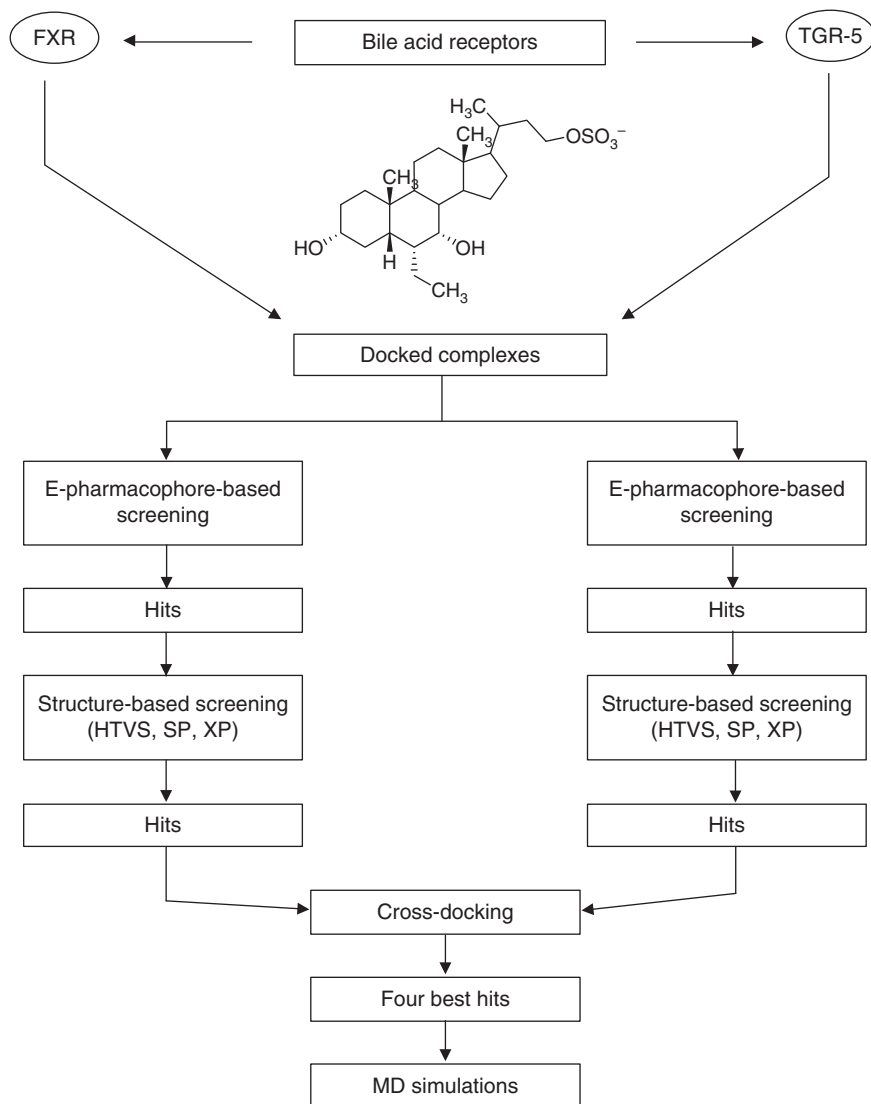


Figure 10.19 Schematic representation of the overall work flow applied for lead identification with e-pharmacophore-based virtual screening.

10.17 Arylboronic Acids as Dual-Acting FAAH and TRPV1 Ligands

Fatty acid amide hydrolase (FAAH) is a member of the amidase signature family of serine hydrolases that cleaves and regulates a broad range of endogenous signaling lipid amides, such as the prototypical endocannabinoid anandamide and anti-inflammatory substance palmitoylethanolamide, the sleep-inducing agent oleamide, and the satiety-regulating mediator oleoylethanolamide [76].

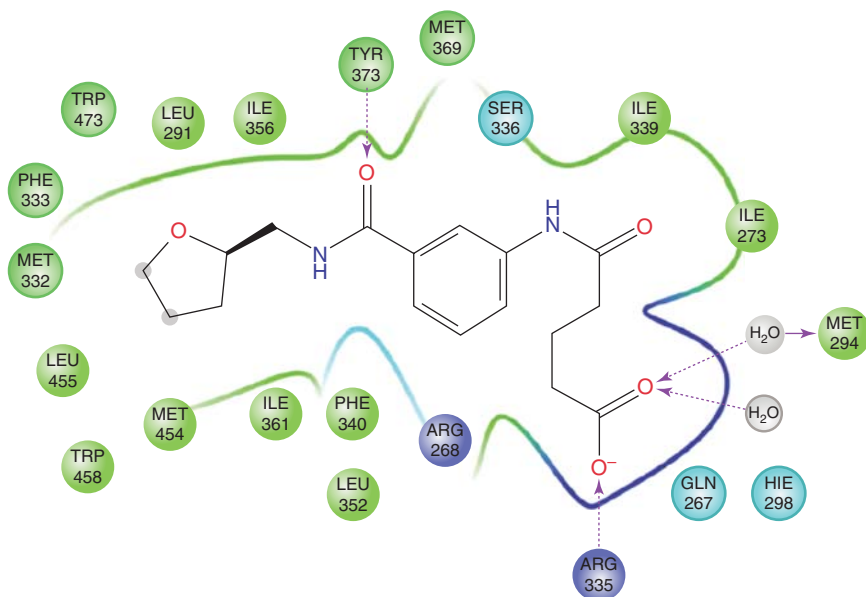


Figure 10.20 Schematic representation of protein–ligand interaction between FXR for the top hit.

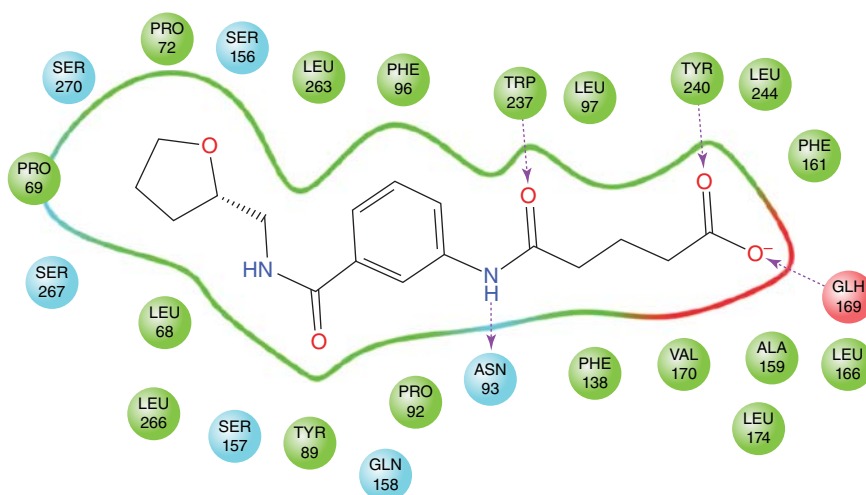


Figure 10.21 Schematic representation of protein–ligand interaction between TGR5 for the top hit.

Thus, blockade of FAAH represents a promising approach for the treatment of various diseases states such as pain, inflammatory, and neuropsychiatric disorders. Recently, a potent and selective FAAH inhibitor turned out to be ineffective in clinical II trials, eventually due to the fact that activation of other non-cannabinoid receptors involved in nociception such as transient receptor potential vanilloid 1 (TRPV1) channel might be activated. Thus, dual

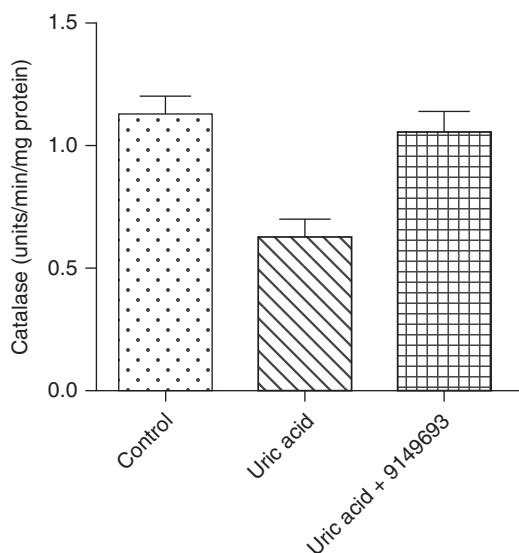


Figure 10.22 Effect of the selected dual agonist on the activities of catalase (CAT) in uric acid-induced MIN-6 pancreatic β cells.

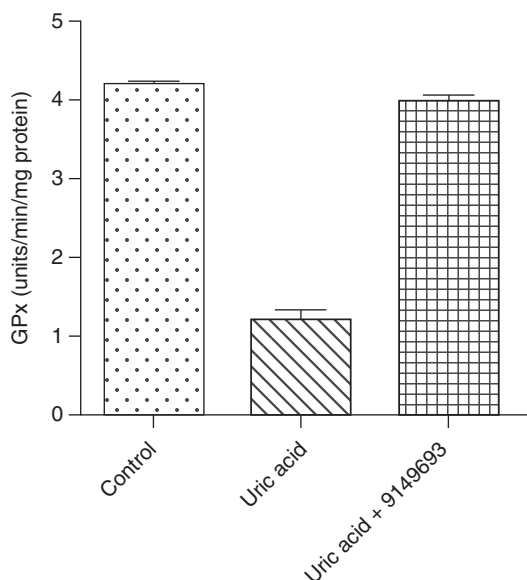


Figure 10.23 Effect of the selected dual agonist on the activities of glutathione peroxidase (GPx) in uric acid-induced MIN-6 pancreatic β cells.

inhibition of FAAH and TRPV1 may be advantageous in pain relief compared with compounds targeting only one protein. From known selective FAAH and TRPV1 inhibitors, a pharmacophore model was suggested, and a boronic acid as the FAAH inhibiting moiety was introduced as also shown in some model structures. The model shows a central hydrogen bond acceptor/donor motif

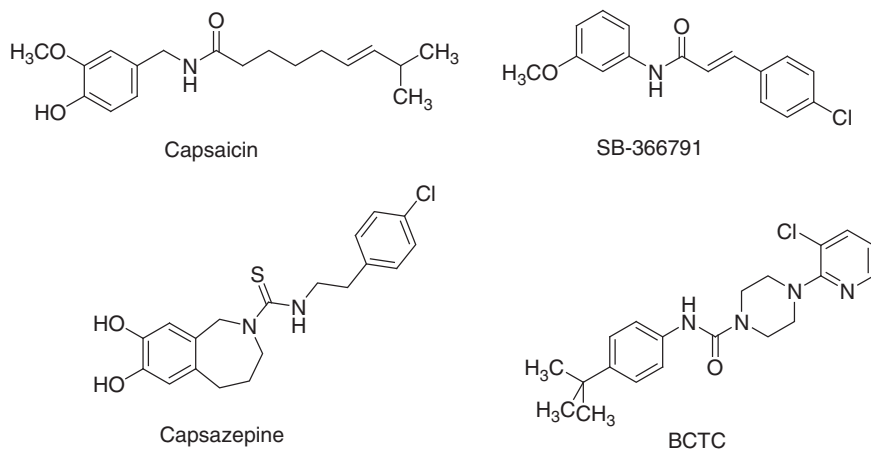


Figure 10.24 Chemical structures of FAAH blockers.



Figure 10.25 Chemical structures of TRPV1 blockers.

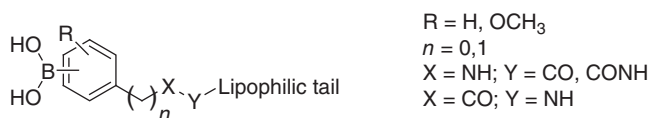


Figure 10.26 Pharmacophore model for arylboronic acid derivatives as dual FAAH/TRPV1 inhibitors.

flanked by a lipophilic tail on one side and an aromatic group with a hydrogen bond acceptor on the other side (Figures 10.24–10.26).

Based on the pharmacophore model, 31 arylboronic acids were prepared and tested on FAAH and TRPV1 channel. Four of them acted as dual FAAH/TRPV1 blockers with IC₅₀ values between 0.56 and 8.11 μM , whereas 10 others inhibited FAAH and activated/desensitized TRPV1. However, these results justify further studies and experiments with these compounds.

10.18 Dual Type II Inhibitors of TGF β -Activated Kinase 1 (TAK1) and Mitogen-Activated Protein Kinase 2 (MAP4K2)

The aim of the work was to develop efficient kinase inhibitors to enable the development and characterization of further kinase targets and drugs [77]. Kinases play a pivotal role in several mechanisms of physiology and pathology; therapeutically relevant ones have been extensively investigated, like breakpoint cluster region-Abelson murine leukemia viral oncogene homolog (BCR-ABL),

EML4-ALK, endothelial growth factor (EGFR), rapidly accelerated fibrosarcoma isoform B (B-RAF), and c-KIT. The adenosin triphosphate (ATP) binding site is a major target of known small molecule inhibitors thus blocking their catalytic function (type I inhibitors), whereas type II inhibitors bind to the ATP binding pocket in addition to an adjacent hydrophobic pocket, which is created when the activation loop containing the conserved Asp–Phe–Gly amino acid motif (DFG) is in an “out” conformation. Imatinib, nilotinib, and sorafenib are crystallographically proven type II inhibitors. Accordingly, a pharmacophore was developed for search of further type II blockers. The pharmacophoric features are a heterocyclic head with one to three hydrogen bonds for addressing the adenosine binding pocket, a linker moiety that traverses the region occupied by the gatekeeper residue, a hydrogen bond acceptor/donor motif, and a hydrophobic tail (Figures 10.27 and 10.28).

Based on this model a library of potential type II inhibitor was constructed and tested against a panel of over 420 kinases. Two compounds (**28** and **29**) could be identified showing dual affinity for TAK1 and MAP4K2 (germinal center kinase (GCK)) and blocked phosphorylation of proteins predicted to be

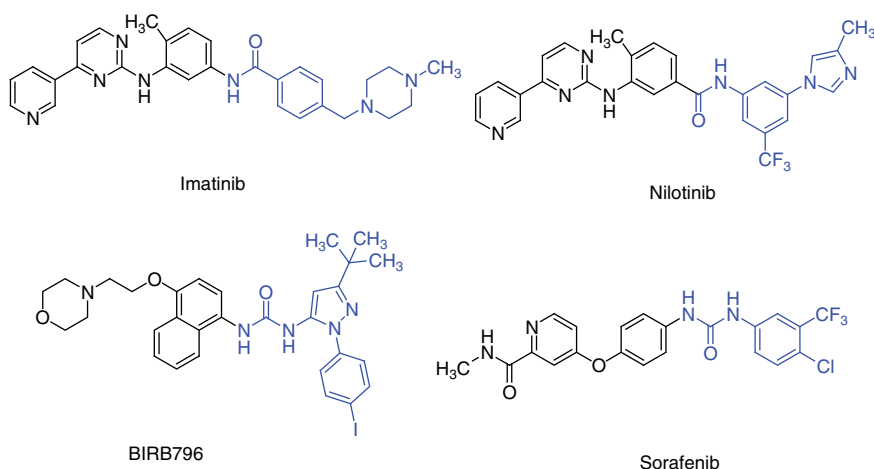


Figure 10.27 General pharmacophore model for the rational design of type II inhibitors: examples of known type II inhibitors, which can be divided in a “type I” head (black) attached to a “type II” tail (blue).

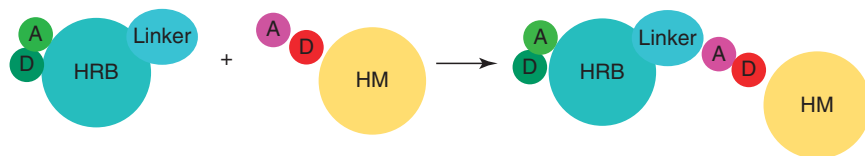


Figure 10.28 Schematic representation of the rational design of new type II kinase inhibitors: A, hydrogen bond acceptor; D, hydrogen bond donor; HRB, hinge-region binding; HM, hydrophobic motif.

downstream of these kinases, such as inhibitor of kappa B kinase (IKK), p38, and c-Jun N-terminal kinases (JNK) at concentrations of less than 100 nM. Some of the candidates exhibited good pharmacokinetic properties enabling further *in vivo* studies. A broader kinase selectivity profiling was conducted using KiNativ, and it could be shown that additional kinases such as ABLs, Abelson-related gene (ARG), p38, SRC, C-terminal Src kinase (CSK), FER, feline sarcoma oncogene (FES), and erythropoietin-producing human hepatocellular receptors (EPH) family kinases may be addressed by the subset of these inhibitor, a potential starting point for further kinase inhibitor development. On the other hand, relatively selective inhibitors against MAP4K2 (**32** and **33**) could be detected, where especially compound **33** exhibited excellent selectivity and potency against MAP4K2 in cellular assays. In contrast modification of the tail moiety may introduce TAK1 selectivity into the basic 4-substituted 1*H*-pyrrolo[2,3-*b*]pyridine molecular pattern. Further details on activity and selectivity could be found in literature [77] (Figure 10.29).

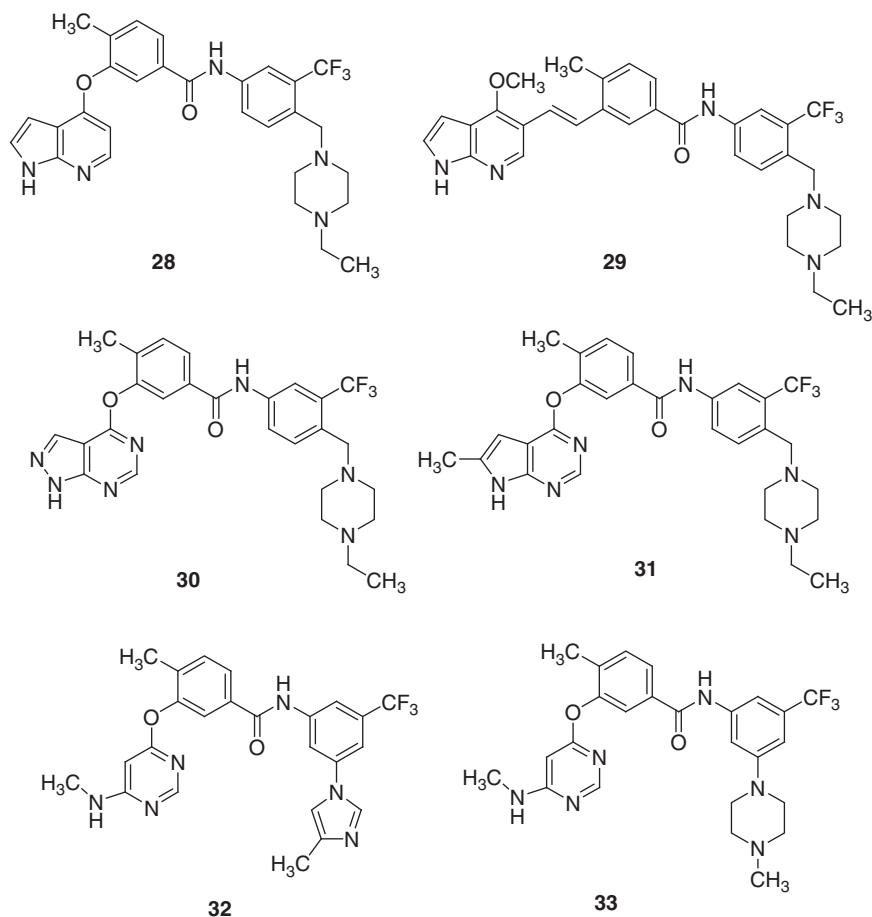


Figure 10.29 Chemical structures of lead compounds with TAK1/MAP4K2 dual activity) [77].

10.19 Conclusion and Outlook

Pharmacophore-based methods are essential in modern pharmaceutical research and can be used for a broad range of tasks in drug discovery like virtual screening, *de novo* ligand design, lead identification and optimization, SAR studies, and so on. To successfully implement a good pharmacophore model, an iterative process with several cycles of improvements, changes, and refinement according to the specific requirements is essential. Thus, multiple quality metrics like the enrichment factor, yield of actives, specificity, sensitivity, and the area under the curve of the ROC plot (ROC-AUC) may be applied. Together with complementary methods (e.g., docking and molecular dynamics (MD) calculation) and post-processing activities like implementation of filters (e.g., applying Lipinski's rule of 5, solubility cutoffs, etc.), an effective and versatile drug discovery method may be developed. As demonstrated in this chapter, the pharmacophore model approach can also be used for designing multiple (in particular dual) ligands following adapted pathways. Again, a thorough creation and repeated adaption of the model combined with complementing methods and filters may lead to successful identification of desired multitarget molecules with biological activity.

As discussed in this chapter, pharmacophore-based methods have their limits and weaknesses (which also apply for other virtual screening techniques), but are valuable and efficient tools for identifying multiple ligand scaffolds; however, it can be assumed that the methods will further be improved due to the dynamic development of computational power, innovative algorithms, and new scientific approaches. To date, they already play a vital role in the drug discovery cascade, and their importance is supposed to be increasing further in the future.

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11

Cellular Assays*Ye Fang*

*Corning Incorporated, Biochemical Technologies, Science and Technology Division, 1 Science Center Drive,
Corning, NY 14831, USA*

11.1 Introduction

Cell-based assays have been dominating in early drug discovery since 1990s. Molecular cloning and gene editing have made it possible to achieve high sensitivity and molecular specificity for drug screening using living cells [1]. Miniaturization, together with large-scale cell culture, has significantly reduced the cost of screening using living cells, whereas the integration with automation has enabled drug screening with unprecedented throughput [2]. Advances in fluorescence techniques, together with the development of fluorescent proteins covering a wide range of spectrum, have enabled high resolution visualization of protein interactions, protein trafficking, and phenotypes in living cells [3]. Advances in chemoproteomics [4] and chemogenomics [5] have rendered the possibility to investigate drug–target binding across the human proteome and drug pharmacology at the human genome level in cells, respectively. Advanced cell culture technologies such as three-dimensional (3D) organotypic culture, together with primary and stem cells, have dramatically expanded the list of disease-relevant cells and models with previously unmatched fidelity for drug profiling and screening [6].

Cell-based assays can be classified into two major types, molecular and phenotypic assays [7]. These assays differ greatly in detection principle, information content, and target coverage, although both can be developed for high throughput (Table 11.1). Cell-based molecular assays mostly measure the effects of compounds in living cells in the context of a predefined and target-based hypothesis [8, 9], whereas phenotypic assays examine the effects of drugs based on the specific phenotypes of cells [10]. Given that cellular assays are extremely diverse, this chapter only serves an introductory overview of current major types of cellular assays used in drug discovery. Each assay is discussed in the context of assay principle and primary applications.

Table 11.1 Common cell-based assays for drug discovery.

Assay	Detection	Information	Target coverage
Ligand binding	Fluorescence	Binding property	Probe limited
Chemoproteomics	MS	Binding	Human proteome
Second messenger	Fluorescence	Signaling	GPCRs
APC	Electrophysiology	Ion flux	Ion channels
PPI	Fluorescence/colorimetric	Protein interaction	Human proteome
Protein trafficking	Fluorescence	Protein translocation	Traffic protein
Chemogenomics	Microarray/NGS	Gene expression	Signaling pathways
Reporter gene	Fluorescence/colorimetric	Gene expression	Receptors
HCS	Fluorescence imaging	Phenotypes	Human proteome
Label-free	RWG	DMR	Receptors
Label-free	Electric biosensor	Impedance	Receptors

MS, mass spectrometry; GPCRs, G protein-coupled receptors; APC, automated patch clamping; PPI, protein–protein interaction; NGS, next gene sequencing; HCS, high content screening; RWG, resonant waveguide grating; DMR, dynamic mass redistribution.

11.2 Cell-Based Molecular Assays

Molecular assays using living cells can be further classified into cell-based ligand binding, chemoproteomics, signaling, protein–protein interaction (PPI), protein trafficking, and chemogenomic assays. Common to these assays is that they measure specific molecules in the receptor signaling cascades to infer the mechanism of action (MOA) of drug molecules. Originating from the bias toward specific MOAs, molecular assays have high throughput for screening and enable medicinal chemistry optimization. However, for novel MOAs the pharmacological profiles of drugs obtained using these molecular assays generally have limited power for predicting their therapeutic effects, thus often resulting in high attrition rate in clinical trials [10].

11.2.1 Ligand Binding Assays

The binding to specific target(s) is vital to the pharmacological mechanism of drug molecules. The equilibrium affinity of a drug binding to its primary target has been used as a surrogate for its *in vivo* efficacy. However, the increasing number of evidences suggests that the residence time of the binary drug–target complex is more closely associated with the therapeutic potential of drug molecules than binding affinity [11]. On the other hand, ligand binding affinity, potency, and kinetics are historically determined based on the competitive binding of drug molecules against labeled ligands to purified or enriched proteins or the direct binding to an immobilized protein using surface plasmon resonance [12]. However, drug binding dynamics is sensitive to the cellular texture associated with target proteins, as proteins typically function as components of extended molecular complexes [13, 14]. Therefore, it is critical to study ligand binding directly in living cells and to correlate binding interactions with physiological outcomes within a common physiological context.

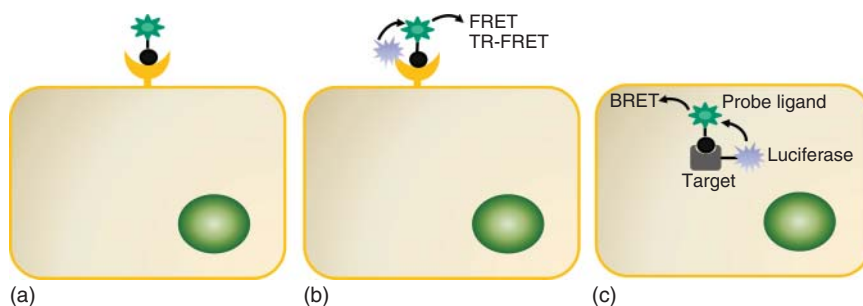


Figure 11.1 Three different cell-based ligand binding assays. (a) Fluorescence imaging after fluorescent-labeled ligands bind to a cell surface receptor. (b) Fluorescence resonance energy transfer (FRET) from a donor fluorescent protein tagged to the target receptor to an acceptor fluorescent ligand. Time-resolved FRET occurs when the donor is a lanthanide label. (c) Bioluminescence resonance energy transfer (BRET) from a donor bioluminescent protein (e.g., luciferase) tagged to the target intracellular protein to a fluorescent-labeled probe ligand acceptor.

In recent years, several cell-based ligand binding assays have been developed to examine target–drug interactions in intact cells (Figure 11.1). The first approach is to use confocal microscopy to directly measure the cell-associated fluorescence after fluorescent probe ligands bind to the receptors expressed in the cells [15]. The second approach is to use a plate reader or microscopy to measure the fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET), respectively, arising from the binding of a fluorescent or bioluminescent ligand to a receptor tagged with a fluorescent protein [15]. Time-resolved FRET (TR-FRET) using lanthanide-labeled ligands was developed to improve assay sensitivity by minimizing the autofluorescence background of living cells [16]. Of note, cell-based ligand binding assays depend on the availability of high affinity labeled ligands, which are often difficult to obtain.

Cell-based ligand binding is useful for studying the pharmacology of drug molecules targeting G protein-coupled receptors (GPCRs) [15], which are the most important class of druggable targets in the human genome and represent over 25% of all proven drug targets [17, 18]. Besides cell surface receptors such as GPCRs and transporters, other target classes including intracellular enzymes can also be assayed using these approaches. For instance, Robers *et al.* developed BRET approach based on the competitive binding of a cell-permeable fluorescent ligand to histone deacetylase (HDAC) fused to NanoLuc luciferase [19]. BRET analysis revealed remarkably long intracellular residence time for the clinically approved prodrug FK228 (Istodax/romidepsin) at HDAC1, explaining the protracted intracellular behavior of this prodrug.

11.2.2 Chemoproteomic-Based Assays

Most drugs, if not all, exert their effects via interacting with multiple targets, a phenomenon termed “polypharmacology” [20]. Polypharmacology may result in beneficial effects via a synergistic mechanism or detrimental effects via off-target(s) [21]. A drug molecule may bind to multiple targets within the same

family or among different families. For instance, a kinase inhibitor drug may also bind to a GPCR or an enzyme, besides kinases. Thus, it is critical to determine target engagement across the human proteome.

In recent years, several mass spectrometry (MS)-centric proteomic approaches have been developed as an unbiased means to determine target engagement in cells (Figure 11.2). First, the shotgun global approach is to use liquid chromatography and mass spectrometry (LC-MS) to scan all possible ions, and the changes in protein expression, posttranslational modifications, or protein thermal stability in the drug-treated cell are compared with the untreated cell. For thermal proteome profiling (TPP) assay, cell or cell extract samples are heated at different temperatures, allowing assessment of drug-induced changes in protein thermal stability across the proteome using quantitative proteomics [22]. This approach is useful for identifying drug targets and off-targets and determining target occupancy and downstream effectors of drug treatment. A recent TPP analysis showed that in human chronic myeloid leukemia K562 cell, the pan-specific kinase inhibitor staurosporine binds to two heme biosynthesis enzymes coproporphyrinogen-III oxidase and ferrochelatase, besides 51 kinases [23]. Ferrochelatase was found to be the common off-target for vemurafenib and alectinib, but not crizotinib. Ferrochelatase deficiency is genetically linked to protoporphyria, suggesting that the photosensitivity clinically associated with vemurafenib and other drugs is mediated by ferrochelatase. This approach can measure endogenous targets without drug modification; however, it is prone to false negative results arising from insufficient abundance, poor solubility, or the

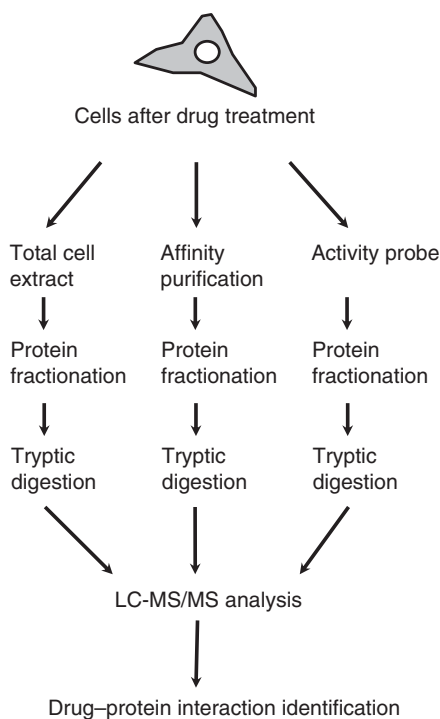


Figure 11.2 Three mass spectrometry-based proteomic techniques for drug–target interaction analysis across the human proteome. The shotgun global approach profiles the entire proteome (left). The affinity purification-based proteomics investigates the proteins that bind to a capture agent (e.g., kinobeads) (middle). The activity-based proteomics investigates the proteins that bind to a chemoproteomics probe, or are associated with a specific organelle, or involve in a specific signaling complex (right).

absence of a significant ligand thermal effect [23]. This approach was initially limited to soluble proteins, but recently was extended to detect membrane protein–small molecule interactions in cells [24, 25].

Second, affinity purification-based mass spectrometry (AP-MS) approach uses LC-MS to identify and quantify specific segments of a proteome based on affinity purification. This approach is ideal to determine target engagement in cells, to study PPIs and biological networks, and to correlate dynamic network rewiring with a specific phenotype [25]. Here, the target engagement is determined by using LC-MS/MS to analyze the interacting proteins captured by a ligand-conjugated solid substrate (e.g., resin or bead). By leveraging the well-known polypharmacology of kinase inhibitors, Bantscheff *et al.* recently developed an affinity resin (“kinobeads”) whose surface was conjugated with seven kinase inhibitors [26]. This kinobead was found to be capable of capturing 307 kinases as well as other ATP- and purine-binding proteins from 14 different human and mouse cell lines. AP-MS analysis showed that in K562 cells imatinib binds to two novel targets (discoidin domain receptor tyrosine kinase 1 and quinone oxidoreductase), besides 13 known protein targets such as ABL/BCR-ABL.

Third, the activity-based protein profiling (ABPP) approach is to use LC-MS to identify and quantify segments of a proteome based on activity fractionation, enrichment of specific organelles, or protein modifications. For the activity-based fractionation, a chemical probe is used to covalently capture one class of biological targets [27, 28]. For instance, Niphakis *et al.* developed a set of lipid-based chemical proteomic probes and used them to identify the off-target of two nonsteroidal anti-inflammatory drugs, rofecoxib and flurbiprofen [28]. These lipid probes were found to bind to over 1000 proteins with particularly high proportions of enzymes and transporters. Pathway analysis revealed the enrichment of proteins involved in protein transport, lipid metabolism, and host–virus interactions. Aldose reductase-related protein 2 was found to be an off-target for rofecoxib, but not ($\pm$)-flurbiprofen. Recently, we used the ABPP approach to compare the proteins presented in isolated mitochondria from cells after cultured in a purine-depleted medium followed by chemical cross-linking with the proteins present in cytosolic fractions [29]. The purine-depleted medium was used to stimulate the formation of purinosome, a dynamic cellular body formed by purine biosynthetic enzymes [14]. MS analysis showed that adenylosuccinate lyase (ASL or ADSL), a known purinosome protein that catalyzes the 8th step in de novo purine biosynthesis, was colocalized with mitochondria, demonstrating a physical link between purinosomes and mitochondria.

11.2.3 Signaling Assays

After activation by an extracellular signaling molecule, many receptors can transmit signal transduction, resulting in a biochemical chain of events inside the cell, many of which have been used as the basis to develop signaling molecular assays. This is best elicited by GPCRs, which transmit signals mainly through G proteins, leading to changes in intracellular levels of second messengers such as cyclic

adenosine monophosphate (cAMP), inositol triphosphate (IP₃), diacylglycerol (DAG), or calcium ions (Ca²⁺). Second messengers are responsible for relaying the signals received by the membrane receptors to the intracellular downstream factors and amplifying the signal strength. Assays that directly measure second messengers are the most popular functional assays for cell-based GPCR screens. For this reason, multiple assay formats have been developed to measure each second messenger. In general, screens for G_{αq}-coupled receptors mostly measure IP₃ turnover or Ca²⁺ mobilization, whereas screens for G_{αs}- and G_{αi}-coupled receptors mostly measure global changes in the concentration of intracellular cAMP [30, 31]. Notably, calcium flux assays such as fluorometric imaging plate reader (FLIPR) have been extended to receptors that are artificially coupled to a chimeric G_{qo5} or G_{qi5} or a promiscuous G_{15/16} protein.

Assays that measure two other second messengers, nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), have also been developed for high throughput screening (HTS) [32]. NO is a reactive nitrogen species produced by NO synthase and is a key signaling molecule involved in several physiological processes in the nervous, immune, and cardiovascular systems. cGMP is implicated in a variety of cellular pathways such as smooth muscle relaxation, cell growth, and cell differentiation.

Receptor signaling also leads to phosphorylation of intracellular protein kinases, which act as downstream effectors for the signal transduced by second messengers and small GTPases. Kinase assays can measure the translocation to/from the nucleus of a particular kinase or the phosphorylation state and activity of kinases. For instance, Bio-Plex Pro magnetic cell signaling assays are multiplexed immunoassays for the detection of intracellular phosphoproteins and total target proteins in cell and tissue lysates [33].

11.2.4 Automated Patch Clamping

Ion channels represent about 8–10% of all proven drug targets [17, 18]. The primary function of ion channels is to regulate the flow of ions across the plasma membrane and the membranes of intracellular organelles of both excitable and non-excitable cells. Whole-cell patch clamp electrophysiology is a gold-standard technique for ion channel drug profiling. Traditionally ion channels were assayed using manual patch clamping. Over the last decade automated planar patch clamp technology has been emerging as the leading platform for ion channel screening with high quality data and high throughput capabilities, at great efficiency and reliability [34, 35]. Common to automated patch clamping is to use a planar patch clamp electrode to position and capture a cell to the recording aperture. After a gigohm sealing is established, a compound is applied and the whole-cell current is recorded. This approach enables screening using overexpressed ion channels in cell lines or native and primary cells. Many platforms are available today including NPC-16 Patchliner and SyncroPatch 96 (Nanion Technologies GmbH, Munich), CytoPatch (Cytocentrics AG, Rostock), PatchXpress 7000A and IonWorks (Molecular Devices, LLC), Dynaflo HT (Celletricon AB, Mölndal), QPatch HT (Sophion A/S, Copenhagen), and IonFlux HT (Fluxion Bioscience Inc., USA).

11.2.5 Protein–Protein Interaction Assays

Proteins perform their biological functions primarily through PPIs. Aberrant PPIs are the bases of multiple diseases such as cancer. Characterizing PPIs is critical to understand protein function, to elucidate the biology of the cell, and to identify new therapeutic targets. Several approaches have been developed to study PPIs in living cells.

The first popular PPI assay is to use resonance energy transfer. Here, the first target protein is fused with a luminescent protein as the donor, while the second protein is fused with a second luminescent protein as the acceptor. Both fusion proteins are stably expressed in the cell at a comparable level. When a stimulation promotes the interaction between the two target proteins, the close proximity (1–10 nm) brought by the interaction results in a resonance energy transfer from the donor to excite the acceptor. FRET occurs when both donor and acceptor are fluorescent molecules, whereas BRET occurs when the donor is a bioluminescent molecule. The currently available fluorescent proteins are diverse and cover nearly the entire visible spectrum, providing numerous possibilities for multicolor labeling and studies of protein interactions [36]. The development of various luminescent proteins with novel characteristics and enhanced properties has come to an increasing number of assays for visualization of structural organization and dynamic processes of protein complexes in living cells. For instance, using the photoactivatable fluorescent protein mEos2-tagged formylglycinamidine ribonucleotide synthase, 3D stochastic optical reconstruction microscopy (3D STORM), a super-resolution fluorescence imaging method, was used to ascertain the colocalization of the purinosome with mitochondria [29].

The second PPI assay is based on protein fragment complementation [37, 38]. This approach uses two rationally designed and inactive fragments of a reporter protein (e.g., β -galactosidase, green fluorescent protein (GFP), or luciferase), each being recombinantly attached to the bait or prey proteins. The interaction between the bait and prey proteins drives the functional complementation of the two split reporter fragments, leading to the recovery of its native structure and activity. Since protein fragment complementation results in an amplified signal, exquisite detection sensitivity can be achieved, permitting analysis of protein interactions at concentrations approximating their normal levels of expression. This approach has been used to perform genome-wide screening of PPIs, to study biochemical networks, and to screen protein inhibitors. Combining FRET/BRET with protein complementation can further increase the power of PPI assays to investigate drug effects [39].

11.2.6 Protein Trafficking Assays

Proteins often translocate between membrane-bound organelles inside membrane vesicles upon stimulation. Intracellular membrane trafficking is essential to the exchange of material between organelles, communication between the cell and its external environment, and organelle biogenesis, structure, and function [40]. Protein traffic disorders can lead to a variety of human diseases including cystic fibrosis (CF) and early-onset severe obesity. Cell-based assays have been developed to identify drugs that can pharmacologically correct the traffic

defects. Direct visualization of protein trafficking using microscopy is the most common approach. For instance, Botelho *et al.* developed a traffic-based assay on the physiologically relevant CF bronchial epithelial (CFBE) cell line [41] that stably expresses a double-tagged reporter, mCherry-Flag-cystic fibrosis transmembrane conductance regulator (CFTR) traffic reporter, for either wild type or F508del-CFTR under an inducible promoter. This assay allows for ratiometric readout of traffic efficiency on a single cell basis with high throughput.

Protein translocation is common to receptor signaling, thus can be developed as the basis for drug screening. For GPCRs, receptor internalization and β -arrestin recruitment assays are two of the most common protein traffic assays [42, 43]. Receptor internalization is an essential component of receptor desensitization process, where cytosolic β -arrestins are recruited to the agonist-activated receptors, causing the receptors to be internalized and mediating their own signaling. These traffic assays require no prior knowledge of the interacting G protein(s), enabling screening against almost all GPCRs. Furthermore, both traffic assays are useful for elucidating biased agonism. Biased agonism or functional selectivity is common to GPCR ligands, which describes the ligand-directed selective activation of a portion of overall signaling pathways that can be activated by the receptor.

11.2.7 Chemogenomic-Based Assays

The ability to perform genome-wide expression analysis has transformed how we address biological problems and discover drugs. Chemogenomics describes the development of target- and/or function-specific chemical ligands and the use of these ligands to globally study gene and protein functions. Chemical genetic data hold strong promise for determining target engagement as it directly relates to drug action, and changes in gene expression upon drug treatment have yielded signatures that can connect unknown MOA to the ones previously established [44]. Based on this promise, Connectivity Map has been developed to discover the functional connections among diseases, genes, and drug action [45]. Central to this approach is to correlate patterns of small molecule sensitivity with differential basal gene expression profiles across cell lines [44] or a gene expression response with a reference set of existing perturbation signatures usually generated in cell lines with a relevant signaling pathway activated [45]. Two workflows are generally used to infer the MOA of drugs. In this first procedure, differentially expressed genes are identified upon drug treatments, and enrichment analysis is then performed to identify biological processes, signaling pathways, or other gene sets that are likely to be deregulated by the drugs. The gene sets could be either gene ontology terms or genes that are deregulated upon perturbation with known, very specific probe molecules. In the second procedure, prior knowledge of signaling networks or transcription regulation is used to assist the predication based on the gene expression data. These chemogenomic approaches allow for generating new biological hypotheses [46], discovering potential probes and drugs, predicting new indications for existing drugs and side effects for new drugs [47], and identifying drug-specific pathways [48]. However, given that many compounds

can bind to multiple targets within cells and only a few are mechanistically associated with the relevant phenotype, target engagement determination based on gene expression profiles alone can be challenging and complicated. Of note, since gene expression signatures associated with drug perturbations can be considered a phenotype, the chemogenomic approach can also be viewed as a phenotypic assay.

11.3 Cell Phenotypic Assays

Phenotypic assays directly quantify a disease-linked phenotype or pathway in physiologically relevant systems with limited assumptions as to the molecular basis of how the system works [10]. Phenotypic screen was historically used in drug discovery before target-based strategy became popular. The recent renaissance of the interest in phenotypic screen is fueled in part by the fact that phenotypic screen is more productive in identifying first-in-class drugs than target-based approaches [10, 49] and in part by the availability of more relevant cell types and cell modes and the advances in high content screening (HCS) techniques. Phenotypic assays hold promise to bridge the *in vitro* test results to *in vivo* efficacy gap. However, phenotypic screen encounters obstacles in fully understanding the biological endpoints, deconvoluting and validating targets, and performing lead optimization [10, 50].

Critical to the success of phenotypic screen is to use disease-relevant cells and models. Suitable cells for phenotypic screen include human primary cells, stem cells, and induced pluripotent stem (iPS) cell-derived phenotype models, besides the commonly used transformed cell lines. Cells with gain-of-function, loss-of-function, or point mutants that are obtained directly from patients or indirectly via genetic engineering can provide functional genetic evidence for target engagement and on-target effects of drugs [51]. 3D cell models including spheroids, organoids, and organs on chips may further increase the likelihood of accurate translation to human biology and diseases [52, 53]. The choice of cell models is dependent on many factors. However, a good cell model requires the pathways of interest to be intact within the system.

Common to phenotypic screens is a well-defined phenotype in one or more disease-relevant model systems. The biological importance of the phenotype examined, and how perturbations might change it, is critical to design a good assay and to interpret results [54]. A wide range of cellular phenotypes have been used as the basis to discover new medicines. The choice of cellular phenotypes is usually dependent on the MOA investigated or the clinical phenotype of interest. For instance, cell death has an important role in many human diseases including cancers and neurodegenerative diseases. Clinically promising cytotoxic and cytoprotective agents can be identified using apoptosis or proliferation as a good phenotype [55]. On the other hand, anti-angiogenesis inhibitors can be identified based on the assessment of the invasion and tube formation of human umbilical vascular endothelial cells or bovine aortic endothelial cells grown on an extracellular matrix or *in vitro* models of neovascularization utilizing a coculture of endothelial and stromal cell precursors in collagen–fibrin matrix [56, 57].

Phenotypic screens are also divergent in the type of readout used (e.g., reporter genes, high content imaging, or label-free biosensors), which is strongly dependent on the cell phenotypes examined. This section reviews three major types of phenotypic assays, reporter gene assays, and label-dependent and label-independent cell phenotypic assays.

11.3.1 Reporter Gene Assays

Receptor signaling often induces transcriptional changes via the binding of transcription factors to transcription response elements within the promoter region of the target genes. This has provided the basis for the development of a number of reporter gene assays, where a reporter gene is under the transcriptional regulation of the regulatory elements of a target gene in cells. These assays have been fruitfully used to discover drug-like molecules targeting receptors that lead to the cellular events associated with signal transduction and gene expression [58].

Reporter gene assays are widely used for GPCR screens. Transcription response elements responsive to second messengers upon GPCR activation include the cAMP response element (CRE), the nuclear factor of activated T-cells response element (NFAT-RE), the calcium-sensitive activator protein 1 (AP1), the serum response element (SRE), and the serum response factor response element (SRF-RE). Commonly used reporter genes include alkaline phosphatase, β -galactosidase, GFP, luciferase, and β -lactamase, all of which can lead to detectable colorimetric, fluorescent, or luminescent signals. Luciferase from *Photinus pyralis* is the most common bioluminescent protein used in reporter gene assays, which catalyzes the oxidation of luciferin in the presence of O_2 , Mg^{2+} , and ATP in a light-producing reaction. Reporter gene assays have advantages in high sensitivity, reliability, convenience, dynamic range, and adaptability to HTS. However, these assays suffer disadvantages in long incubation time, the gene expression signal quite distal from the receptor activation, and the use of highly artificial cell systems. The substrates used may also introduce interference. Recently, D-luciferin, the most widely used luciferase substrate, was found to display partial agonist activity at the GPR35 [59].

Reporter gene assays have also been used to examine previously intractable drug targets or cellular processes. For instance, Fu *et al.* [60] developed a reporter gene assay to screen small modulators to treat diabetes. One of the hallmarks of diabetes is the perturbation of endoplasmic reticulum (ER) function and chronic ER stress. Here, two high throughput functional screening systems were developed to independently measure the free chaperone content and protein-folding capacity of the ER. The first assay employed fusing a gene fragment that encodes a peptide derived from the ATF6 α luminal domain (ATF6LD) to a reporter gene from *Cypridina noctiluca* that encodes luciferase (Cluc), driven by the cytomegalovirus promoter, to measure the free chaperone content and reserve capacity of the ER. The second assay employed the membrane protein asialoglycoprotein receptor 1 (ASGR1)-Cluc fusion protein to monitor ER folding capacity. Azoramide was found to improve ER protein-folding ability and activate ER chaperone capacity to protect cells against ER stress in multiple systems. This compound also exhibited potent antidiabetic efficacy.

11.3.2 High Content Imaging Assays

Most cell phenotypic assays rely on labels for detection. HCS is the workhorse for label-dependent cell phenotypic assays. In principle, HCS employs automated microscope to measure simultaneously multiple fluorescent features of individual cells in multi-well plates, which, in turn, are used to infer the biological activity of compounds. Besides target-based applications, HCS is now primarily used for phenotypic screens, in particular, where single cell analysis is a prerequisite for phenotype(s) examined (e.g., the movement within a cell or between cells or the phenotypes associated with specific subpopulations of cells in a heterogeneous mix).

HCS is centered on automated microscopic imaging techniques and statistical tools for normalization, clustering, and visualization of multidimensional data obtained. In the past decades, there are many commercial vendors of instruments in the HCS arena. These automated microscopes not only are capable of using fluorescent and transmitted light to image cells but also provide sophisticated image and data analysis capabilities. The instruments used in HCS include wide-field imagers, confocal imagers, and laser scanning cytometers [reviewed in details in [61].

HCS is powerful in that it can yield biologically relevant, statistically robust data with high throughput. Furthermore, HCS allows for multiplexing in examining cell phenotypes including novel phenotypes within a single screen campaign [62, 63]. Examining multiple phenotypes permits the fine classification of drugs based on MOAs and toxicity. In principle, any phenomenon that can be seen reproducibly in a microscope can ultimately be assayed with HCS. Phenotypes that can be addressed by HCS have been dramatically increased over the past decades, ranging from translocation to trafficking, neurite outgrowth, tube formation, mitosis, infection, differentiation, wound healing, migration, chemotaxis, proliferation, death, epithelial–mesenchymal transition, cell size, and many others [60, 64].

HCS is well suited to examine biologically more relevant systems such as coculture, primary cells, and stem cells, which, in turn, leads to increased power for predicting *in vivo* effects and ultimately reduces attrition rate of drugs in clinical trials. In particular, advanced cell models such as 3D organotypic niches can restore many unique aspects of *in vivo* cellular physiology [52]. For instance, selective targeting of cancer stem cells (CSCs) may represent a novel druggable MOA for developing anticancer therapeutics. Hartwell *et al.* screened 14,718 compounds in a leukemia-stroma coculture niche system for inhibition of cobblestone formation [65]. The cobblestone phenotype is characterized by the burrowing of primitive cells beneath a layer of stromal fibroblasts, forming phase dark areas arising from the organization in tight association of cobblestone area-forming cells. Lovastatin, a clinically used cholesterol-lowering drug, was found to effectively inhibit the growth of leukemia stem cells *in vitro* and *in vivo* via inhibition of HMG-CoA reductase, but not hematopoietic stem and progenitor cells.

HCS has been used in almost all stages in early drug discovery process, ranging from target prediction and validation, hit identification and confirmation, lead

optimization and prioritization, toxicity assessment, and clinical data analysis. HCS-based target engagement determination can be achieved by combining with functional genetic approaches (e.g., gain or loss of function) [51] or by cell phenotype clustering, which compares the similarity in profiles of compounds with those of probe molecules with known MOAs [62, 63].

However, HCS has weakness associated with the generation of large sets of image data, which pose challenges in data storage, transfer, annotation, and process. In addition, the consistency and reproducibility of cellular models used may also complicate HCS results, since cells are well known to be susceptible to mechanical forces [66], thermal fluctuation [67], or other conditions (e.g., passages or donor ages for primary cells) [68].

11.3.3 Label-Free Cell Phenotypic Assays

The past decade has witnessed increasing applications of label-free biosensors for cell phenotypic analysis of drug action. Two types of biosensors have been used (Figure 11.3). First, resonant waveguide grating (RWG) uses its characteristic surface-bound evanescent wave under resonance condition as the transduction mechanism for cell phenotypic assays [69], whereas electric biosensor employs the impedance of a cell-electrode system as the transduction mechanism for whole-cell sensing [70]. RWG measures the tiny changes in local refractive index arising from the dynamic mass redistribution (DMR) of cells upon stimulation [71], while electric biosensor monitors the changes in cellular impedance arising from the ionic redistribution surrounding the cells upon stimulation [72]. The DMR is recorded as the shifts in resonance wavelength in picometer (pm), whereas the impedance is recorded as the change in cell index,

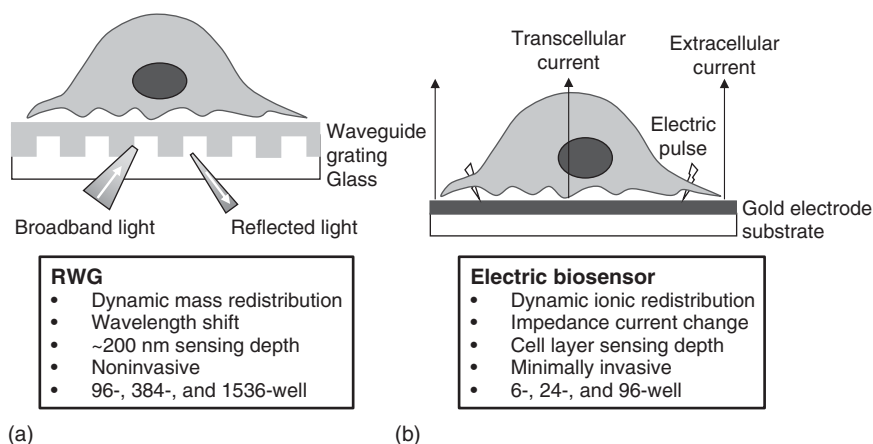


Figure 11.3 Two popular label-free biosensor techniques used for cell phenotypic screening. (a) Resonant waveguide grating (RWG), wherein cells are cultured onto its waveguide surface and only the mass redistribution within the bottom portion of cells is monitored in real-time as the shift in resonance wavelength of the reflected light. (b) Electric biosensor, wherein the cells are cultured on the surface of the biosensor having arrayed gold microelectrodes and both flows of extracellular and transcellular currents are measured in real-time when a low alternating current voltage at variable frequencies is applied to the cell layer.

a unitless parameter obtained after normalization. Common to both is that they record a real-time kinetic phenotypic response of living cells upon drug treatment [73].

Label-free cell-based assays have several unique features. First, RWG is totally noninvasive, whereas electric biosensor is minimally invasive, so it is amenable to study cellular responses in almost all cell types including immortalized, stem, and primary cells, regardless of being anchorage dependent or not [74]. Second, both biosensors measure a kinetic and holistic cellular response, which contains high information content about receptor signaling pathways [29, 75, 76]. Third, both biosensors have a wide coverage in targets, pathways, and cell phenotypes, enabling multi-target screening in the context of various cell phenotypes [77, 78]. Fourth, both biosensors offer an unprecedented flexibility in assay formats to investigate the biochemical, molecular, and cellular MOA of drug molecules [79]. Fifth, RWG and electric biosensor support screening with a throughput of up to 1536-well and 384-well microplates, respectively. Of note, RWG also supports screening based on binding affinity [80], besides cell-based assays.

Label-free cell phenotypic assays have found applications in various early drug discovery processes, including hit identification, lead optimization, target engagement determination, and drug safety assessment [77–79, 81, 82]. Label-free cell phenotypic screens can not only discover different classes of ligands for the same receptor of interest but also identify ligands for multiple targets, all with low false positives and negatives and within a single campaign. Gitschier *et al.* developed a triple-addition screen using RWG to identify novel agonists, allosteric potentiators, and antagonists for muscarinic M_1 receptor [83]. Zhang *et al.* used RWG to screen fractions from two traditional Chinese medicinal (TCM) plants using three cell lines, A431, A549, and HT29, and found a high amount of niacin presented in both plants, besides multiple fractions containing compounds active at the GPR35 [84].

Label-free cell phenotypic assays allows for quantitative structure–activity relationship (SAR) analysis, supporting medicinal chemistry optimization regarding the efficacy, potency, MOA, and off-target effects, all within a single campaign. Schmidt *et al.* screened a targeted library of small carboxylic acids using DMR assays and identified ligands selective to free fatty acid receptor 2 (FFA2; GPR43) [85]. We had combined DMR profiling with ligand similarity search to quickly expand hit library and perform effective SAR, leading to the identification of lead-like compounds for GPR35 [86, 87].

Label-free cell phenotypic assays have been used for lead selection, since they enable analysis of drug pharmacology with fine details in terms of biased agonism, on-target pharmacology, drug residence time, off-target effect, and drug toxicity [77–79]. Effective lead prioritization and selection for preclinical testing is essential to reduce the cost for drug discovery. Current approaches for lead selection are mostly idiosyncratic and unpredictable. Brust *et al.* combined DMR assay with other conventional molecular assays to examine several dopamine 2 receptor ligands [88]. The third generation antipsychotic drug aripiprazole was found to display a unique functional profile at this receptor – a partial agonist for $G_{\alpha i/o}$, a robust antagonist for $G_{\beta\gamma}$ signaling, and a weak partial agonist for both heterologous sensitization and DMR.

Label-free cell phenotypic assays, in combination with functional genetic and chemical biology techniques, can be used to determine target engagement and discover novel biology, in particular signaling pathways. A recent DMR agonist profiling of a library of 72 ion channel ligands showed that pinacidil, a known K_{ATP} channel opener, triggered a robust negative DMR in A431, A549, HT29, and HepG2C3A, but not HepG2 cells [89]. RNAi knockdown of known K_{ATP} channel component and pathway proteins showed that the pinacidil-activated channel is a SUR2-containing K_{ATP} channel and mediates signaling through Rho kinase, Janus kinase-2 and Janus kinase-3 in HepG2C3A cells. Furthermore, DMR profiling studies also suggested a novel slow intracellular signaling, possibly from endosomes, for β_2 -adrenergic receptor in A431 cells [90], and a novel pathway that certain GPCRs (e.g., α_{2A} -adrenergic receptor) regulate the purinosome assembly and disassembly processes [76], possibly via a mechanistic target of rapamycin (mTOR)-mediated mechanism targeting purinosomes to mitochondria in HeLa cells [29].

11.4 Summary

Cellular assays have been and will continue dominating in early drug discovery. A wide range of cellular assays have been implemented in various stages of drug discovery process. In general, cell-based molecular assays are commonly used for identifying follower drugs as they have high molecular specificity, whereas cell-based phenotypic assays are useful for discovering first-in-class drugs as they have higher predictive power for *in vivo* effects. Cellular assays are extremely diverse and continue evolving, as technologies continue being advanced and advanced cell models become available for drug testing.

11.5 Current and Future Perspectives

Drug discovery is a long, costly process. Cellular assays have been the workhorse for drug discovery, and a diverse range of cellular assays have been used in various stages of drug discovery process. However, different assays have their own characteristics and may find primary applications at specific stages. Phenotypic assays including reporter gene, HCS, and label-free cell phenotypic assays are useful for phenotypic screens. Chemoproteomic- and chemogenomic-based approaches are powerful for target deconvolution after phenotypic screens. Label-free cell phenotypic assays, in combination with functional genetic and chemical biology approaches, can also be used for target deconvolution. Cell-based molecular assays including signaling, PPI, and protein trafficking assays are useful for target-based screens. Automated patch clamping is uniquely positioned for screening drugs targeting ion channels. Cell-based ligand binding assays are useful to relate to the residence time at the receptors to the efficacy of drug molecules. The choice of assays is dependent on the nature and purpose of drug discovery campaigns. Molecular assays have been fruitful for

discovering follow-on drugs, where the molecular MOA is known and well characterized. Phenotypic assays are increasingly used in discovering drugs targeting unmet therapeutic needs and unexploited biological mechanisms, where pharmaceutical innovation has become increasingly challenging.

Drug pharmacology is far more complicated than initially thought. The innate complexity in pharmacology of a drug molecule is originated from its possibility to bind to multiple clinically relevant targets (a phenomenon referred to polypharmacology), to trigger pathway-selective activity (biased agonism or on-target pharmacology), to mediate cellular context-dependent responses (phenotypic pharmacology), to modulate multiple targets within a signaling network (network pharmacology), and to reside at its primary target differently from other targets (biochemical MOA). Drug pharmacology is further complicated by the well-known metabolism. Thus, it is critical to combine multiple assay technologies in order to fully comprehend drug–target interactions and their functional consequences. The increasingly realized importance of drug residence time for *in vivo* effects requires the development of innovative cell-based assays to examine the kinetics of drugs binding to their targets in native cellular environment. The commonly occurring polypharmacology of drug molecules demands further improvement of existing chemoproteomic approaches to truly cover the entire human proteome. The biased agonism of drug molecules acting at a target receptor requires the integration of multiple assays with increasing differentiation power to elucidate the exact molecular MOAs of lead-like molecules for better lead prioritization and selection. The presence of divergent phenotypic pharmacology calls for systematic representation of drug action across the different types of cells. Furthermore, to further close the gap between *in vitro* results and *in vivo* effects, it is necessary to use better cell models for drug testing. Fluorescent techniques with better resolution would be greatly beneficial to ascertain data quality and drug action. Combining multiple empirical approaches with computational approaches can also improve the productivity of drug discovery.

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Part IV

Therapeutic Areas for Designed Multiple Ligands

12

Developing Serotonergic Antidepressants Acting on More Than the Serotonin Transporter

Gerard J. Marek

Astellas Pharma Global Development, Inc., Development Medical Science CNS and Pain, 1 Astellas Way, Northbrook, IL 60062, USA

12.1 5-HT Transporter-Based Multiple Ligands for Depression

The first selective serotonin reuptake inhibitors (SSRIs) or serotonin transporter (SERT) inhibitors approved for the treatment of major depressive disorder (MDD) were zimelidine outside of the United States and fluoxetine in the United States in 1982 and 1987, respectively [1, 2]. While zimelidine was withdrawn throughout the world due to rare but potentially lethal effects of a Guillain–Barré-like syndrome, a range of SSRIs including citalopram, escitalopram, fluvoxamine, paroxetine, and sertraline (Figure 12.1) was developed and approved by either the US Food and Drug Administration (FDA) or other regulatory agencies throughout the world. Excluding zimelidine, all of these SSRIs became blockbusters because of significant safety and tolerability compared with tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). However, there is some evidence from head-to-head trials with TCAs by the Danish University Antidepressant Group (DUAG) that SSRIs may be inferior to TCAs with respect to the treatment of MDD [3, 4]. Furthermore, although there are meta-analyses suggesting that SSRIs are equivalent in efficacy compared with TCAs, this viewpoint is compromised by suboptimal dose selection for most industry-sponsored trials directly comparing SSRIs to TCAs [5–7]. However, the Anderson meta-analysis in 2000 did suggest that (i) TCAs are superior to SSRIs for the inpatient population and (ii) amitriptyline (Figure 12.2) is superior to SSRIs, and a trend for higher doses of TCAs (>149 mg) is more efficacious than SSRIs. This latter point is highlighted by the known dose (exposure)-dependent efficacy of TCAs and the likelihood that most industry-sponsored head-to-head SSRI/TCA clinical trials utilized suboptimal TCA dose selection [8–11]. This hypothesis that SSRIs are probably inferior in efficacy to TCAs raises the question as to what pharmacological activities might be added to SSRIs that would result in superior antidepressant activity somewhere between that seen with TCAs and that observed with the treatment by gold standard, electroconvulsive therapy (ECT).

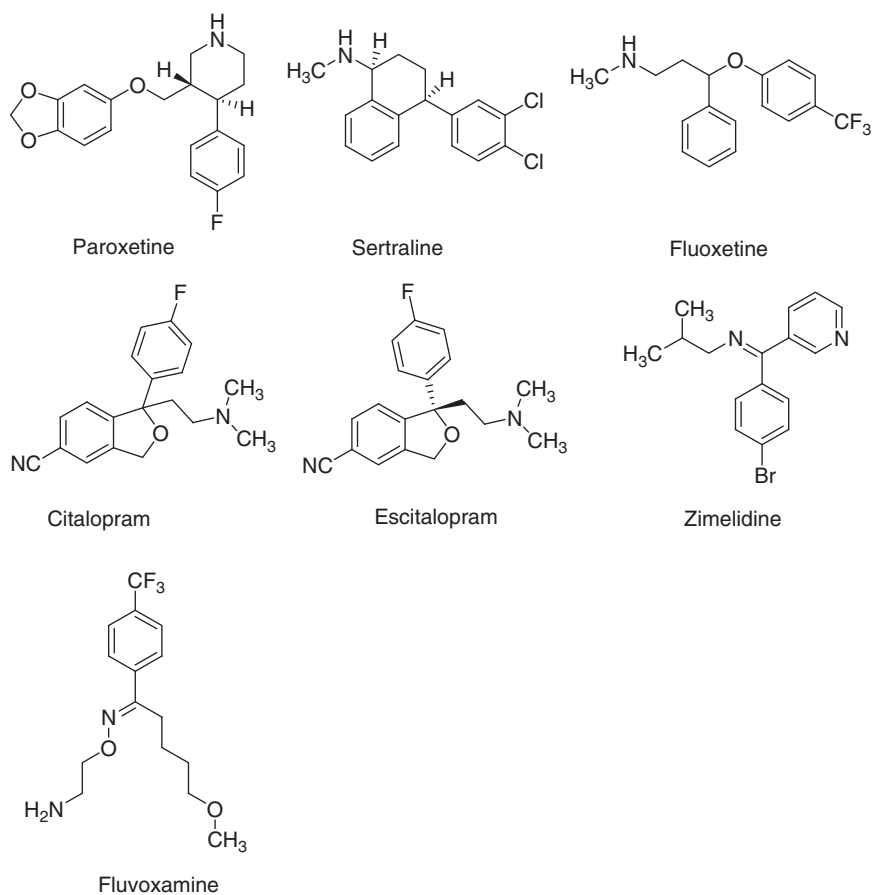


Figure 12.1 Chemical structure of SSRIs. The chemical structure of SSRIs approved for the treatment of MDD is shown: citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, and zimelidine.

12.2 Beyond SSRIs: Strategies to Improve upon SSRI Antidepressant Activity

The overall efficacy of SSRIs has been questioned based on the number of pivotal trials needed to demonstrate antidepressant efficacy for regulatory approval and randomized comparative trials such as the DUAG comparisons of clomipramine with SSRIs (Figure 12.2). This raises three different questions when focusing attention primarily on the serotonergic system. First, are there additional pharmacological actions of TCAs with respect to serotonin that might explain potentially greater pharmacological activity of TCAs than SSRIs? Second, are there pharmacological actions of SSRIs that might actually attenuate antidepressant activity of SSRIs through non-serotonin transporter (non-SERT) targets? Specifically, what receptors indirectly activated by SERT inhibition might oppose the activity of other receptors mediating antidepressant activity?

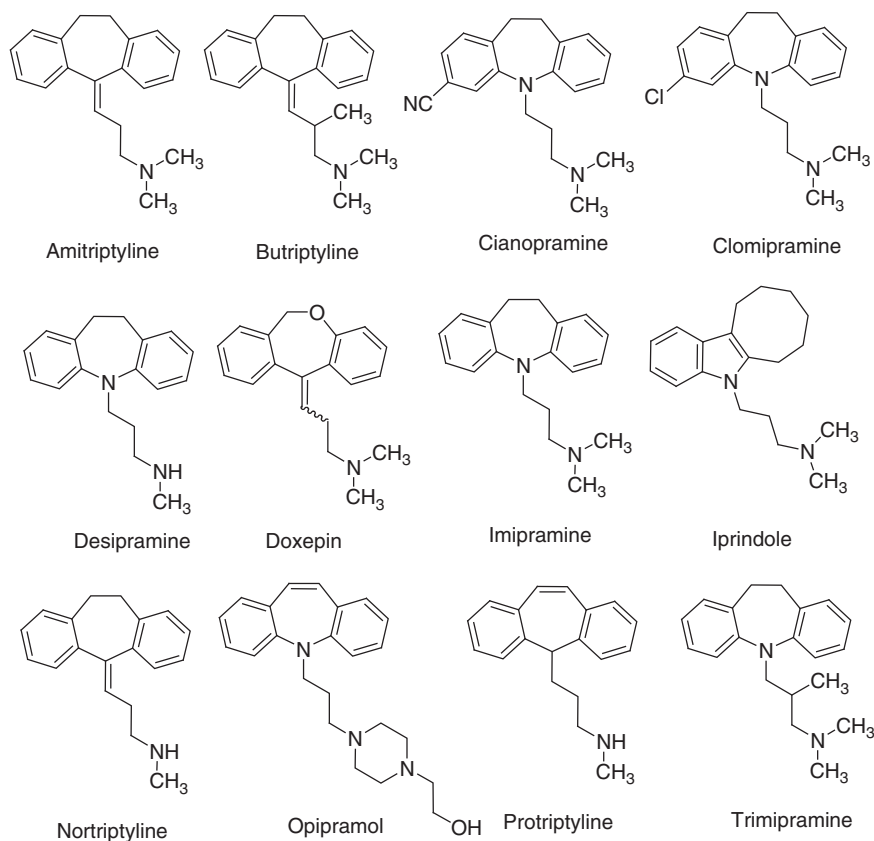


Figure 12.2 Chemical structure of TCAs. The chemical structure of TCAs approved for the treatment of MDD is shown including especially amitriptyline, clomipramine, desipramine, imipramine, nortriptyline, and protriptyline.

Third and related to the first point, are there other serotonergic pharmacological targets that might result in greater antidepressant activity than observed with SSRIs when combined with SERT inhibition or non-SERT serotonergic targets? The purpose of this chapter is to describe the evidence that multiple non-SERT serotonergic targets can be combined with SERT inhibition in discovering novel antidepressant drugs with differentiated clinical activity compared with SSRIs alone.

12.3 Roster of Serotonergic Targets for Drug Developed Outside of the Serotonin Transporter (SERT)

At least 14 receptors make up the serotonin or 5-hydroxytryptamine (5-HT) class of receptors [12]. The 5-HT₁ receptor subclass includes the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors. The 5-HT_{1A} receptor is one of the most studied receptor targets for novel antidepressant drugs either for

monotherapy, combination therapy in a single pill, or combination treatment with SSRIs. Widespread agreement exists that activation of 5-HT_{1A} receptors mediates, at least in part, antidepressant activity of SSRIs [13–15]. The 5-HT₂ receptor subclass includes the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Excluding SSRIs, downregulation/desensitization of 5-HT_{2A} receptors is one of the most common downstream effects of most approved antidepressant drugs, even those that block 5-HT_{2A} receptors [16–18]. 5-HT_{2A} receptor blockade is a shared common pharmacological effect for many approved antidepressant drugs (Figure 12.3) including TCAs, mirtazapine, mianserin, trazodone, nefazodone, atypical antipsychotic drugs, and many first-generation antipsychotic drugs excluding haloperidol [19, 20]. 5-HT₃ receptors are the only ion channel in the 5-HT receptor family. Two closely structural tetracyclic analogs mirtazapine and mianserin as well as the new “SSRI plus” vortioxetine block this receptor. There is not any clear evidence that any of these drugs including vortioxetine possess greater antidepressant efficacy than SSRIs alone. The 5-HT₄ is another receptor for which there has been some interest in developing novel antidepressants in part based on the effects of 5-HT₄ receptor agonists on *in vivo* preclinical screens [21]. However, it is not apparent how activating 5-HT₄ receptors could enhance the effects of SSRIs, assuming that ambient synaptic 5-HT levels following SERT inhibition activate this receptor. The 5-HT_{5A/5B} receptors are relatively poorly characterized compared with many of these other receptors. Both the 5-HT₆ and 5-HT₇ receptors are of interest, especially since atypical antipsychotic drugs with antidepressant activity like clozapine, olanzapine, or risperidone have considerable potency for blocking these receptors [22–24].

12.4 Previously Approved Antidepressants with Multiple Serotonergic Molecular Targets

There are a number of previously approved antidepressant drugs that possess more than one molecular serotonergic target. The TCA clomipramine (Figure 12.4) highlights one spectrum of TCAs with approximately 100-fold greater selectivity for blocking SERT than the norepinephrine transporter (NET), while desipramine (desmethylinipramine, DMI) exemplifies TCAs with approximately 100-fold greater selectivity for blocking NET than SERT [27–29]. With respect to the serotonergic system, clomipramine is as potent at 5-HT_{2A} and 5-HT_{2C} receptors as it is as NET [27, 30]. Since 5-HT_{2C} receptor antagonists generally do not appear to be strong antidepressants in most preclinical models, while 5-HT_{2A} receptor antagonists are efficacious in certain models, 5-HT_{2A} receptor blockade is a viable pharmacological hypothesis to explain the appearance of greater therapeutic efficacy for clomipramine compared with SSRIs. Most of the TCAs are also potent 5-HT_{2A} receptor antagonists though a smaller number possess such potent activity at SERT as does clomipramine [27, 28, 31]. NET inhibition might be responsible for the additional therapeutic activity, or both NET inhibition, and 5-HT_{2A} receptor blockade might contribute to the therapeutic advantage with respect to comparisons of TCAs with SSRIs. In terms of other targets, clomipramine shows no appreciable affinity for 5-HT_{1A}, 5-HT_{1B},

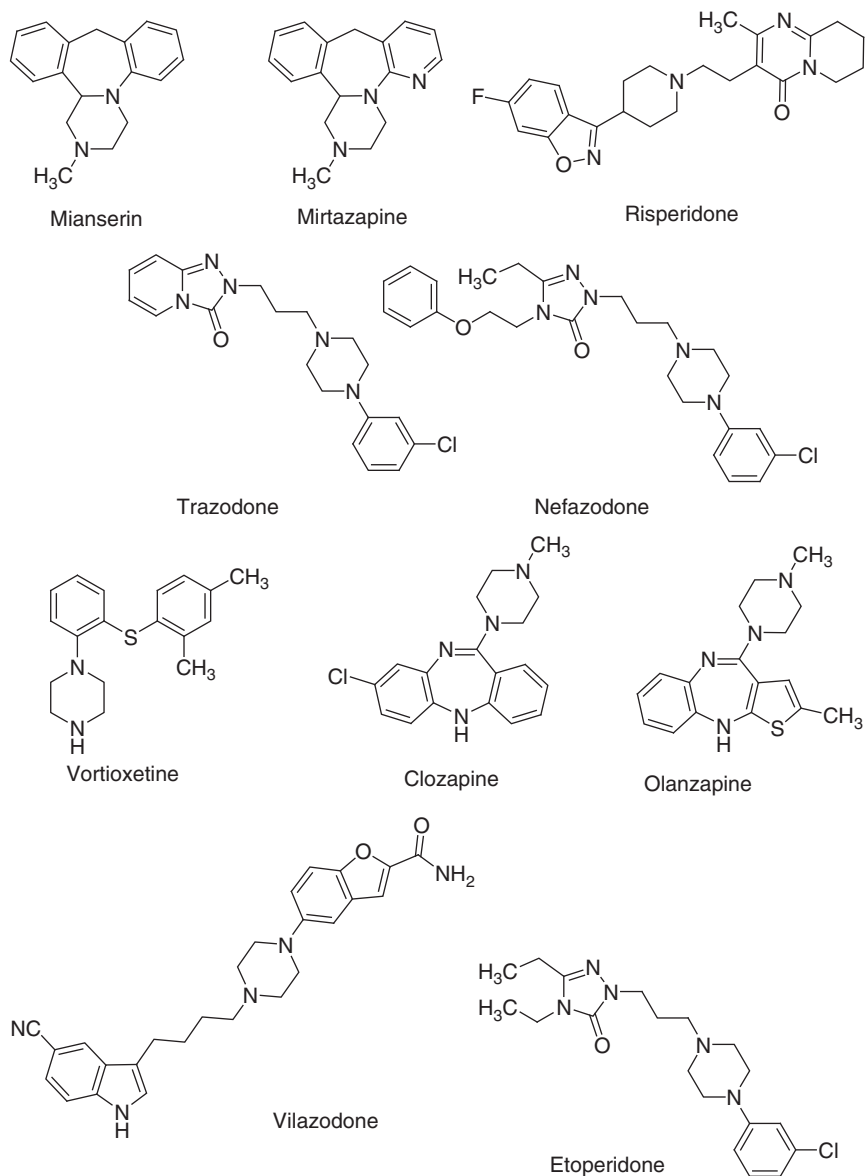


Figure 12.3 Chemical structure of antidepressant and/or antipsychotic drugs acting on multiple serotonergic molecular targets. Included are tetracyclic antidepressants (mianserin and mirtazapine), serotonin antagonist–reuptake inhibitors (SARIs: trazodone, nefazodone, etoperidone), the multimodal serotonergic antidepressant vortioxetine, the SERT inhibitor/5-HT1A receptor agonist vilazodone, and atypical antipsychotic drugs (risperidone, olanzapine, and clozapine).

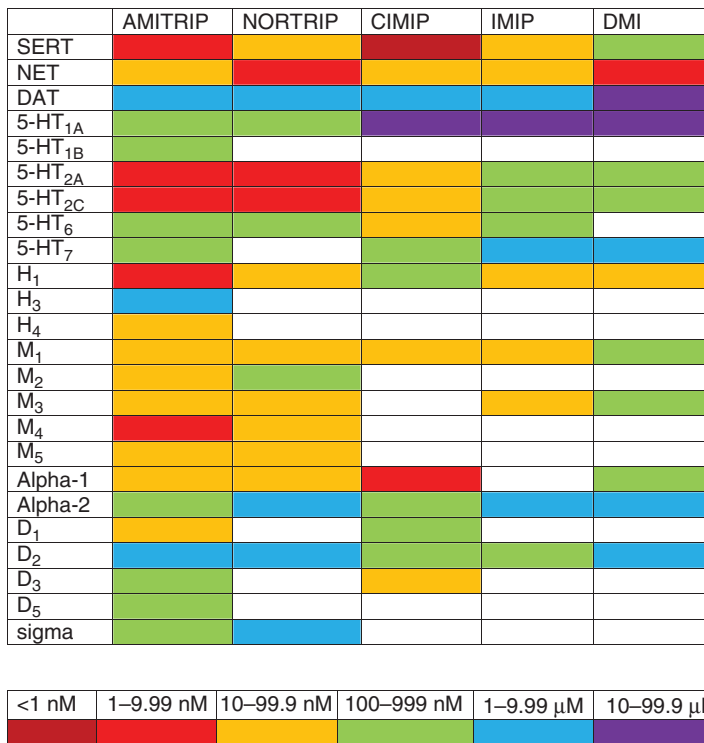


Figure 12.4 Heat map for affinity of representative tricyclic antidepressant drugs at monoamine transporters and receptors. The data plotted for this figure comes from the PDSP K_i database and an additional reference [25, 26]). The tertiary amine TCA amitriptyline (AMITRIP) is metabolized into the secondary amine TCA nortriptyline (NORTRIP). Clomipramine (CIMIP) is the TCA that inspired Arvid Carlsson to champion the development of the first approved SSRI zimelidine. Imipramine (IMIP) is also a tertiary amine TCA that is metabolized into the secondary desmethylimipramine or desipramine (DMI). The color code represents different ranges of potency from <1 nm (dark red) to >10 μ M (purple). Each row shows the affinity of these different tricyclic antidepressants for a range of transporters (SERT, NET, and DAT) as well as serotonergic (5-HT), histaminergic (H), muscarinic cholinergic (M), α -adrenergic (α_1 and α_2), and dopaminergic (D) receptor subtypes as well as the sigma binding site.

or 5-HT_{1D} receptors and little appreciable activity at 5-HT₃ receptors [30]. In addition to the direct pharmacological effect on 5-HT_{2A} receptors, clomipramine like most other TCAs downregulates 5-HT_{2A} receptor binding and downstream functional effect of 5-HT_{2A} receptor activation [32, 33]. This downregulation and blockade of 5-HT_{2A} receptors and biasing serotonergic transmission toward the “inhibitory” actions of 5-HT might be a general feature of TCAs and other antidepressant drugs with potent 5-HT_{2A} receptor antagonist activity. Thus, clomipramine’s apparent increased therapeutic activity compared to SSRIs’ might be related to direct blockade of SERT and blockade/downregulation of 5-HT_{2A} receptors, though other additional pharmacological effects (especially NET inhibition) also could contribute to clomipramine’s favorable efficacy profile.

The multiple serotonergic mechanisms by which the antidepressant effects of a number of TCAs are exerted are similarly highlighted by amitriptyline and nortriptyline (Figures 12.2 and 12.4). Amitriptyline is a tertiary amine that is metabolized by the cytochrome P450 isoenzyme CYP2C19 into another approved secondary amine TCA nortriptyline. The affinity of both drugs for both the 5-HT_{2A} and 5-HT_{2C} receptors appears to be in the range of 4–9 nM [34]. Amitriptyline and nortriptyline also potently inhibit SERT (K_i 's of 3.1 and 16.5 nM) and NET (K_i 's of 22.4 and 4.37 nM). The only receptor site where either of these drugs is as potent an inhibitor as the 5-HT_{2A} receptor is the histamine H₁ receptor for amitriptyline (K_i of 1.1 nM). Several different meta-analyses have found a small though statistically significant advantage favoring amitriptyline compared with all other antidepressants and also SSRIs [5, 35]. This advantage of amitriptyline, however, is tempered by the greater number of early terminations from these trials and a greater number of adverse events.

Vortioxetine (Figure 12.3) is the most recent serotonin reuptake inhibitor approved for the treatment of depression [36, 37]. It not only is most potent at SERT but also interacts in decreasing potency with 5-HT₃ (antagonism), 5-HT_{1A} (agonism), 5-HT₇ (antagonism), 5-HT_{1B} (partial agonism), and 5-HT_{1D} (antagonism) receptors [38]. The rat *in vivo* potency profile is 5-HT₃ > SERT > 5-HT_{1B} > 5-HT_{1A} and 5-HT₇, but the 5-HT_{1A} and 5-HT₇ potency may be underestimated in the rat due to species differences. Any definitive evidence for greater antidepressant efficacy than SSRIs does not appear. However, post hoc analyses of acute treatment MDD clinical trials suggest that vortioxetine does possess pro-cognitive effects as assessed by the digit symbol substitution test [39]. In a placebo-controlled, double-blind flexible dose study in depressed patients with cognitive dysfunction, vortioxetine significantly improved cognitive function, while only a trend for an improvement ($p < 0.1$) was observed for duloxetine, despite numerically outperforming vortioxetine with respect to the change from baseline in MADRS by approximately 33% [40]. Furthermore, actions at these additional serotonin receptors or a lower SERT occupancy required for efficacy (50–80%) may contribute to a relative lack of sexual dysfunction or sleep disruption compared with that for SSRIs [38]. It also remains to be determined exactly which additional serotonergic receptor(s) is modulated by vortioxetine and are responsible for the improved cognitive function.

Vilazodone (Figure 12.3) is a dual-acting SERT inhibitor and 5-HT_{1A} receptor partial agonist with an *in vitro* potency of approximately 1 nM at each site [41]. *In vivo* dialysis and electrophysiological studies confirmed the dual actions of vilazodone [42, 43]. There is no clear evidence that efficacy onset is hastened with vilazodone compared with other SSRs or that overall efficacy is greater compared with SSRIs [44, 45]. Preclinical comparisons of male rat sexual behavior suggested that while citalopram or paroxetine suppressed sexual behavior, vilazodone appeared largely devoid of similar effects [46]. Unlike SSRIs, vilazodone has been reported to have a placebo-like level of sexual dysfunction using a validated instrument to assess sexual function [47, 48].

Trazodone, nefazodone, and etoperidone are closely related structural phenylpiperazine analogs (Figures 12.3 and 12.5) that have been applied with the misnomer of serotonin antagonist–reuptake inhibitor (SARI) due to putative

	TRAZ	NEFAZ	ETOPER	m-CPP	MIAN	MIRT
SERT						
NET						
DAT						
5-HT _{1A}						
5-HT _{1B}						
5-HT _{2A}						
5-HT _{2C}						
5-HT ₃						
5-HT ₆						
5-HT ₇						
H ₁						
α -1A						
α -2A						
α -2C						
β -1						
β -2						
M ₁ or M						
D ₁						
D ₂						
D ₃						
D ₄						
D ₅						
Sigma1						
Sigma2						

<1 nM	1–9.99 nM	10–99.9 nM	100–999 nM	1–9.99 μ M	10–99.9 μ M

Figure 12.5 Heat map for *in vitro* potency of representative atypical antidepressant drugs and a metabolite at monoamine transporters and receptors. The data plotted for this figure comes from the National Institute of Mental Health (NIMH)-sponsored PDSP *K_i* database (trazodone, m-CPP, mianserin, mirtazapine) and additional references [28, 49–52] for the nefazodone and etoperidone data. Trazodone (TRAZ), nefazodone (NEFAZ), and etoperidone (ETOPER) are metabolized into *meta*-chlorophenylpiperazine (m-CPP). These serotonin antagonist–reuptake inhibitors (SARIs) bind most potently to 5-HT_{2A} receptors and then α ₁ adrenergic receptors. These drugs range from 8-fold (nefazodone) to 25-fold (etoperidone) less potency at SERT than 5-HT_{2A} receptors. Mianserin (MIAN) and mirtazapine (MIRTAZ) are tetracyclic structural analogs. The color code represents different ranges of potency from <1 nM (dark red) to >10 μ M (purple). Each row shows the affinity of these different tricyclic antidepressants for a range of transporters (SERT, NET, and DAT) as well as serotonergic (5-HT), histaminergic (H), muscarinic cholinergic (M), adrenergic (α ₁, α ₂ β ₁, β ₂), and dopaminergic (D) receptor subtypes as well as the sigma binding sites.

dual activity at both 5-HT_{2A} receptors and SERT from an *in vitro* perspective [28, 53–55]. Trazodone, nefazodone, and etoperidone appear to be most potent at blocking 5-HT_{2A} receptors. Trazodone and nefazodone are approximately three- to sixfold less potent at 5-HT_{2C} receptors. The rank potency order of trazodone at a number of receptors is 5-HT_{2A} (*K_i* = 35.8 nM) > 5-HT_{2B} (78.4 nM) > 5-HT_{1D} (106 nM) > 5-HT_{1A} (118 nM) > α _{2c}-adrenergic receptor

(155 nM) > α_{1A} -adrenergic receptor (153 nM); histamine H_1 (220 nM) > SERT (367.3 nM) > sigma2 (536 nM) > dopamine D_4 [34]. Both trazodone and nefazodone are approximately 10-fold and 7-fold less potent at inhibiting SERT compared with 5-HT_{2A} receptor blockade. Trazodone is well known and is widely used off-label at low doses of approximately 25–100 mg at bedtime for insomnia in depressed patients on SSRIs or non-depressed patients as a non-benzodiazepine alternative. These beneficial sleep effects of trazodone have been attributed to some combination of 5-HT_{2A}, α_1 -adrenergic, and histamine H_1 receptor blockade [54]. Higher trazodone doses in the range of 150–600 mg/day are required for the treatment of MDD [54, 56], which have been suggested to be mediated by dual actions of these phenylpiperazine-based drugs at 5-HT_{2A} receptors and then SERT [54]. The structurally related etoperidone (Figure 12.2) was approved for the treatment of depression in Europe as early as 1977.

However, there are both preclinical and clinical data that question whether SERT inhibition is involved in the antidepressant-like effects of trazodone and nefazodone. Regarding the preclinical data, trazodone up to 32 mg (ip) was previously demonstrated to be only a “weak and short-acting inhibitor of the uptake of serotonin by comparison with fluoxetine” in rats [57, 58]. In this same study, nearly complete 5-HT_{2A} receptor inhibition was observed with 10–20 mg of trazodone using antagonism of quipazine-induced corticosterone elevations [57]. Similar observations were reported for nefazodone [59]. Additional preclinical results suggest that antidepressant-like activity of trazodone in rodents is due to blockade of 5-HT_{2A} receptors in the rat differential-reinforcement-of-low rate 72-s (DRL 72-s) operant behavioral screen. Namely, the potency of trazodone’s antidepressant-like effects (5–20 mg, ip) is consistent with known *in vivo* activity at 5-HT_{2A} receptors [60–62]. In addition, the trazodone metabolite *meta*-chlorophenylpiperazine (m-CPP), a 5-HT_{2A} receptor partial agonist/5-HT_{2C} receptor agonist/5-HT_{1B} agonist, fails to exert antidepressant-like activity on DRL 72-s behavior [60]. Moreover, blocking metabolism of trazodone to m-CPP by a non-selective cytochrome P450 inhibitor enhances the potency trazodone on the DRL 72-s schedule [60]. While some discussion of potential antidepressant action of 5-HT_{2C} receptor antagonists has been proposed, it is also notable that preclinical antidepressant screens point toward 5-HT_{2C} receptor agonism and not 5-HT_{2C} receptor antagonism as an important pharmacological lead to be exploited in developing novel antidepressant drugs [63, 64]. Thus, preclinical evidence appears to support a role for 5-HT_{2A} receptor antagonism in the antidepressant action of trazodone and nefazodone.

Clinical biomarker and efficacy data also casts doubt on the SERT inhibition, significantly mediating the antidepressant activity of trazodone and nefazodone. First, a small double-blind, placebo-controlled trial of trazodone (mean daily dose 235 ± 10 mg $\times$ 10 weeks) for the treatment of obsessive compulsive disorder (OCD) failed to observe a reduction of its symptoms [65], contrary to the known effectiveness of SSRIs for this indication. Second, uptake of 5-HT into platelets following 10 weeks of trazodone treatment in this study was nonsignificantly decreased by only a mean of 26%. This stands in stark contrast to previous

reports by this same group that fluoxetine (70 ± 10 mg/day $\times$ 10 weeks) and clomipramine (205 ± 20 mg/day $\times$ 10 weeks) decreased 5-HT uptake by over 95% in patients with OCD [66]. A similar finding has been reported for nefazodone treatment of patients with major depression where this study included an internal SSRI-positive comparator arm [67]. Six subjects with MDD were treated twice daily for 6 weeks (except for one patient treated for 2 weeks) on nefazodone (mean 292 ± 111 mg/day with flexible dosing based on tolerability from 200 to 450 mg/day). This nefazodone treatment decreased the mean 5-HT platelet level at treatment endpoint by 3%. In contrast, the mean 5-HT platelet level in 10 depressed subjects treated for 6 weeks on either fluoxetine (20 mg QD, $n = 4$), paroxetine (20 mg, $n = 4$), or sertraline (100 mg, $n = 2$) was decreased by 88%. These results are consistent with the observation that over 80% brain SERT occupancy is associated with therapeutic doses of SSRIs [68, 69]. No brain receptor or transporter occupancy studies have been published for trazodone or nefazodone except for a single study suggesting that a single 200 mg nefazodone dose is associated with a $39 \pm 17\%$ decrease in the 5-HT_{2A} receptor binding potential measured near the nefazodone $T_{\max}$ [70]. Given the reliable agreement that the potency of either trazodone or nefazodone for SERT is close to 10-fold lower than that for the 5-HT_{2A} receptor, this result also casts doubt on a contributory role of SERT inhibition, mediating the antidepressant action of trazodone or nefazodone.

Relatively, little work has been directed at understanding both benefits and risks of concurrent treatment of trazodone and SSRIs. A single study that has been conducted does not allow for determining whether pharmacokinetic or pharmacodynamics or both factors play a role in an apparent greater antidepressant response to the combined treatment in MDD with trazodone and fluoxetine [71, 72]. While it is commonly assumed that some combination of 5-HT_{2A}, histamine H₁, and α_1 -adrenergic receptor blockade is responsible for beneficial sleep effects of trazodone at relatively low doses (25–100 mg), this does not rule out the hypothesis that greater occupancy of these same receptors at higher doses (150–600 mg/day) might be playing a role in the antidepressant effects of trazodone. For example, human histamine H₁ receptor occupancy studies have suggested that only about 50% of histamine H₁ receptors are required to induce the onset of sleep [73]. Adequate receptor occupancy studies are required to supplement known dose (exposure) relationships to clinical response. Theoretical modeling of extrapolations based on *in vitro* receptor affinity data and animal and human pharmacokinetic data with a number of incumbent assumptions do not provide adequate confidence and often have been proven to be in error compared with optimal measurement of target engagement from human positron emission tomography (PET)/SPECT neuroimaging studies. Thus, it remains to be determined what combination of 5-HT_{2A} and non-5-HT_{2A} receptors, if any, may contribute to therapeutic effects of trazodone or nefazodone in the treatment of MDD.

Mirtazapine and mianserin (Figures 12.3 and 12.5) are another pair of closely related tetracyclic compounds that most mainly bind to histamine H₁, α_2 -adrenergic, 5-HT_{2A}, 5-HT_{2C}, and 5-HT₃ receptors [34]. Mianserin was originally developed by Organon as an antidepressant drug based on sleep

polysomnographic (PSG) effects similar to a range of approved antidepressant drugs, though mianserin did not inhibit monoamine uptake or block monoamine oxidase. The most frequently discussed mechanism underlying the antidepressant action of mianserin is blockade of α_2 -adrenergic autoreceptors and heteroreceptors, as these effects increase ambient norepinephrine, 5-HT, dopamine, and acetylcholine throughout the limbic forebrain [74]. In addition to antidepressant activity during monotherapy, a number of studies have provided evidence for enhanced efficacy compared with monotherapy when adding mianserin to either SSRI treatment that is ongoing or an SSRI that has begun [75–77]. However, not all studies have observed an enhanced combination/augmentation effect when adding mianserin to SSRIs [78].

Mirtazapine is the 6-aza analog of mianserin and was approved by the FDA as an antidepressant drug in 1996. A meta-analysis of antidepressants developed between 1991 and 2007 found that mirtazapine (in addition to venlafaxine, sertraline, and escitalopram) was more efficacious than duloxetine (Figure 12.5), fluvoxamine, paroxetine, and reboxetine [79]. Mirtazapine was found to augment the effects of SSRIs and serotonin–norepinephrine reuptake inhibitors (SNRIs) in depressed patients [80]. Combination of mirtazapine plus paroxetine treatment of MDD was suggested to be more efficacious than either monotherapy [81].

12.5 Tested and Failed/Technically Difficult Dual-Acting Serotonergic Compounds

Flibanserin (Figure 12.6) is a dual-acting 5-HT_{2A} receptor antagonist/5-HT_{1A} receptor agonist that was approved by the FDA in 2015 for the treatment of hypoactive sexual desire disorder in premenopausal women after a failed initial development program for MDD. The initial phase 2 trials of flibanserin were conducted in 892 patients with major depression [82]. In none of the four placebo-, fluoxetine-, or paroxetine-controlled trials did flibanserin significantly outperform the placebo treatment arm. Fluoxetine failed to separate from placebo in two of these trials. However, flibanserin did appear to outperform placebo in response to the question “how strong is your sex drive.” This was the genesis of the flibanserin sexual dysfunction development effort. While both 5-HT_{2A} receptor antagonism and 5-HT_{1A} receptor agonism have been suggested to be potential antidepressant targets, it is not completely surprising that combining these actions failed to result in antidepressant activity in clinical trials. There is at least one preclinical antidepressant drug screen, the DRL 72-s operant schedule in rodents, for which adding a 5-HT_{1A} receptor agonist to 5-HT_{2A} or 5-HT_{2A/2C} antagonists (ketanserin and trazodone) suppresses the antidepressant action of the 5-HT₂ receptor antagonist (G. Marek, unpublished results). Flibanserin itself demonstrated antidepressant-like effects in olfactory bulbectomized rats and in the learned helplessness paradigm, but not on DRL 72-s behavior [83]. Flibanserin appears to be an intriguing example where combining two putative serotonergic mechanisms of antidepressant action might have canceled out the effects of the other since activation of presynaptic

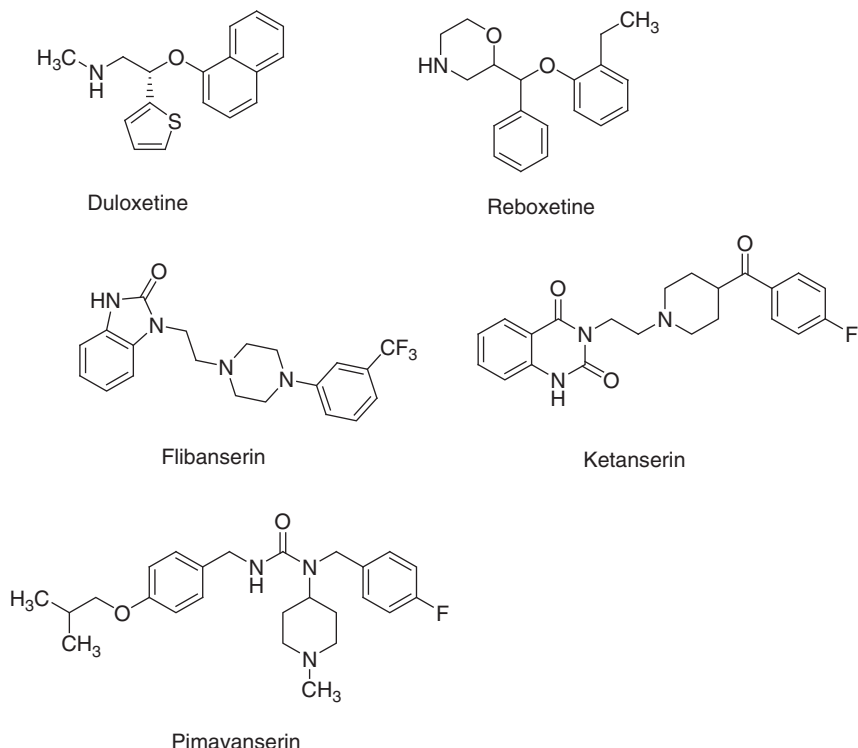


Figure 12.6 Chemical structure of the SNRI duloxetine, the NET inhibitor reboxetine, and three other drugs blocking 5-HT_{2A} receptors. These 5-HT_{2A} receptor antagonists include ketanserin, pimavanserin, and the failed antidepressant flibanserin (also a 5-HT_{1A} receptor agonist).

5-HT_{1A} receptors with coincident decreases in dorsal raphe firing rate would decrease ambient stimulation of 5-HT_{2A} receptors. Thus, the 5-HT_{2A} receptor blockade would be diminished under conditions of reduced firing of brainstem 5-HT-containing cells that project throughout the limbic forebrain.

12.6 Technical Challenges to Developing New Chemical Entities with Multiple Mechanisms of Action

The medications discussed above have been either serendipitous (e.g., clomipramine, mianserin) or rationally designed (e.g., flibanserin) new chemical entities that were either found to possess antidepressant action in patients or tested for the treatment of MDD. A number of substantial challenges exist when rationally designing NCEs acting on multiple serotonergic sites. These dual- or multiple-mechanism-acting NCEs generally will be considerably more resource intensive than a typical discovery project directed at a single molecular

mechanism of action. First, more molecules will need to be screened to identify a molecular acting with approximately equal potency at two molecular sites than a single molecular site. The hurdle is further increased if trying to combine several different activities with another activity like SERT inhibition. Second, a major challenge arises when considering off-target activity that may compromise safety margins from toxicological effects to pharmacological effects putatively associated with clinical efficacy. For example, citalopram has been associated with increases in the corrected QT interval (QTc) related to cardiac repolarization [84]. Increases in QTc are hypothesized to increase the risk of fatal cardiac arrhythmias such as Torsade de Pointes [85]. An unfortunate overlap of chemical pharmacophores exists between potassium channels (rapid delayed rectifier potassium currents (I_{kr}) translated from the human ether-a-go-go-related gene (*hERG*)) underlying risk to fatal cardiac arrhythmias and G-protein-coupled receptors (GPCRs) including certain serotonin receptors (e.g., 5-HT₂ receptor family). Thus, any project targeting 5-HT_{2A} or 5-HT_{2C} receptor ligands is at an even greater risk for QTc issues if a combined SERT inhibitor/5-HT_{2A} receptor antagonist is desired given separate propensity toward off-target hERG activity by both 5-HT₂ receptor antagonists and SERT inhibitors. Other toxicological liabilities such as phospholipidosis are present when combining pharmacophores (SERT and 5-HT_{2A} receptor) that each may possess unique liability (e.g., fluoxetine and pimavanserin) toward these untoward effects and increased development risks [86, 87]. Third, when trying to develop drugs targeting 5-HT_{2A} or 5-HT_{2C} receptors, lack of specificity for the 5-HT_{2B} receptor is a significant liability as agonist or partial agonist activity at this latter receptor has been associated with the development of pulmonary hypertension [88, 89]. These are some reasons why a greater percentage of the dual-acting serotonergic antidepressants have been discovered in a serendipitous fashion either when following clinical effects found testing different clinical indications (imipramine's initial trials in schizophrenia) or when they were applied to preclinical antidepressant drug screens and/or later administered to depressed patients (mianserin). For all of the reasons discussed, rational drug design with projects designed to target dual-acting serotonergic targets is significantly easier to develop when having at least a few prototype molecular scaffolds with some degree of activity at several different serotonergic-related sites (e.g., SERT, 5-HT receptors).

Given the number of hurdles in rationally developing antidepressants targeting more than one pharmacological action on the serotonergic system discussed above, it is of significant heuristic value to acknowledge the clinical evidence present or absent for combining a range of serotonergic targets with SSRIs. This is important because some clinically relevant differentiation must be present for the dual-acting serotonergic NCE compared with what is observed for SSRIs. Given the general acceptable safety profile of SSRIs, this generally requires a clear benefit with respect to the magnitude of antidepressant efficacy. Without such important clinical differentiation, payers have little incentive to allow relatively expensive recently approved drugs into their formulary if generic SSRIs are equally effective.

12.7 Clinical Experiments with SSRIs and 5-HT_{1A} Agonists/Antagonists

Clinical precedence in the form of clinical experiments combining different pharmacological activities is an important argument for establishing new development programs targeting novel antidepressants directly modulating two or more serotonergic molecular sites. The initial randomized, double-blind, placebo-controlled trials for three drugs from the azapirone class of 5-HT_{1A} receptor partial agonists in patients with MDD were initially reported in the 1990s. A review and meta-analysis identified 15 RCTs comparing buspirone, gepirone, ipsapirone, or zolospirone with placebo [90]. Each of these partial agonists (excluding the single zolospirone trial) separated from placebo as did this entire 5-HT_{1A} agonist class in the meta-analysis. The first of these drugs were approved by the FDA for the treatment of MDD in March 2016 despite an FDA advisory panel voting against approval 9–4 based on lack of substantial evidence indicating antidepressant efficacy. This same review and meta-analysis failed to find convincing evidence of antidepressant activity for buspirone and tandospirone in four trials where the azapirone plus an SSRI (three studies) or TCA was compared with the SSRI or TCA alone [90]. Thus, the clinical data with the azapirones does not support developing combined SERT inhibitors/5-HT_{1A} receptor agonists when seeking greater clinical efficacy than standard of care monotherapy. Furthermore, the azapirone antidepressant clinical trial literature for the combination of SSRIs with partial 5-HT_{1A} receptor agonists is consistent with the apparent similar efficacy effect size for vilazodone compared with SSRIs.

An extensive literature from an initial report dating back to 1994 exists for combining 5-HT_{1A} receptor antagonists with SSRIs, suggesting both promise and pessimism that this is a fruitful avenue to pursue in developing antidepressants with superior efficacy [91]. The bulk of this data concerns pindolol, a 5-HT_{1A} receptor antagonist/partial agonist that also is a β_1 -adrenergic receptor antagonist [92, 93]. Interpretation of these studies needs to take into account that pindolol appears to occupy human 5-HT_{1A} autoreceptors in the human dorsal raphe compared with postsynaptic 5-HT_{1A} receptors in the cortex [94]. However, neuroimaging of depressed patients suggest that this preferential autoreceptor occupancy by pindolol may be attenuated by disease state and/or medication [95]. In addition, pindolol does appear to possess weak partial agonist efficacy at 5-HT_{1A} receptors under at least some conditions. Nevertheless, a meta-analysis suggests that pindolol does appear to result in earlier (within 2 weeks) sustained antidepressant efficacy [96]. Conflicting results from meta-analyses suggest that sustained antidepressant efficacy (4–6 weeks of treatment) for pindolol added to SSRIs, if present, is less robust than the early improvement [96, 97]. Clinical pindolol and fluoxetine studies by Eli Lilly examining a dose range from 4 to 32 mg/day found no evidence of an earlier onset of action or greater overall antidepressant efficacy [98]. Thus, efficacy for pindolol has been examined by exploring doses that produce ~40% occupancy (7.5 mg) and ~60% occupancy of dorsal raphe autoreceptor [94, 95]. Post hoc testing in several studies has suggested that pindolol may be more effective in producing a greater antidepressant

response when added to SSRIs in patients during their first episode of MDD [97, 99].

Several selective 5-HT_{1A} receptor antagonists have also been synthesized and tested in the clinic [15]. Robalzotan (NAD-299 or AZD 7371) is a 5-HT_{1A} receptor antagonist with over approximately 250-fold greater selectivity at this site compared with other serotonergic and non-serotonergic receptors [100]. Informing phase 2 dose selection, a PET neuroimaging study demonstrated that a single 10 mg oral dose appeared to occupy ~62–85% and 68–75% of midbrain dorsal raphe and cortical 5-HT_{1A} receptors [101]. Robalzotan monotherapy (5, 10, and 20 mg bid) was tested in 385 patients with MDD in a randomized, double-blind, placebo-controlled, paroxetine-controlled study [102]. Robalzotan did not possess antidepressant activity in this study, even for a post hoc test in those centers demonstrating a 2-point improvement on the HAMD scale for paroxetine compared with placebo (Tables 12.1 and 12.2).

DU-125530 is another selective 5-HT_{1A} receptor antagonist that appears, unlike pindolol, to be highly potent at both presynaptic and postsynaptic 5-HT_{1A} receptors in both rodents and humans [103]. A 50-patient randomized, double-blind, placebo-controlled trial examining the addition of DU-125530 to fluoxetine in patients with MDD failed to observe either a rapid onset or a more efficacious action compared with the placebo-treated group [103]. The authors concluded that potently blocking postsynaptic 5-HT_{1A} receptors may cancel out any benefit of antagonizing presynaptic 5-HT_{1A} receptors. Another implication from this trial is the suggestion that non-5-HT_{1A} receptors can at

Table 12.1 Serotonergic receptor characteristics.

Receptor	Transduction path	MDD efficacy	Safety/tolerability
5-HT _{1A}	Inhibit AC	Postsynaptic agonists Presynaptic antagonists	Agonists improve sexual dysfunction, dizziness
5-HT _{1B}	Inhibit AC	Presynaptic antagonists?	
5-HT _{1D}	Inhibit AC	?	Triptan cardiovascular issues
5-HT _{1E}	Inhibit AC	?	
5-HT _{1F}	Inhibit AC	?	
5-HT _{2A}	Activates PLC/PKC	Antagonists	Edema (antagonist); hallucinations (agonist)
5-HT _{2B}	Activates PLC	?	Pulmonary hypertension (agonist)
5-HT _{2C}	Activates PLC/PKC	Agonists (preclinical)	Dyspepsia and diarrhea
5-HT ₃	Opens cation channels	Antagonists?	
5-HT ₄	Activates AC/PKA	Agonists (preclinical)	
5-HT _{5A/B}	Inhibit AC	?	
5-HT ₆	Activates AC5	Antagonists?	Urinary tract infection
5-HT ₇	Activates AC5/1/8	Antagonists?	

? -unknown relationship therapeutic efficacy

Table 12.2 Non-serotonergic receptor characteristics.

Receptor	Transduction path	MDD efficacy	Safety/tolerability
H ₁	Activates PLC/PKC	?	Sedation (antagonists)
H ₂	Activates AC/PKA		
H ₃	Inhibits AC/opens CACNA1B		Insomnia, anxiogenic (antagonists)
H ₄	Inhibits AC		
α _{1A} Adrenergic	Activates PLC/PKC	?	
α _{1B} Adrenergic	Activates PLC/PKC		Postural hypotension and priapism (antagonists)
α _{2A} Adrenergic	Inhibits AC		Enhance (agonists) and impair (antagonists) cognition
α _{2C} Adrenergic			
β ₁ Adrenergic	Activates AC	Agonists	Worsen depression (antagonists); tachycardia and increased contractility (agonists)
β ₂ Adrenergic	Activates AC/PKA/directly activates L type Ca ²⁺ channels		Tachycardia, tremor, excessive sweating, anxiogenic, insomnia, agitation (agonists)
Dopamine D ₁	Activates AC/PKA		Hypotension (D _{1/5} agonist)
Dopamine D ₂	Inhibits AC		Tremors, akathisia, tardive dyskinesia, galactorrhea (antagonists)
Dopamine D ₃	Inhibits AC	Preclinical (agonists)	Nausea, constipation, decreased appetite (antagonists)
Dopamine D ₄	Inhibits AC		Seizures? (antagonists)
Dopamine D ₅	Activates AC/PKA		Hypotension (D _{1/5} agonist)
Sigma-1			
Sigma-2			

?- potentially relate to antidepressant efficacy.

least partially mediate the therapeutic effects from increasing ambient serotonin throughout the CNS with sub-chronic SSRI treatment. Work by a number of industry sponsors in developing either selective 5-HT_{1A} receptor antagonists or SERT inhibitors with 5-HT_{1A} receptor antagonism has been previously reviewed [15]. The clinical study with DU-125530 suggests that novel antidepressants seeking faster onset and sustained antidepressant efficacy would need to inhibit SERT and 5-HT_{1A} autoreceptors while largely sparing postsynaptic 5-HT_{1A} receptors. Thus, the implication of the literature for preclinical and clinical studies involving 5-HT_{1A} receptor antagonism and SERT inhibition is that the scientific and commercial feasibility of combination molecules possessing antagonist action at both SERT and 5-HT_{1A} receptors with a desired greater efficacy than SSRI monotherapy is a formidable task.

12.8 Clinical Experiments with SSRIs and Drugs Possessing 5-HT_{2A} Receptor Blockade

Substantial, though certainly not conclusive, evidence suggests that adding 5-HT_{2A} receptor antagonism to SERT inhibition may result in a clinically meaningful increase in antidepressant efficacy compared with SERT inhibition alone. First, pipamperone (5 mg bid) has been reported to increase the antidepressant response when added to paroxetine compared with paroxetine during the early weeks of treatment, but not at the study endpoint [104]. In this study, pipamperone did or tended to improve problems in sleeping, concentration, and pessimistic thoughts both at weeks 4 and 8 study endpoints. Unfortunately, the interpretation of this study was compromised by the desire to minimize arrhythmia risk through potentially suboptimal dose selection [105]. The significance of this dose is that the recommended pipamperone antipsychotic dose is 120–360 mg. Dose selection in this study to avoid toxicity may have led to inadequate exposures to reach maximal brain 5-HT_{2A} receptor occupancy as the target 5-HT_{2A} receptor occupancy was only around 60%. Thus, inadequate dose selection may have precluded a clear definitive test that pipamperone combination treatment with SSRIs is greater than SSRI treatment alone. This pipamperone plus paroxetine combination data is interesting given an older report that monotherapy with pipamperone (~80 mg × 1 week) in depressed subjects resulted in improvements in mood and sleep [106].

5-HT_{2A} receptor blockade (Figure 12.7) appears to be the most potent single-shared pharmacological mechanism accounting for increased antidepressant efficacy when adding atypical antipsychotic drugs (aripiprazole, brexpiprazole, olanzapine, quetiapine, and risperidone) to ongoing or the beginning of SSRI or SNRI treatment of refractory patients [56, 113]. The atypical antipsychotic drug clozapine (Figures 12.3 and 12.7) is also a potent 5-HT_{2A} receptor antagonist that may possess antidepressant properties, but there has been relatively little studied for augmentation of antidepressants in patients with MDD (Figure 12.7). These atypical antipsychotic drugs also potentially act on a number of other sites that may contribute to antidepressant action including 5-HT_{2C}, 5-HT₆, 5-HT₇, dopamine D₂, D₄, α_{1A}-adrenergic, α_{1B}-adrenergic, α_{2C}-adrenergic, and histamine H₁ receptors (Figure 12.7). Typical antipsychotic drugs, many of which are potent 5-HT_{2A} receptor antagonists (see chlorpromazine and thioridazine in Figure 12.7), are also known to possess antidepressant efficacy as previously reviewed [114]. Given the empirically demonstrated increased liability of patients with mood disorders toward extrapyramidal symptoms when treated with typical antipsychotic drugs, it is understandable that first-generation antipsychotic drugs have not been tested either in combination or augmentation paradigms with SSRIs or SNRIs in patients with MDD. Notably absent from the Robertson and Trimble review was the butyrophenone haloperidol, a drug relatively weak at binding to 5-HT_{2A} receptors compared with the dopamine D₂ family of receptors.

As discussed above, a second class of drugs, the tetracyclic mianserin and mirtazapine, may also enhance the effects of SSRIs in depressed patients by

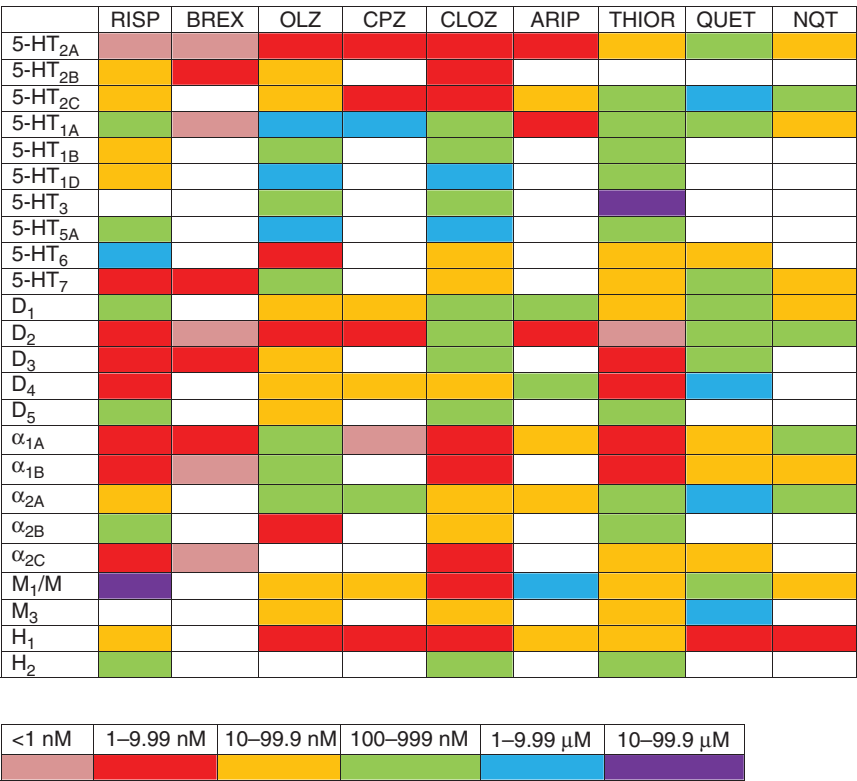


Figure 12.7 Heat map for *in vitro* potency of representative atypical and typical drugs at a range of monoamine receptors. The risperidone, olanzapine, clozapine, chlorpromazine, and thioridazine data plotted for this figure comes from the PDSP K_i database. The remaining data are derived from the NIMH PDSP K_i database and additional references [107–112]. The atypical antipsychotics or second-generation antipsychotics include risperidone (RISP), brexpiprazole (BREX), olanzapine (OLZ), clozapine (CLZ), aripiprazole (ARIP), quetiapine (QUET), and the quetiapine metabolite nor-quetiapine (NQT). The typical antipsychotic drugs exemplified in this figure are chlorpromazine (CPZ) and thioridazine (THIOR). The color code represents different ranges of potency from <1 nm (pink) to >10 μM (purple). Each row shows the affinity of these different tricyclic antidepressants serotonergic (5-HT), dopaminergic (D), histaminergic, α-adrenergic (α₁ and α₂), muscarinic cholinergic (M), and histaminergic (H) receptor subtypes.

blocking 5-HT_{2A}/5-HT_{2C} and/or 5-HT₃ receptors. Blockade of 5-HT_{2C} receptors would appear to be a less likely explanation for the augmenting effects of atypical antipsychotic drugs or mirtazapine/mianserin upon SSRIs in depressed patients. One can question whether many of the atypical antipsychotics and mirtazapine/mianserin would be more effective if they did not block 5-HT_{2C} receptors as potently as many of them do. As noted earlier, 5-HT_{2C} agonists appear to have robust antidepressant effects in a wide range of preclinical antidepressant drug screens [63, 64]. In fact, over a decade ago DuPont was working on developing novel antidepressant drugs that would block 5-HT_{2A} receptors while simultaneously activating 5-HT_{2C} receptors. This direction by

DuPont appears supported by the finding that potent biologically important effects on impulsivity as well as some antidepressant drugs screens (DRL 72-s schedule, olfactory bulbectomy) are modulated by 5-HT_{2A} and 5-HT_{2C} receptors in the opposite direction. Namely, these models predict that blockade of 5-HT_{2A} and activation of 5-HT_{2C} receptors are related to antidepressant-like activity or anti-impulsive-like activity. A potential clinical trial to test whether the activation of 5-HT_{2C} receptors by WAY-163909 or vabicaserin would exert a frank monotherapy antidepressant action appears to have been scrapped following the acquisition of Wyeth by Pfizer. However, given that SSRIs would already activate most serotonergic receptors in the brain, it is less clear whether a 5-HT_{2C} agonist like vabicaserin could enhance the antidepressant action of SSRIs or SNRIs.

This clinical hypothesis that selective blockade of 5-HT_{2A} receptors might enhance the antidepressant action of SSRIs can now be tested in the clinic by double-blind, placebo-controlled, randomized clinical trials comparing the off-label combination of pimavanserin (Figure 12.6) with SSRIs compared with SSRIs alone. Pimavanserin is a 5-HT_{2A} inverse agonist (biologically acting as 5-HT_{2A} receptor antagonist even if frank inverse agonist activity is not present) that was recently approved by the FDA and launched in 2016 for the treatment of Parkinson's disease psychosis [115].

12.9 Non-SERT Serotonergic Targets Mired in Phase 2/3

The 5-HT_{1B} receptor is distributed widely throughout the rodent and primate brain as it plays major roles as both a serotonergic autoreceptor on serotonergic axons and a heteroreceptor modulating the release of other neurotransmitters [116, 117]. Several 5-HT_{1B/1D} receptor antagonists have been tested as antidepressants in phase 2 proof-of-concept (PoC) trials. Elzasonan or CP-448,187 was initially found to have antidepressant efficacy at 3 mg in the phase 2 PoC trial, potentially suggesting an inverted U dose–response curve (information on NIH NCATS website); however efficacy for this compound was not replicated in a later study. No results have been provided for an additional 262-patient phase 2 MDD trial for elzasonan comparing a sertraline/elzasonan combination with sertraline alone and with placebo alone over a treatment duration of 8 weeks (www.ClinicalTrials.gov, identifier NCT00275197). Subsequently, elzasonan was discontinued by Pfizer in 2008 [118] and in 2013 for the treatment of OCD. It also appears to possess reasonably potent 5-HT_{1A} receptor activity, and activity at this site may be associated with dizziness (<https://ncats.nih.gov/files/CP-448187.pdf>). With respect to testing of CP-448187, a dose of 3 mg daily has been reported to be associated with 70–0% occupancy of the brain 5-HT_{1B} receptors in a PET neuroimaging study (<http://ncats.nih.gov/files/CP-44187>).

A second selective 5-HT_{1B} receptor antagonist, AR-A000002 (AZD8129), also was moved into a phase 2 MDD trial based on encouraging preclinical results [119–121]. AstraZeneca developed a radiotracer for this site, and the clinical MDD study(ies) likely used this critical biomarker data for dose selection

[122–124]. It discontinued development of this compound on July 28, 2005. Thus, on the basis of clinical work performed with AZD8129 and elzasonan, including a trial examining the combination of an SSRI and elzasonan in depressed patients, the combination of SERT inhibition and 5-HT_{1B} receptor blockade in the same molecule does not appear to be a viable direction toward developing novel antidepressant drugs with superior clinical activity than seen with SSRIs.

The 5-HT_{1F} receptor is clearly expressed in CNS vasculature, making testing of 5-HT_{1F} agonists for migraine a readily obvious clinical experiment given nearly equipotent effects of the antimigraine drug sumatriptan on 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors [125]. Lasmiditan is a 5-HT_{1F} agonist currently in the midst of phase 3 testing [126]; the limited CNS distribution of this receptor makes it not consistent with this receptor mediating a major role in the antidepressant effects of SSRIs or SNRIs.

The 5-HT₆ receptor is another 5-HT receptor distributed in the limbic forebrain [127, 128] and is potently blocked by atypical antipsychotic drugs like clozapine and olanzapine (Figure 12.7). Several selective 5-HT₆ receptor antagonists have been tested as cognitive enhancers for Alzheimer's disease in phase 2 (SB-742457) and phase 3 (Lu AE58054) with questionable clinically relevant pro-cognitive efficacy. However, there does not appear to be a strong rationale for combining such drugs with SSRIs to pursue more efficacious treatment of MDD.

Like the 5-HT₆ receptor, the 5-HT₇ receptor is distributed in the limbic forebrain [128–130] and is potently blocked by atypical antipsychotic drugs such as clozapine and risperidone (Figure 12.6). One 5-HT₇ receptor antagonist, JNJ-18038683, was investigated in a placebo-controlled trial in patients with MDD, but equivocal results were obtained [131]. Nevertheless, 5-HT₇ receptor blockade is one in a number of additional pharmacological actions that the antidepressant vortioxetine possesses in addition to SERT inhibition. This receptor may have contributed to some degree to the augmentation or combination antidepressant effects of atypical antipsychotic drugs and SSRIs.

12.10 Conclusions and Outlook

SSRIs have been established for over 40 years as a treatment for MDD with indiscriminate activation of 14 different receptor subtypes. Activation of at least postsynaptic 5-HT_{1A} receptors appears to mediate, at least in part, the therapeutic effects of SSRIs. Activation of 5-HT_{2A}, 5-HT_{2C}, and 5-HT₇ receptors may attenuate the therapeutic effects of SSRIs. The improved therapeutic efficacy of certain TCAs (amitriptyline) compared with SSRIs may be due to blockade of 5-HT_{2A}, 5-HT_{2C}, NET, or other non-serotonergic receptors. While the addition of pindolol and 5-HT_{1A} weak partial agonism/antagonism clearly appears to decrease the onset latency of SSRI therapeutic efficacy, development of dual-acting SSRI/5-HT_{1A} partial agonists/antagonists has not led to more effective antidepressants. Clinical experiments adding atypical

antidepressants (trazodone, mirtazapine, mianserin) or atypical antipsychotic drugs to SSRIs/SNRIs suggest highlighting a potential role for blocking 5-HT_{2A}, 5-HT_{2C}, 5-HT₆, or 5-HT₇ receptors. Blockade of 5-HT_{2A} receptors appears to be the most potent shared pharmacological effect of these drugs. The hurdles in bringing compounds to the clinic combining multiple serotonergic mechanisms of action (including SERT inhibition) are challenging. However, no novel dual- or triple-acting serotonergic antidepressants with improved antidepressant efficacy compared with SSRI monotherapy have been brought to phase 3 trials or approved thus far. Nevertheless, medications combining SERT inhibition with blockade of 5-HT_{2A} and/or 5-HT₆ and/or 5-HT₇ receptors and/or presynaptic 5-HT_{1A} receptors appear to be candidates for novel antidepressant drugs with greater antidepressant efficacy than SSRIs. In addition, drugs combining SERT inhibition with blockade of 5-HT_{2A} and/or 5-HT₃ and/or 5-HT₆ and/or 5-HT₇ receptors may also lead to novel antidepressant medications with improved efficacy for treating cognitive dysfunction associated with depression.

List of Abbreviations

5-HT	5-hydroxytryptamine (a.k.a., serotonin)
AC	adenyl cyclase
DUAG	Danish University Antidepressant Group
DRL 72-s	differential-reinforcement-of-low rate 72-s
ECT	electroconvulsive therapy
FDA	US Food and Drug Administration
MAOIs	monoamine oxidase inhibitors
MDD	major depressive disorder
NET	norepinephrine transporter
NIMH	National Institute of Mental Health
OCD	obsessive compulsive disorder
PDSP	Psychoactive Drug Screening Program
PLC	phospholipase C
PKC	protein kinase C
SARI	serotonin antagonist–reuptake inhibitors
SERT	serotonin transporter
SNRIs	serotonin–norepinephrine reuptake inhibitors
SSRIs	selective serotonin reuptake inhibitors
QTc	corrected QT interval of electrocardiograms
TCAs	tricyclic antidepressant

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13

Multiple Ligands Targeting the Angiotensin System for Hypertension

Agustin Casimiro-Garcia

Medicine Design, Pfizer Worldwide Research and Development, 610 Main Street, Cambridge, MA 02139, USA

Angiotensin II (AII) is an octapeptide that is considered as one of the main effectors of the renin–angiotensin–aldosterone system (RAAS) [1, 2], one of the most important systems controlling blood pressure (BP) and cardiovascular and renal function in man. In the classical pathway of the RAAS, the cascade begins with cleavage of the precursor angiotensinogen, produced by the liver, into the biologically inactive angiotensin I (AI) by renin, which is secreted by the juxtaglomerular cells of the kidney. Enzymatic conversion of AI into AII is primarily carried out by angiotensin-converting enzyme (ACE), a membrane-bound zinc metalloprotease. In the heart, kidney, and brain, other proteases like cathepsin G, chymases, and endopeptidases also contribute to AII production [1]. AII is a potent vasoconstrictor with a plethora of additional actions including hormonal, neuronal, and renal tubular effects. It acts in many tissues including the kidney, heart, blood vessels, brain, and lymphatic organs. The actions of AII are exerted by binding to the AII type 1 (AT_1) and type 2 (AT_2) receptors that belong to the G-protein-coupled receptors (GPCRs) [1, 3]. It has been long recognized that the AT_1 receptor mediates the actions of AII on the cardiovascular system in both physiological and pathological states, including vasoconstriction, BP increase, water and sodium retention, growth promotion, fibrosis, and inflammation.

Pharmacological intervention of the actions of AII has led to two effective and widely used classes of antihypertensive agents, including ACE inhibitors (ACEi), which inhibit the formation of AII from AI, and angiotensin receptor blockers (ARBs), which prevent the binding of AII to AT_1 receptors. The ARBs currently in use are considered as very well-tolerated antihypertensive agents that also provide protection against stroke, renal damage, and cardiovascular disease [4]. The ability to design compounds that not only block the AT_1 receptor but also target other pathways involved in the control of BP, cardiovascular disease, and other disease states has led to novel ARBs. The following sections describe recent progress with this new class of bifunctional agents. A review of advances in the structural understanding of AT_1 receptor ligand binding is discussed first to provide a general understanding of the AT_1 pharmacophore and the key structural features of the interaction with the receptor. A number of recent reviews have

discussed the design of compounds that target ACE inhibition in combination with other pathways, and these dual agents are not reviewed here [5, 6].

13.1 Recent Advances in the Structural Basis for AT₁ Receptor Ligand Binding

The structure of the AT₁ receptor and binding mode of ARBs have remained unknown despite decades of research efforts. Recent advances in the crystallography of GPCRs and, in particular, the application of recently developed crystallography methods have provided the first crystal structure of the human AT₁ receptor in complex with ZD7155 (**1**), a high-affinity ARB, at 2.9 Å resolution (Figure 13.1) [7]. A second crystal structure of the AT₁ receptor in complex with the inverse agonist olmesartan (**2**) was also reported recently [8]. The availability of these crystal structures has revealed key structural features of the interaction, and mutagenesis studies have further corroborated their critical role. A conserved binding mode for other clinically used ARBs was proposed based on these structures. Both ligands are anchored to the receptor through three key residues: Arg167^{ECL2}, Tyr35^{1.39}, and Trp84^{2.60}. An extensive network of ionic interactions is formed between the guanidine group of Arg167^{ECL2} and the tetrazole moiety of the ligands. Mutation of this residue to Ala was found to abolish ligand binding. An acidic moiety is well established as a key component of the pharmacophore among ARBs, further confirming the critical role of this interaction. In addition to its role for ligand-binding affinity, Arg167^{ECL2} may be relevant for selectivity, as it is a unique residue of the AT₁ receptor when compared with other structurally similar GPCRs [7]. Another key interaction is the hydrogen bonding between Tyr35^{1.39} and a nitrogen from the C4' heteroaryl moiety (see biphenyl ring numbering; Figure 13.2). Diverse heteroaryl groups are tolerated at this position, as observed with the two different heteroaryls incorporated in **1**

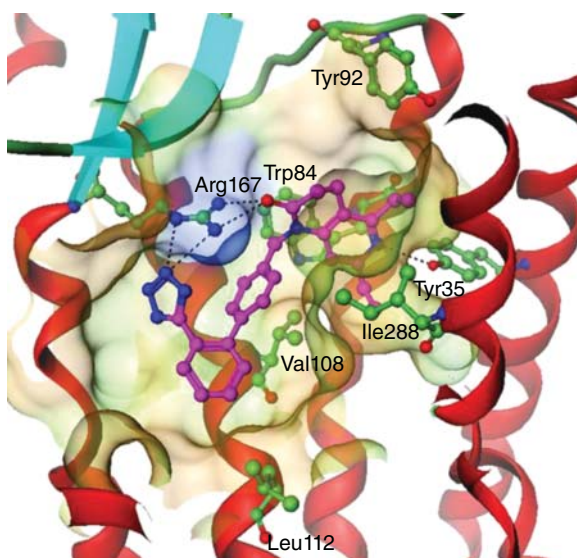


Figure 13.1 Crystal structure of the human AT₁ receptor in complex with **1** (magenta). Key residues from the AT₁ receptor involved in the interaction with ARBs are shown in green. The structure was retrieved from the Protein Data Bank using accession code 4YAY.

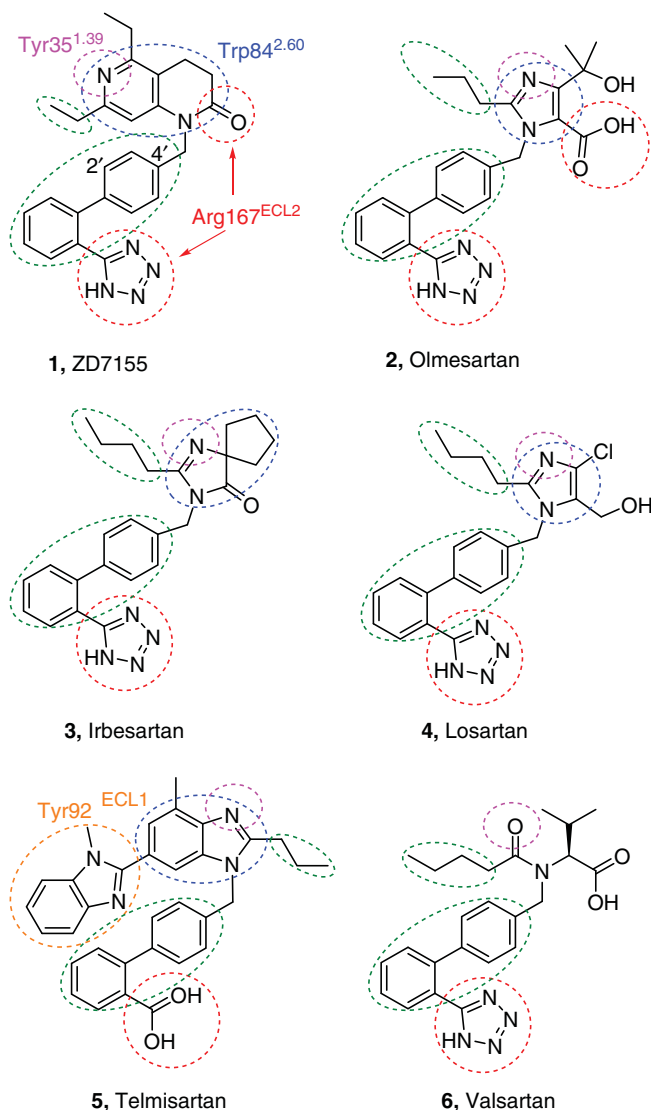


Figure 13.2 Common binding mode of ARBs to the AT₁ receptor reported by Zhang *et al.* includes interactions with three key residues: Arg167^{ECL2} (red), Tyr35^{1.39} (magenta), and Trp84^{2.60} (blue) [7]. In addition, hydrophobic contacts are formed between the biphenyl motif and the alkyl tail with a set of lipophilic residues from the receptor (green). Interactions depicted for 1 or 2 are taken from their corresponding crystal structures (PDB accession codes 4YAY and 4ZUD, respectively). Docking simulations were used to propose the binding mode of other compounds.

(naphthyridin-2-one) and 2 (imidazole), as long as they retained a properly positioned nitrogen capable of engaging in hydrogen bonding. Mutation of Tyr35^{1.39} to Ala was found to abolish binding capability. The heteroaryl group of the ARBs also engages in π - π interaction with Trp84^{2.60}, and mutation of this residue to Ala also led to abolishing the binding to the receptor. Hydrophobic

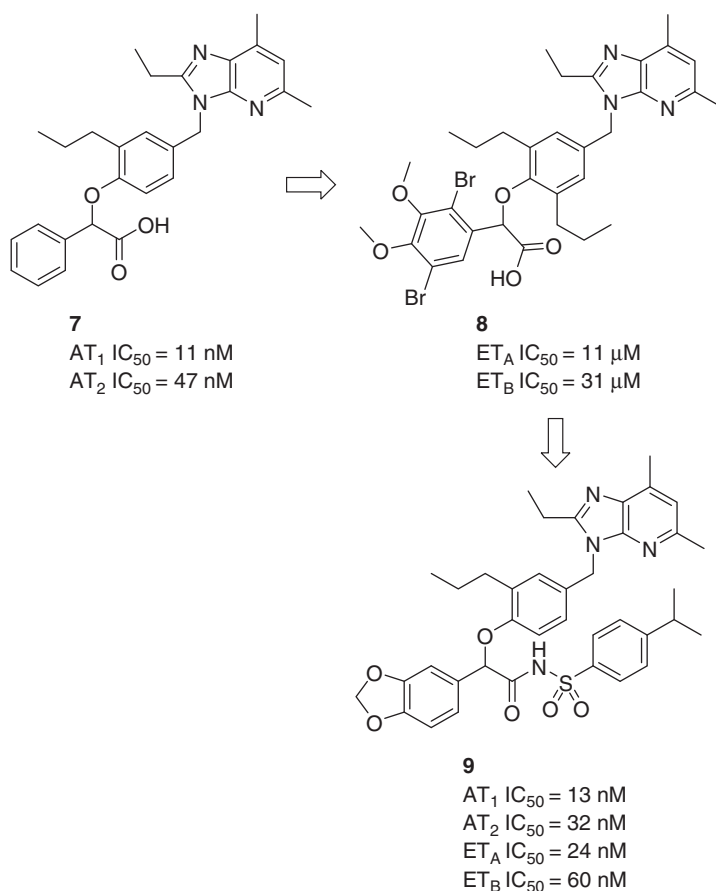
interactions are also formed between the biphenyl moiety of the ligands and lipophilic residues on the receptor, including Val108^{3.32} and Leu112^{3.36}. Additional hydrophobic contacts are formed between the short alkyl tail attached to the heteroaryl moiety in **1** and **2** and a narrow hydrophobic pocket formed by Tyr35^{1.39}, Phe77^{2.53}, Val108^{3.32}, Ile288^{7.39}, and Tyr292^{7.343}.

Docking simulations were performed using the new crystal structure of **1** bound to the AT₁ receptor to analyze the binding mode of clinically relevant ARBs [7]. Selected examples are depicted in Figure 13.2. A common binding mode was proposed that includes interaction with the three key residues, Arg167^{ECL2}, Tyr35^{1.39}, and Trp84^{2.60}, as well as hydrophobic contacts between lipophilic residues and the biphenyl group or the short alkyl tail, both of these motifs being retained in all these compounds. Given the structural differences between these ARBs, additional interactions were proposed for selected compounds that are likely to contribute to potency. One example is the additional hydrophobic interactions and π - π contacts formed between Tyr92^{ECL1} and the second benzimidazole ring of telmisartan (**5**).

13.2 Design of Dual AT₁ and Endothelin A Receptor Antagonists

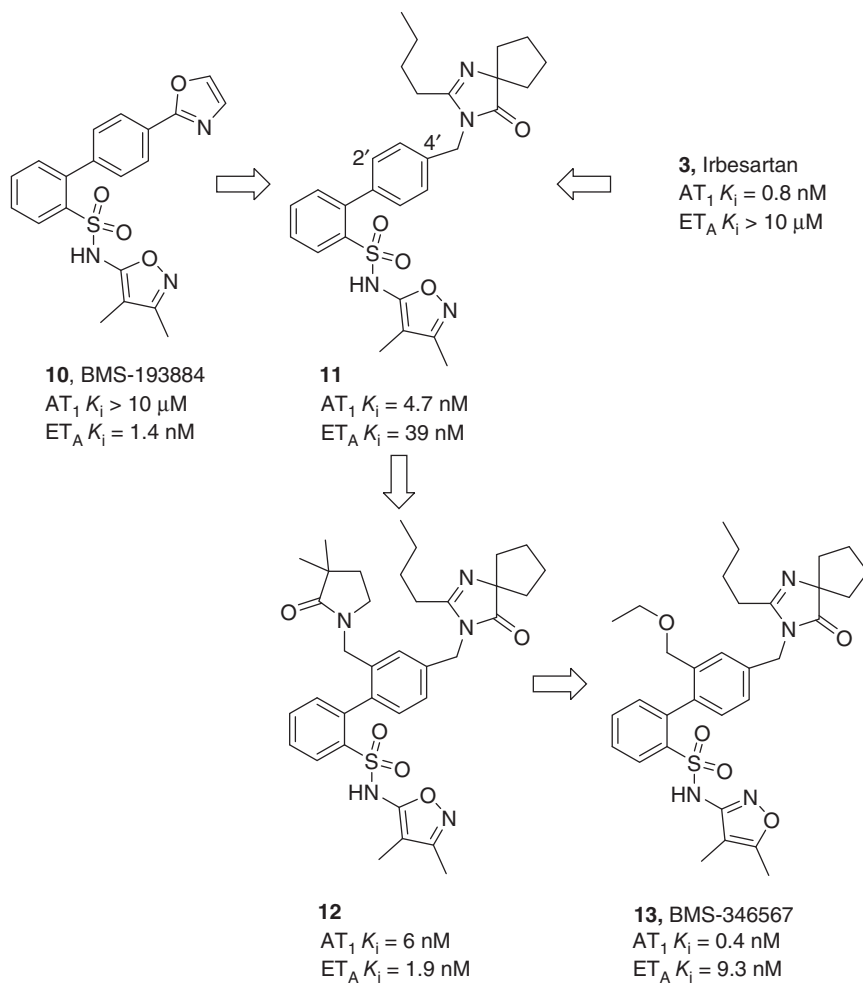
The endothelins (ET-1, ET-2, ET-3) is a family of peptides first isolated from endothelial cells that mediate their actions by interaction with two specific GPCRs (ET_A and ET_B) [9]. ET-1 is the predominant isoform produced by the vascular endothelium and controls cardiovascular and respiratory functions via ET_A receptors. Both ET-1 and AII are known to be involved in the pathogenesis of hypertension and cardiovascular disease [10], and thus, simultaneous blocking of the actions of these peptides has been pursued as an approach to target hypertension. Studies in animal models have demonstrated that combined treatment with an ARB and ET blocker produced additive antihypertensive effects [11]. It has been also recognized that there are structural and functional similarities between the AII and ET receptors, and this has provided the opportunity to target both receptors with a single molecule.

The identification of the first dual AT/ET receptor blockers was reported by Walsh *et al.* (Scheme 13.1) [12]. A search from the Merck AII antagonist collection afforded compounds that also displayed binding to ET receptors. From a class of α -phenoxyphenylacetic acids like **7**, previously identified with potent mixed AT₁/AT₂ activity [13], the lead **8** was identified with modest binding to both ET receptors. Optimization of this lead was pursued through a number of modifications, including (i) simplification of the dibromophenyl ring, (ii) replacement of the carboxylic acid with an acylsulfonamide to retain an acidic functionality with reduced rate of *O*-glucuronidation, and (iii) monoalkyl substitution at the central phenyl ring to eliminate unproductive interactions between the alkyl groups and the acylsulfonamide. From these efforts, acylsulfonamide **9** emerged as a compound with potent AT₁ and AT₂ activity that also exhibited good activity at both ET receptors. These results confirmed the possibility to obtain the desired dual pharmacology in a single molecule.



Scheme 13.1 Design of dual AT₁ receptor antagonist/ET receptor blocker **9**.

The discovery of the first selective dual AT₁/ET_A receptor blockers, also referred to as dual-action receptor antagonists (DARAs), was reported by Murugesan *et al.* (Scheme 13.2) [14]. The structural features required for ET_A antagonism were derived from **10**, a potent ET_A antagonist previously identified by this group [15], and were combined with the scaffold of the potent ARB irbesartan (**3**; Figure 13.2) to give the novel biphenylsulfonamide **11**. This compound exhibited dual activity at AT₁ and ET_A receptors and confirmed that the isoxazole sulfonamide is an adequate replacement for the tetrazole group of **3** to retain potent AT₁ activity. Substitution at C2' position of the biphenyl scaffold was utilized for optimizing ET_A receptor affinity, an approach based on previous SAR from the ET_A receptor antagonist series. In this way, **12** was identified as a potent dual AT₁ and ET_A receptor blocker possessing pharmacokinetic (PK) properties in rat suitable for *in vivo* evaluation. Oral dosing of **12** to normotensive rats demonstrated its unique ability to block the vasopressive effects of both AII and big ET-1 (induced pressor model), while neither **10** nor **11** alone could block the effects of both of these two agents.



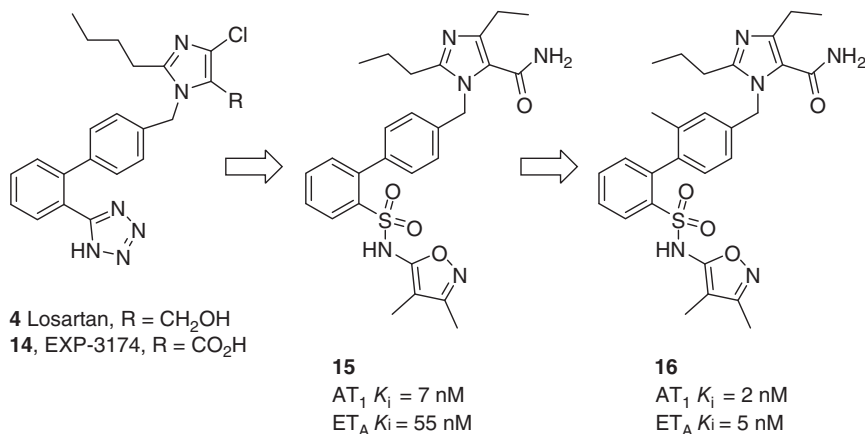
Scheme 13.2 Design of dual AT_1/ET_A receptor blockers 11–13.

Second-generation DARAs were pursued to improve potency and PK properties of **12** [16]. It was identified that although **12** exhibited moderate bioavailability in rat ($\%F = 38$), its bioavailability in higher species was lower ($\%F < 10\%$). In addition, potency for both receptors was below the potency observed for reference compounds (e.g., **3** and **10**), providing room for optimization. The medicinal chemistry strategy to improve **12** was focused at removing potential sites of metabolism, lowering molecular weight, and reducing the number of H-bond donor/acceptor, as improving these two properties is well known to increase the oral bioavailability of drugs. Modification of the C2' group was used to improve potency. These efforts led to the identification of BMS-346567 (**13**) that was characterized as a potent DARA, with improved PK profile over **12**. It was also more efficacious and longer acting than **3**, **10**, and **12** in the induced pressor model. Further evaluation of **13** was carried out in the spontaneously

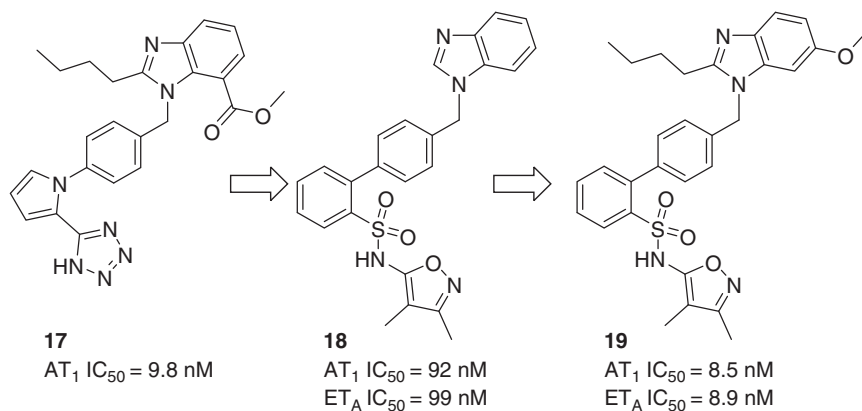
hypertensive rat (SHR) model in which **13** clearly demonstrated superiority over **3** by essentially transforming the SHR into normotensive rats. **13** (also known as PS433540) was advanced to clinical trials, and results from phase IIb studies were reported in 2009 [17, 18]. In these trials, the high dose of **13** (800 mg) produced a statistically significantly greater reduction in BP than treatment only with the ARB **3**. The compound was found to be generally well tolerated with no serious adverse effects, with edema being the most frequent at high dose. This agent, now called sparsentan, is currently in phase III clinical trials.

In a continued effort to identify additional DARAs possessing oral bioavailability, a second series of compounds was designed with the main focus on decreasing molecular weight as an approach to improve PK properties [19]. The key structural features required for the dual activity were incorporated in a design that departed from the ARB losartan (**4**) or its active metabolite, EXP-374 (**14**) (Scheme 13.3). **4** was selected because its C4' imidazolylmethyl moiety was identified as one of the smallest high-potency AT₁ active groups. Implementation of this strategy led to **15** that exhibited the desired dual activity. Incorporation of a C2' substituent was then employed to improve ET_A activity. A small methyl group was sufficient to increase both ET_A and AT₁ potency as observed in **16**. *In vivo* evaluation of **16** in the AII-induced pressor model demonstrated efficacy that was comparable with **12**, but without reaching the efficacy level obtained with the ARB **3** alone. Relatively poor absorption of **16** was suggested as a potential reason for the limited *in vivo* efficacy.

There are other ARB scaffolds that have been utilized as the starting point to design new dual AT₁/ET_A receptor blockers. Bai *et al.* modified ARB **17**, previously reported by Xu *et al.* [20], to incorporate a phenyl isoxazolyisulfonamide leading to a new series of DARAs (Scheme 13.4) [21]. Initial compounds in this series like **18** confirmed the design strategy to obtain the desired dual activity. Optimization for binding at both receptors was achieved by substitution on the C4' benzimidazole ring, leading to their best compound, **19**. The *n*-butyl tail attached to the benzimidazole is likely to contribute to AT₁ potency through



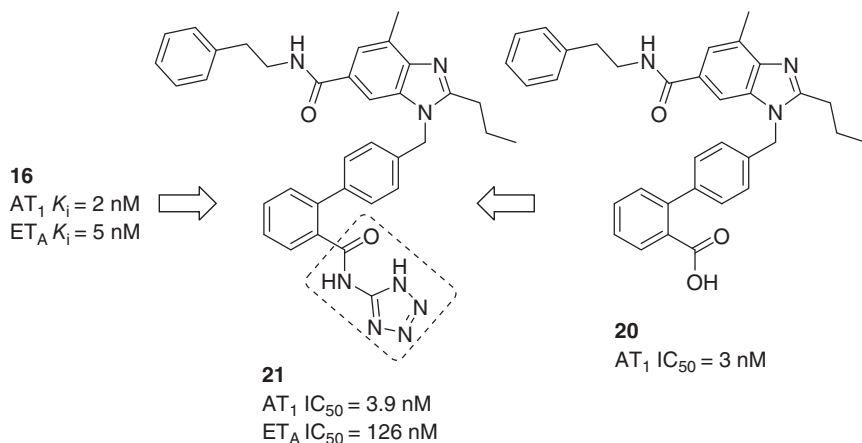
Scheme 13.3 Design of dual AT₁/ET_A receptor blockers **15** and **16**.



Scheme 13.4 Design of dual AT₁/ET_A receptor blockers **18** and **19**.

interactions with the narrow hydrophobic pocket in the AT₁ receptor as was discussed in Section 13.1. *In vivo* evaluation in renal antihypertensive rats (RHRs) demonstrated that **19** delivered efficacious reduction of BP and did not cause changes in heart rate.

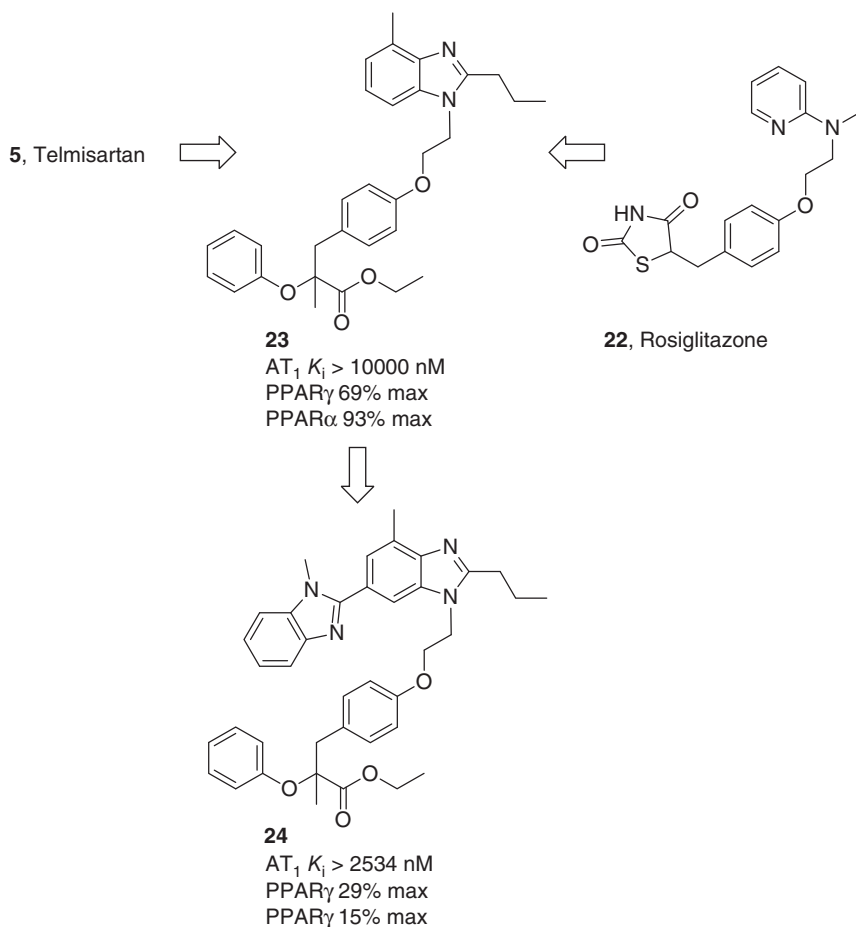
Departing from a series of 6-substituted aminocarbonyl benzimidazoles with established AT₁ activity previously reported by Xue *et al.* [22], a hybrid approach was pursued by Han *et al.* (Scheme 13.5) [23]. The scaffold was modified with the incorporation of *N*-(1*H*-tetrazol-5-yl)-amide as an isostere of *N*-(3,4-dimethyl-5-isoxazolyl)sulfonamide which is the typical acidic moiety found in most DARAs leading to novel agents with dual activity. Among the more promising compounds from this work, **21** was identified with optimal balance of AT₁ and ET_A activity. Similar to compounds described previously, evaluation in SHR_s demonstrated that **21** delivered robust reduction of BP that lasted longer than the reference ARB **3** and heart rate was also effectively controlled.



Scheme 13.5 Design of dual AT₁/ET_A receptor blocker **21**.

13.3 Design of Dual AT₁ Receptor Antagonist/PPAR γ Partial Agonists

Telmisartan (**5**, Figure 13.2) is a unique ARB that, in addition to its potent selective AT₁ receptor antagonism, has been reported to have partial activity at the nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR γ) [24, 25], an established target for thiazolidinedione drugs like pioglitazone and rosiglitazone (**22**; Scheme 13.6) [26], a class of antidiabetic agents. The use of **5** is not associated with the adverse effects observed with the utilization of full PPAR γ agonists like body weight (BW) gain [27]. These observations have led to the search of agents possessing the dual pharmacology of AT₁ receptor antagonism and partial PPAR γ agonism as they could potentially treat several recognized cardiovascular risk factors including hypertension, insulin resistance, and hypertriglyceridemia. However, this hypothesis may need review based on data from large clinical trials with **5**. Results from these studies suggested that this

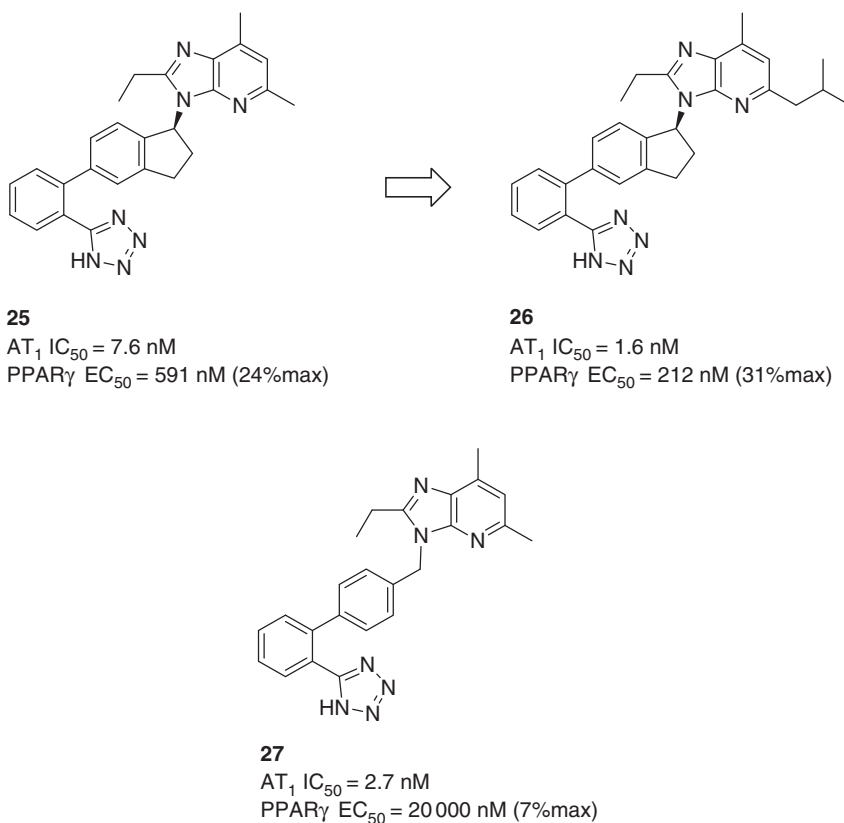


Scheme 13.6 Design of dual AT₁ antagonist/partial PPAR γ agonist **24**.

ARB may have only modest effects, at best, at reducing the risk of cardiovascular events or new-onset diabetes [28, 29].

The structural resemblance between **5** and the full PPAR γ agonist **22** when superimposed inside the PPAR γ ligand-binding domain (LBD) led Mizuno *et al.* to design new benzimidazole analogs as dual AT $_1$ receptor antagonist/PPAR γ partial agonists (Scheme 13.6) [30]. A structure-based design approach was pursued for the design of the new compounds using an AT $_1$ receptor homology model and the crystal structure of **22** bound to PPAR γ LBD. The need for the *N*-methylbenzimidazole moiety of **5** to retain AT $_1$ activity was suggested by **23**, a compound that exhibited PPAR γ activity, but was devoid of AT $_1$ activity. Incorporation of the aforementioned functionality led to **24**, which was one of the most potent AT $_1$ antagonists from this study that also exhibited PPAR γ activity. Interestingly, this series also exhibited moderate activity at PPAR α (15% activation at 10 μ M) that could be derived from the fibrate-like moiety (2-methyl-2-phenoxypropanoate ethyl ester) incorporated in these compounds. Removal of the ester in **24** to give the corresponding carboxylic acid maintained the AT $_1$ activity, but this acid was completely devoid of PPAR γ activity.

A different strategy for the identification of dual AT $_1$ antagonist/partial PPAR γ agonists was reported by Casimiro-Garcia *et al.* (Scheme 13.7) [31]. A search

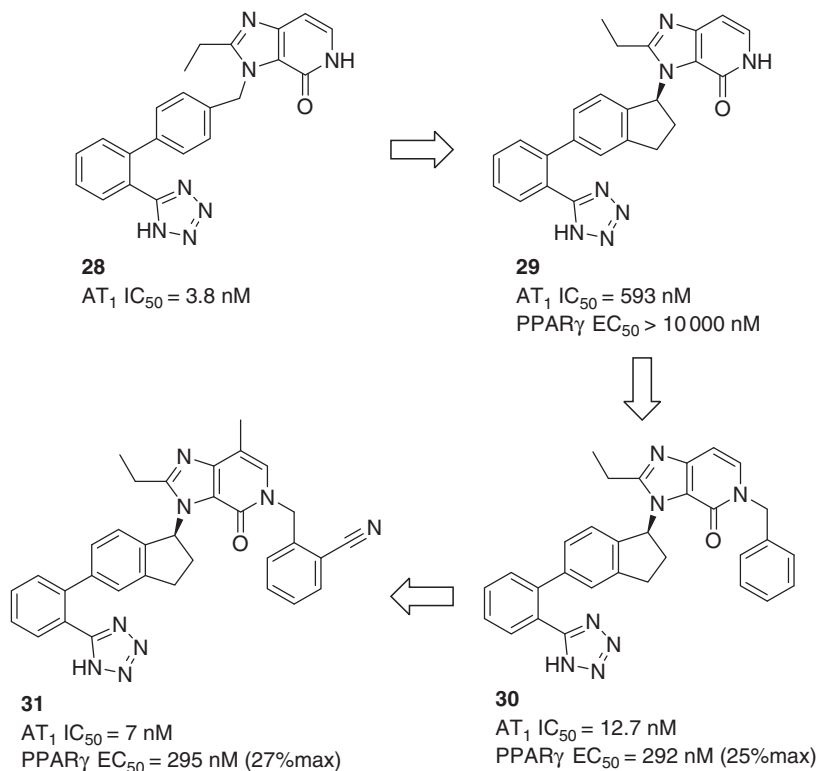


Scheme 13.7 Dual AT $_1$ antagonist/partial PPAR γ agonists **25** and **26**.

of Pfizer proprietary collection of compounds and data from previous ARB and PPAR_γ programs in combination with a cross-screening approach led to the identification of **25** possessing robust AT₁ activity and partial activation of PPAR_γ. **25** was derived from a previous ARB program, and the SAR associated with AT₁ binding of this class of compounds was well understood [32]. A crystal structure of **25** in complex with PPAR_γ LBD guided efforts to improve interactions with this receptor. The presence of the indane ring was determined to be important for the PPAR_γ agonist activity, as demonstrated with **27** that retained potent AT₁ activity, but was devoid of PPAR_γ activity. Thus, conformational restriction derived from the indane ring was a key design element required for PPAR_γ activity. Optimization efforts led to **26** that was characterized as a potent AT₁ receptor blocker with partial PPAR_γ agonism, highly selective over related receptors (AT₂, PPAR_α, and PPAR_β), and with oral bioavailability in rat. The dual pharmacology of **26** was demonstrated using two animal models. In the SHR, **26** produced BP lowering for a full 24 h, an effect comparable with that observed with **5**. In the male Zucker diabetic fatty (ZDF) rat model of insulin resistance, treatment with **26** led to glycemic improvements equivalent to that of the full PPAR_γ agonist pioglitazone, but with a significantly reduced incidence of BW gain. Even more interestingly, plasma concentrations achieving efficacy in the SHR and ZDF models were within threefold that demonstrated the potential utility of this compound to treat multiple cardiovascular risk factors like hypertension and insulin resistance at a single dose level.

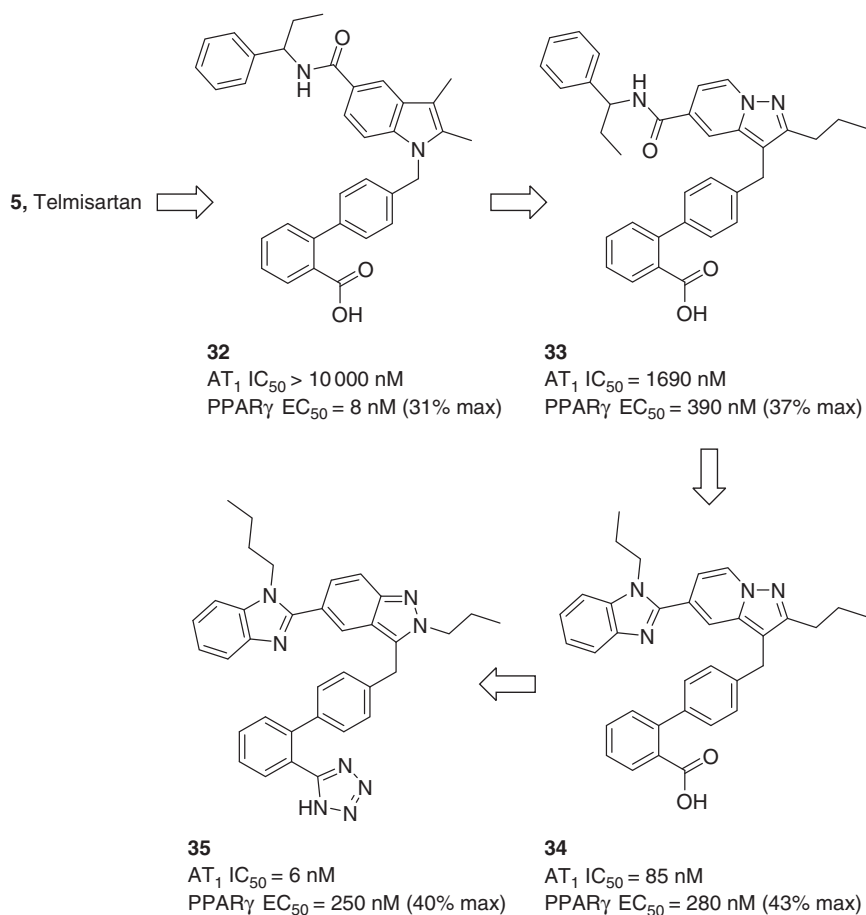
A complementary series of dual AT₁ blocker/partial PPAR_γ agonist was designed by Casimiro-Garcia *et al.* (Scheme 13.8) [33]. As discussed earlier, conformational restriction derived from the indane ring was identified as a critical design component required for PPAR_γ activity. In addition, substitution at C5 of the imidazopyridine was identified as a preferred position to modulate activity at both receptors. From a review of ARBs [34], scaffolds suitable to display the design components were identified. A series of imidazopyridines previously reported as potent AT₁ receptor blockers was selected as the starting point [35]. Modification of the unsubstituted imidazopyridone **28**, previously reported as a potent AT₁ receptor blocker [35], to incorporate the indane ring in **29**, led to significant loss of AT₁ activity and no PPAR_γ activity. Interestingly, addition of a single benzyl group at the pyridone NH drastically turned this compound into **30**, a potent dual AT₁ receptor antagonist with partial PPAR_γ activity. The design of optimized compounds was based on three approaches. First, structure-based optimization of PPAR_γ activity was pursued after obtaining a crystal structure of **30** bound to PPAR_γ LBD. Second, modifications of the benzyl ring were utilized to better understand structural requirements for both AT₁ and PPAR_γ activity. Third, modulation of physicochemical properties was used to identify compounds with a good balance between potency and ADME properties. These efforts provided imidazopyridone **31** that was identified combining potent activity at both receptors and good ADME and physicochemical properties.

Modification of the scaffold of the ARB **5** was utilized by Lamotte *et al.* for the identification of the potent PPAR_γ agonist **32**. These alterations unfortunately also led to losing AT₁ activity [36]. The efforts to restore AT₁ antagonism, while maintaining PPAR_γ activity, led to the identification of potent dual AT₁ antagonist/partial PPAR_γ agonists (Scheme 13.9) [37]. The approach to improve AT₁



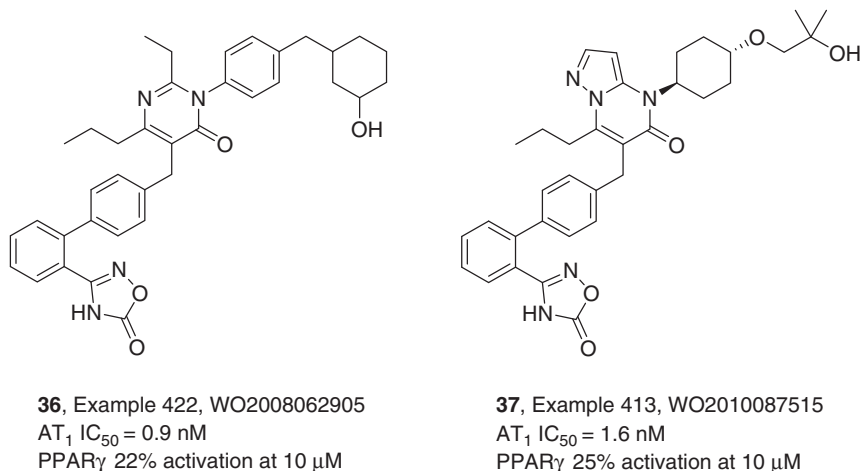
Scheme 13.8 Design of dual AT₁ antagonist/partial PPAR_γ agonists **30** and **31**.

activity was based on SAR knowledge from previous ARBs. A nitrogen at position 3 of the C4' heteroaryl group serving as H-bond acceptor was emphasized as a key component of the AT₁ receptor pharmacophore. Interestingly, this hypothesis is now supported with the recent structural understanding of AT₁ receptor ligand binding. As discussed in Section 13.1, hydrogen bonding between Tyr35^{1.39} and the nitrogen from the C4' heteroaryl moiety represents one of the key interactions with ARBs, and mutation of this residue led to complete loss of binding capability. New compounds were designed incorporating heteroaryl groups possessing this functionality. Pyrazolopyridine **33** showed a significant gain in AT₁ activity while keeping PPAR_γ partial agonism and validated this approach. Further modification of the central scaffold to a 2*H*-indazole and of the left-hand side of the compound provided **34** with a more balanced dual profile. Interestingly, replacement of the carboxylic acid with a tetrazole was utilized to further improve AT₁ activity. In this way, indazole **35** was identified possessing potent dual activity, selectivity versus other PPAR isoforms, and good PK properties. Evaluation of **35** in the SHR model demonstrated efficacy for BP lowering. In the Zucker fa/fa rat model, **35** demonstrated glycemic improvements equivalent to those observed with a full PPAR_γ agonist, but with no BW gain, representing a highly interesting result from this study.



Scheme 13.9 Design of dual AT₁ antagonist/partial PPAR_γ agonists **33–35**.

Researchers at Takeda Pharmaceutical Company have disclosed potent AT₁ antagonists possessing partial PPAR_γ activity in several patent applications [38–40]. Selected examples, namely, **36** and **37**, are shown in Scheme 13.10. Details of the design of these compounds have not been reported in the peer-reviewed literature. The incorporation of an alcohol connected to the C4' heterocycle required for AT₁ activity using a spacer of different size is a structural element found in several of the disclosed compounds. This functionality is likely relevant for PPAR_γ activity and possibly was designed to engage in hydrogen bonding with residues in the “charge clamp” of this receptor, like His323, Tyr327, His449, and Tyr473. This region in PPAR_γ is engaged by full agonists to build a hydrogen bonding network important for binding to this receptor [41, 42]. *In vivo* data for **37** and a few additional compounds was described [40]. Evaluation of **37** in SHR demonstrated antihypertensive effects, while testing in the Wistar fatty rat showed dose-dependent inhibition of plasma triglycerides and rate of plasma glucose, confirming the expected dual pharmacology.

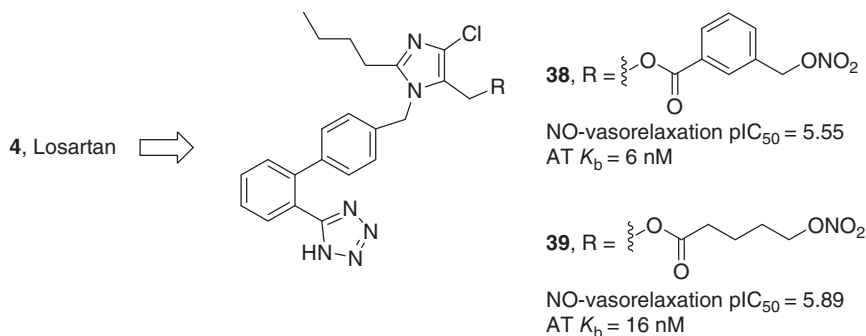


Scheme 13.10 Dual AT_1 receptor blocker/partial $PPAR_\gamma$ agonists **36** and **37**.

13.4 Design of Dual AT_1 Receptor Blocker/NO-Releasing Agents

The chemistry and biology of nitric oxide (NO), a small signaling molecule derived from the amino acid L-arginine by NO synthase, is complex [43]. It is a molecule with very short half life and generated in response to a variety of stimuli by different cell types. In the endothelium, stimulation by the biologically active peptide bradykinin causes NO release and leads to a number of biological effects, including vascular smooth muscle relaxation through activation of guanylate cyclase and the production of cyclic guanosine monophosphate (cGMP) as the second messenger. This results in the activation of a variety of cGMP-dependent protein kinases that mediate downstream actions. The physiological levels of endogenous NO mediate multiple processes in the cardiovascular system, and dysregulation of the NO system is associated with cardiovascular disease [44].

The design of dual AT_1 receptor blockers/NO-releasing agents has been pursued to enhance the antihypertensive effects of the ARBs. Breschi *et al.* reported the design of the first dual-acting molecules **38** and **39** that departed from the ARB **4** (Scheme 13.11) [45]. **4** was selected based on its high AT_1 activity and structural features suitable for introduction of a NO donor moiety (i.e., an alcohol group). The NO-releasing groups were selected to evaluate how different linkers change stability toward ester bond cleavage and impact NO release. The vasorelaxing effects of **38** and **39** were demonstrated in vascular tissue assays with KCl or AII stimulation. The effect with KCl was strongly inhibited by an inhibitor of guanylate cyclase and thus confirmed that the effect was derived from the release of NO. The NO-releasing activity from **38** did not require previous hydrolytic removal of the side chain, as no change in NO release was observed when an esterase inhibitor was included in the assay. However, the AT_1 antagonist potency of **38** was lowered (K_b = 40 nM) in the presence of the esterase inhibitor, demonstrating that AT_1 antagonism is derived from **4** itself, after hydrolytic removal

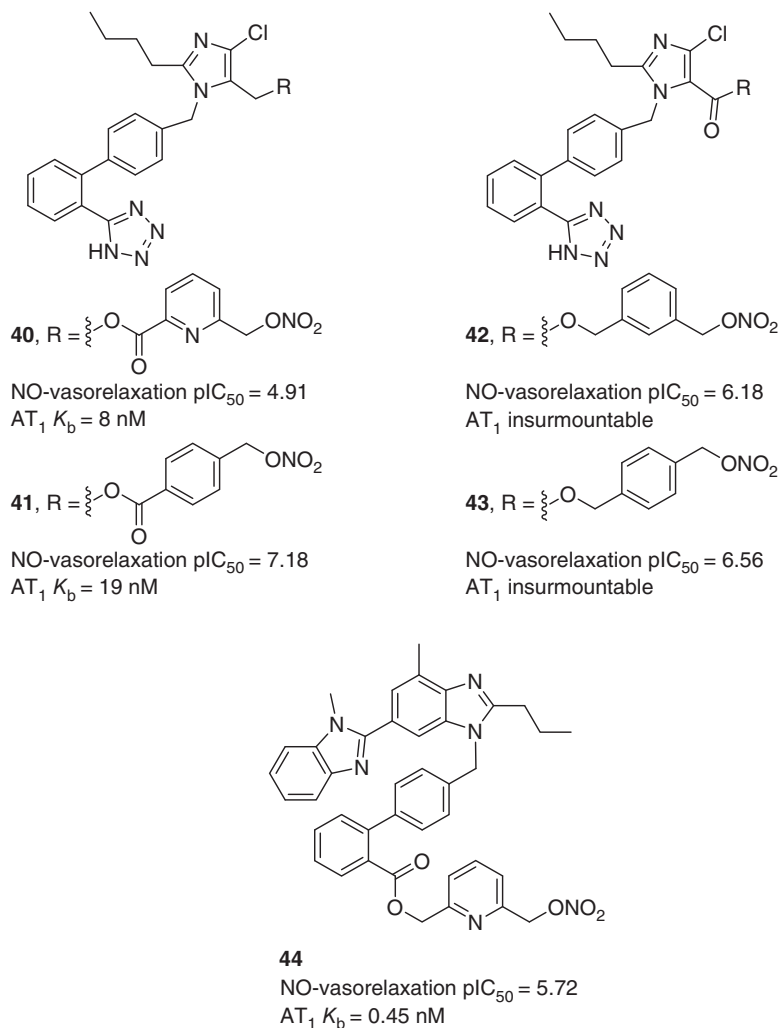


Scheme 13.11 Design of dual AT₁ receptor blocker/NO-releasing agents **38** and **39**.

of the side chain. *In vivo* evaluation of **38** in SHR caused a significant reduction in BP that was comparable with the effect obtained with **4**. Evaluation of **38** in an experimental model of cardiac ischemia–reperfusion demonstrated improved cardioprotective effects when compared with **4** [46].

In an effort to further investigate the role of the linker in the modulation of NO release, Breschi *et al.* reported a series of additional analogs derived from either **4** or its active metabolite, **14** [46]. Selected examples are included in Scheme 13.12. Replacing the phenyl ring of the NO-releasing moiety in **38** with a pyridine afforded **40** that displayed reduced vasorelaxing activity but retained potent AT₁ antagonist activity equal to **38**, suggesting rapid hydrolytic cleavage of the side chain. Moving the nitrooxymethyl chain from the *meta* position to the *para* position gave **41**, which was the compound with the highest vasorelaxing effect in the series. This change led to a small drop in AT₁ antagonism as compared with **38**, suggesting a slower release of the free **4** moiety. The benzylic esters **42** and **43** retained potent vasorelaxing effects, but did not show AT₁ activity under the standard incubation period of the assay (20 min). Upon longer incubation (60 min), both compounds exhibited insurmountable AT₁ activity, typical of nonreversible receptor antagonists. This data suggested that the inverse benzylic ester slowed down the hydrolytic cleavage to provide free **14**.

Li *et al.* reported the design and characterization of the NO-releasing agent WB1106 (**44**; Scheme 13.12), a derivative of telmisartan (**5**; Figure 13.2) [47]. In this approach, and in contrast with the strategy described by Breschi *et al.*, the benzoic acid group of the ARB was used to introduce the NO-releasing moiety as an ester derivative. Evaluation of **44** using vascular tissue assays confirmed that this compound acts as a dual AT₁ receptor antagonist and NO-releasing agent. **44** was also characterized as a telmisartan prodrug, and its AT₁ antagonist activity was dependent on metabolic hydrolysis. *In vivo* evaluation of **44** in SHR demonstrated antihypertensive effects comparable with those of **5**. Interestingly, measurement of cGMP levels in the aorta of rats treated with **44** showed a significant increase of this mediator as compared with those of vehicle-treated animals. This data supported the generation of NO from **44**, leading to cGMP formation and vasorelaxation. Ester **44** was also evaluated in rats fed a high-fat and carbohydrate diet. The results from this study, like reduction of BW and



Scheme 13.12 Dual AT_1 receptor blocker/NO-releasing agents **40–44**.

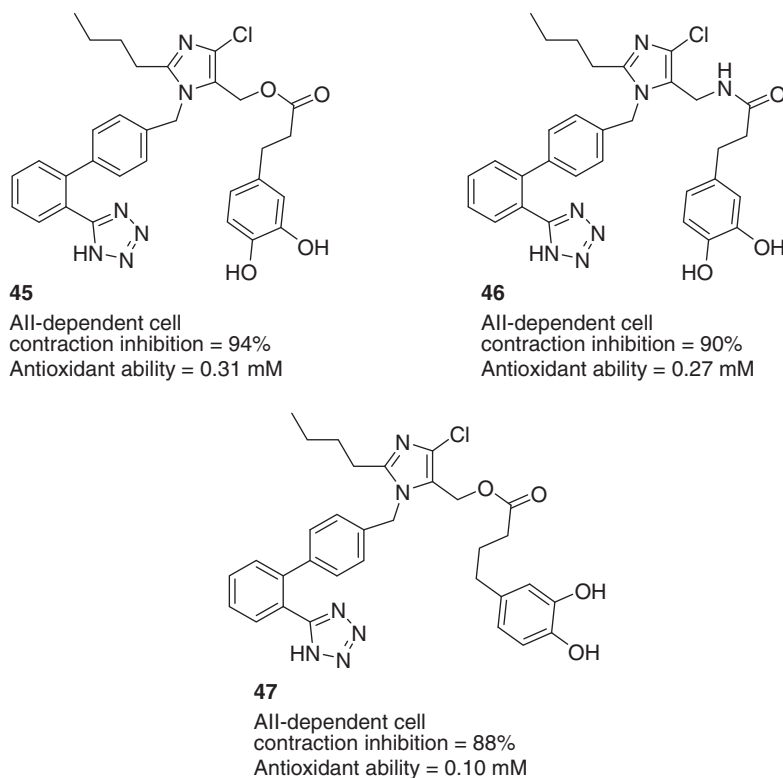
blood glucose, were ascribed to the synergistic effects of the ARB moiety and NO-releasing group, although the known $\text{PPAR}\gamma$ activity derived from the ARB **5** scaffold likely contributed to this outcome.

13.5 Design of Dual AT_1 Receptor Blocker/Antioxidant Activity Agents

The oxidative stress that takes place at the vascular level represents a well-known mechanism that is responsible for the development of vascular damage and contributes to the progression of cardiovascular disease [48]. The localized formation of reactive oxygen species induces significant endothelial dysfunction and alters

the normal balance in the heart and arterial walls. Studies in animals and in patients with cardiovascular disease have shown that a number of drugs in clinical use as well as other compounds in different stages of development effectively prevent oxidative stress and presumably may prevent vascular disease; however verification of their therapeutic potential for cardiovascular diseases needs to be demonstrated [49]. Interestingly, a recent small clinical trial showed that irbesartan (**3**; Figure 13.2) appears to have beneficial effects on oxidative stress [50]. Incorporation of an antioxidant moiety into an ARB has been postulated to augment the potential benefits of this class of agents based on the rationale that this would provide a more specific approach to target antihypertensive or antioxidant molecules to cardiovascular cells.

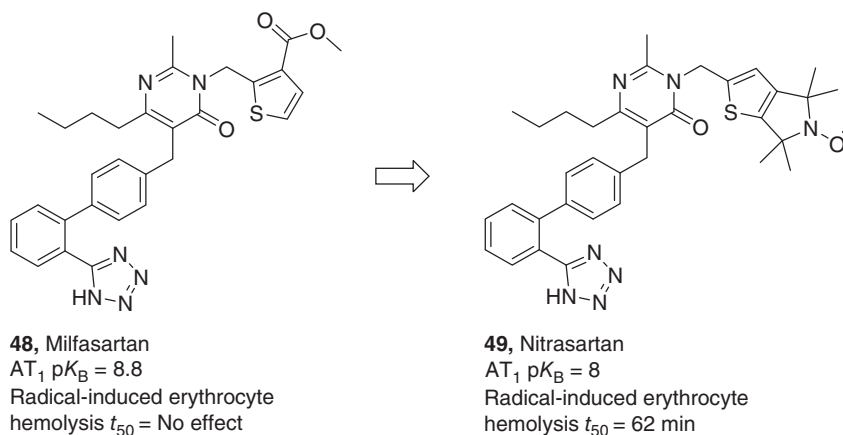
Dual agents possessing AT₁ antagonism and antioxidant activity have been reported by Garcia *et al.* [51, 52]. The design of these compounds departed from **4** and followed a similar strategy as discussed for other dual-acting agents. The alcohol functionality of **4** was utilized to attach a catechol moiety, which was selected based on their well-known redox properties. Among the synthesized compounds, phenols **45–47** (Scheme 13.13) demonstrated enhanced antioxidant ability than **4** that displayed minimal effects (antioxidant ability = 0.03 mM), while all these phenols exhibited AT₁ antagonist activity that was comparable with **4** (AII-dependent cell contraction inhibition = 93%). *In vivo* evaluation of



Scheme 13.13 Dual AT₁ receptor blocker/antioxidant agents **45–47**.

45 using a rat model of hypertension not only decreased systolic BP but also led to reaching control values, while treatment with **4** alone did not reach normalization [51]. Ester **45** was also able to normalize the changes induced in vascular walls, as determined by measuring the levels of extracellular matrix proteins and the local protein oxidative damage, outcomes that were not achieved with **4**. The authors suggest that these results imply that treatment with this type of dual-acting agents may be more efficacious to prevent the cardiovascular damage induced by hypertension than treatment with an ARB alone. The ester bond in **45** was found to exhibit acceptable metabolic stability, leading to relatively high plasma concentration (171 nM) of the intact compound in rat plasma. The high compound level has been suggested to explain the observed antioxidant effect.

Additional compounds possessing dual AT₁ antagonism and antioxidant activity have been reported by Jani *et al.* [53]. In this design, modification of the thiophene ring of milfasartan (**48**) [54], an ARB that reached phase I clinical trials [55], was utilized to incorporate moieties with antioxidant activity (Scheme 13.14). These modifications included sulfur/selenium exchange, thiophene to benzothiophene, or substituted benzothiophene, and thiophene to the more elaborated ring in nitrasartan (**49**) containing a nitroxide group. The prevention of radical-induced erythrocyte hemolysis (time to 50% lysis, t_{50}) was utilized in combination with other assays to differentiate compounds for antioxidant activity, while a vascular tissue assay was used to determine AT₁ activity. The results of these assays demonstrated that **49** possessed AT₁ antagonism comparable with the unmodified **48**, but differentiated from this compound and other analogs by its antioxidant capacity. Further evaluation of **49** confirmed both its AT₁ and antioxidant activities; in particular this compound was able to decrease NADPH-mediated superoxide production in rat aorta, while no effect was observed with **48**. The results from these assays indicated that AT₁ antagonist activity occurs at much lower concentrations (~3–10 nM) than antioxidant activity (1–10 μ M). The authors suggested that matching the potency for these activities may not be as important as ensuring that nitroxide gets released to the key sites of free radical generation.



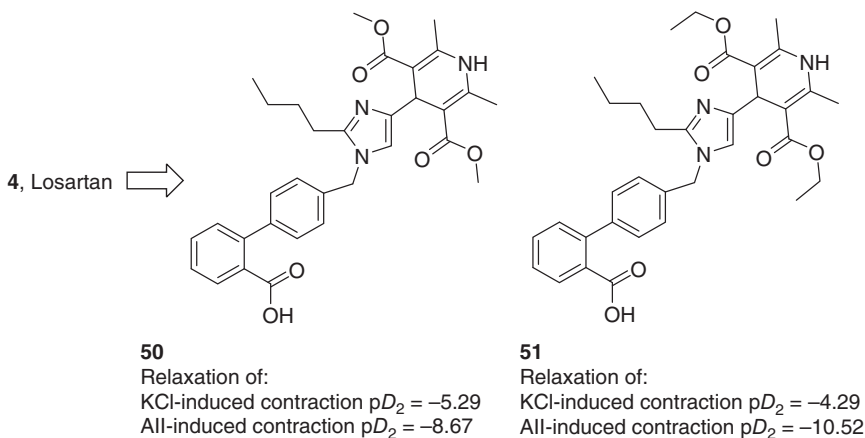
Scheme 13.14 Design of dual AT₁ receptor blocker/antioxidant agent **49**.

13.6 Design of AT₁ Receptor Antagonists with Additional Activity in Other Pathways

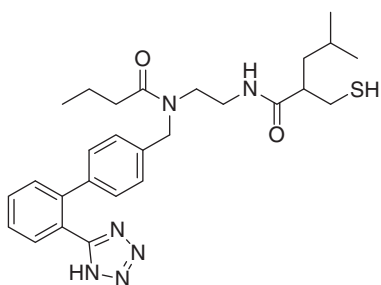
Additional compounds possessing AT₁ antagonism along with activity in other pathways have been reported. Due to the limited number of these reports, they are collectively reviewed in this section.

Modification of the imidazole moiety of **4** to introduce a dihydropyridine group and replacement of the tetrazole ring with a carboxylic acid was utilized in the design of the dual AT₁ antagonist/L-type calcium channel blockers **50** and **51** (Scheme 13.15) [56]. The vasorelaxing effect of these compounds was demonstrated in vascular tissue assays using KCl or AII stimulation. **50** showed an effect comparable with that of the well-known L-type calcium channel blocker nifedipine ($pD_2 = -5.58$) with KCl stimulation, while both **50** and **51** were reported to have higher potency than **4** ($pD_2 = -5.66$) when AII was used as the stimulant. The enhanced vasorelaxant effect observed with AII stimulation was attributed to blocking both AT₁ receptor and L-type calcium channel.

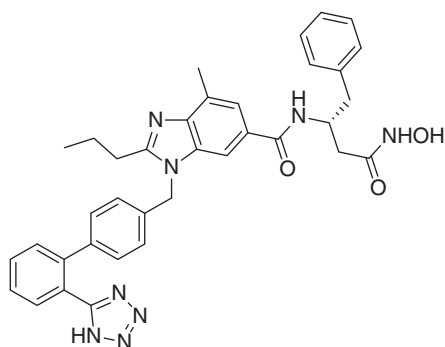
Neutral endopeptidase (neprilysin or NEP) is a zinc metallopeptidase that metabolizes a number of vasoactive peptides with important roles in BP homeostasis, including the vasorelaxant peptide bradykinin and the atrial natriuretic peptide that lowers BP by increasing diuresis [57]. It has been shown that pharmacological inhibition of NEP increases the actions of the natriuretic peptide, resulting in BP reduction. Dual blockade of the AT₁ receptor and NEP has been proposed as an approach to achieve superior antihypertensive efficacy than with ARB therapy alone, but without the serious adverse effects (angioedema) observed with vasoepitidase inhibitors like omapatrilat that target inhibition of NEP and ACE. The concept has been already demonstrated in animal models of hypertension in which the combination of an ARB and a NEP inhibitor resulted in comparable effects as those observed with a vasoepitidase inhibitor [58]. Furthermore, Novartis has recently gained FDA approval of the drug Entresto (previously known as LCZ-696), a 1:1 molar complex of the ARB **6**, and the NEP inhibitor sacubitril [59] for the treatment of heart failure [60].



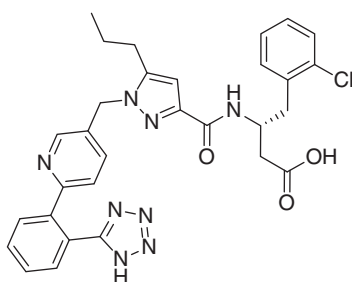
Scheme 13.15 Design of dual AT₁ receptor antagonist/calcium channel blockers **50** and **51**.



52, Example 1, US20080188533

AT₁ pK_i > 7NEP pIC₅₀ ≥ 7

53, Example 2, WO20090149521

AT₁ pK_i ≥ 5NEP pIC₅₀ ≥ 5

54, Example 10, WO2011005674

AT₁ pK_i ≥ 8NEP pIC₅₀ ≥ 7**Scheme 13.16** Dual AT₁ receptor blocker/NEP inhibitors **52–54**.

Researchers at Theravance Inc. have reported diverse series of dual AT₁ receptor blocker/neprilysin inhibitor in several patent applications [61–69]. Selected examples of disclosed compounds are provided in Scheme 13.16. A discussion of the design of these dual-acting compounds has not yet been reported in the peer-reviewed literature. In general, these compounds are characterized by the presence of an aryl acid group and a zinc-chelating moiety like a thiol or hydroxamic acid moiety as observed in **52** and **53**, likely introduced to obtain NEP activity. In the most recent patent applications, a large set of the exemplified compounds incorporate a 1,2-disubstituted butanoic acid that appears to appropriately function as a replacement for the thiol or hydroxamic acid moiety, as suggested by the robust dual activity disclosed for compound **54**.

13.7 Summary

After decades of investigation, angiotensin and its receptors and ligands continue to be the focus of intense research across academia and industry. This

is highlighted with the recent report of the first crystal structure of the AT₁ receptor in complex with an ARB [7] and the recent FDA drug approval of azilsartan medoxomil that represents the newest addition to this family of therapeutic agents [70] and Entresto [60]. The ability to modulate the AT₁ receptor in combination with other pathways using a single ligand was reviewed in the previous sections. These dual-acting agents retain the key pharmacophore required for AT₁ binding: generally a biphenyl group, an acidic moiety, a heteroaryl ring attached to the biphenyl scaffold, and a short alkyl tail. The role of each one of these components was discussed using structural data. The capability of other protein families of therapeutic interest to accommodate this pharmacophore is noteworthy. These include members from other GPCRs (ET_A and ET_B), nuclear hormone receptors (PPAR γ), ion channels (L-type calcium channel), and metalloproteases (NEP). As research in this field continues to evolve, more protein families are likely to demonstrate the ability to bind to the AT₁ pharmacophore, leading to the identification of new dual-acting agents. For most of the ligands discussed in this chapter, the dual activity profile was primarily demonstrated in preclinical studies, including the use of *in vitro* assays and, for selected examples, *in vivo* animal models. The ability to achieve efficacy at a single dose in multiple animal models required to characterize the dual activity was demonstrated with examples from the dual AT₁ antagonists/partial PPAR γ agonists. Characterization of the dual activity profile in human clinical trials has been limited to only an example from the dual AT₁/ET_A receptor blocker category (currently in phase III clinical trials). Interestingly, this agent demonstrated statistically significant greater reduction in BP than treatment with the ARB alone [17, 18]. Taken together, these results are encouraging to continue the search for novel agents possessing dual activity at the AT₁ receptor in combination with other pathways of medical relevance.

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14

Multiple Peroxisome Proliferator-Activated Receptor-Based Ligands

Dmytro Kukhtar¹, Miquel Mulero¹, Raul Beltrán-Debón¹, Cristina Valls¹, Gerard Pujadas^{1,2}, and Santiago Garcia-Vallve¹

¹ Universitat Rovira i Virgili (URV), Facultat de Química, Departament de Bioquímica i Biotecnologia, Cheminformatics and Nutrition Group, c/Marcel·lí Domingo, 1, 43007, Tarragona, Catalonia, Spain

² EURECAT, TECNIO, Technological Center of Nutrition and Health (CTNS), Avinguda Universitat, 1, 43204, Reus, Catalonia, Spain

14.1 Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that regulate glucose and lipid metabolism [1]. PPARs form obligate heterodimers with the retinoid X receptor (RXR), and, upon binding a ligand, the PPAR/RXR heterodimer binds to specific response elements located in the 5'-flanking region of target genes, thereby acting as a transcriptional regulator. Similar to the other members of the nuclear receptor superfamily, the conformation of the receptor changes upon binding to a ligand, forming high-affinity interactions with coactivators that remodel chromatin and activate the transcription of the target genes [2].

PPARs were first identified as receptors that were activated by several ligands that caused the proliferation of peroxisomes [3, 4]. PPARs share the organization of the nuclear receptor superfamily [5] that consists of several functional domains: (i) a domain located at the N-terminal end called A/B domain that is responsible for the transcriptional activation function (AF-1), (ii) a highly conserved DNA-binding domain (DBD) that contains two zinc-finger motifs that are responsible for DNA binding, and (iii) a ligand-binding domain (LBD) implicated in receptor heterodimerization, ligand binding, the interaction with coactivators and corepressors, and also the ligand-dependent transcriptional AF (AF-2). The PPAR LBD shares a common fold with the nuclear receptor superfamily that consists of 13 α -helices and four small β -sheets [5]. The ligand-binding pocket of PPARs is quite large compared with other nuclear receptors, allowing them to interact with a broad range of ligands [1]. Although a wide range of compounds, particularly saturated and unsaturated fatty acids (FAs) and eicosanoid derivatives, have been identified as natural ligands of PPARs, their intracellular concentration ranges are not always known and their physiological relevance is not always clear [1]. In the last decades, several potent synthetic PPAR ligands



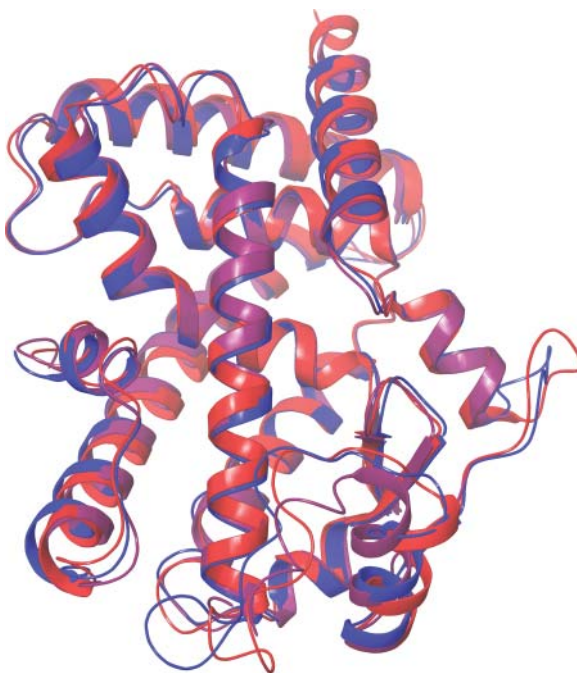
Figure 14.1 Multiple sequence alignment of the primary structures of the LBDs of PPARα, PPARδ, and PPARγ. Numbers above and below the alignment represent the numeration of the residues in PPARα and PPARγ isoform 1, respectively.

have been developed. Some of these synthetic ligands, such as thiazolidinediones (TZDs) and fibrates, are being used successfully for the treatment of diabetes and dyslipidemia. There is an increasing number of evidence supporting novel therapeutic perspectives for PPAR ligands. In addition, the potential beneficial roles of several PPAR ligands in nonalcoholic fatty liver disease, asthma, psoriasis, cancer, diabetic nephropathy, drug addiction, Alzheimer's disease, Parkinson's disease, and other conditions are being investigated [6–15].

Three subtypes of PPARs that are encoded by separate genes have been identified [1]: PPAR α , which is expressed in the liver, kidney, heart, muscle, and adipose tissue, among others; PPAR δ , which is expressed ubiquitously, but is markedly expressed in the brain, adipose tissue, and skin; and PPAR γ , which is abundantly expressed in both white and brown adipose tissues. The LBDs of the three subtypes of PPARs share a high level of sequence homology (Figure 14.1) and a common general 3-D structure (Figure 14.2). It is now established that covalent modifications by phosphorylation, ubiquitination, or SUMOylation are implicated in the regulation of PPAR α and PPAR γ functions [16, 17], whereas information about such modifications for PPAR δ is still limited [18].

PPAR α is a nuclear receptor that is a master regulator of hepatic nutrient metabolism during fasting, inducing hepatic FA oxidation and ketogenesis and regulating hepatic glucose production [19]. PPAR α regulates the expression of genes encoding the enzymes and transport proteins that control lipid homeostasis, thereby stimulating FA oxidation and improving lipoprotein metabolism [19]. FAs are able to activate PPAR α , with a general preference for long-chain

Figure 14.2 Structural alignment of the protein backbones of the LBDs of PPAR α (from PDB file 3VI8, shown in purple), PPAR δ (from PDB file 2GWX, shown in blue), and PPAR γ (from PDB file 2ZK0, shown in red).

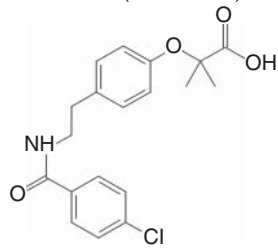
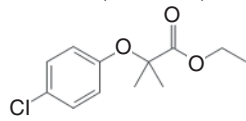
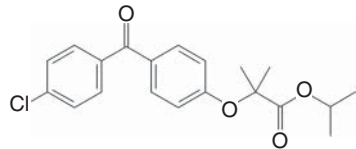
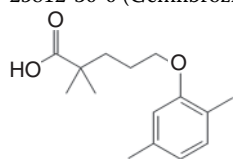
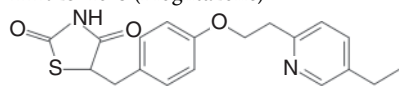
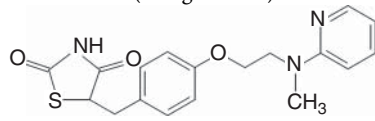


poly-unsaturated FA, numerous FA-derived compounds, and compounds showing a structural resemblance to FA, including oxidized FAs, endocannabinoids, and eicosanoids [19]. Fibrates, such as fenofibrate and the other drugs shown in Table 14.1, are PPAR α ligands that are used to treat atherogenic dyslipidemia by reducing the plasma triglyceride levels and increasing the high density lipoprotein (HDL) levels [20]. PPAR α also exerts pleiotropic anti-inflammatory and antiproliferative effects, prevents the proatherogenic effects of cholesterol accumulation on macrophages by stimulating cholesterol efflux, and exhibits an anti-atherogenic action [21]. Several clinical studies have shown the beneficial effects of several PPAR α agonists on reducing coronary heart disease, myocardial infarction, and stroke in type 2 diabetes (T2D) patients, preventing microvascular complications and ameliorating coronary reverse flow velocity and arterial stiffness, particularly in patients with high triglyceride and low-HDL cholesterol levels (both of which are risk factors for cardiovascular diseases) [22–24].

PPAR δ (also known as PPAR δ/β) is the least studied receptor among the PPARs. It is ubiquitously expressed in multiple tissues, most of which are involved in aspects of lipid metabolism, such as skeletal and cardiac muscles, hepatocytes, adipocytes, and macrophages [25]. It regulates the expression of many genes involved in lipid metabolism, glucose homeostasis, and inflammation [18]. Its activation is associated with anti-inflammatory processes in the treatment of autoimmune diseases, such as arthritis, eczema, and psoriasis, and anti-obesity processes in the treatment and prevention of obesity-induced insulin resistance, T2D, and cardiovascular disease [18, 26, 27]. Although some PPAR δ agonists have reached clinical trials, there are still no marketed drugs targeting PPAR δ . Some of the experimental data have suggested that when fully activated, the PPAR δ pathway could promote or accelerate the incidence of severe pathologies, such as colonic inflammation, colorectal cancers, and psoriasis [18, 28]. Other results show that PPAR δ antagonists may be beneficial under certain pathophysiological conditions [29].

PPAR γ is a nuclear receptor that is abundantly expressed in both white and brown adipose tissues, where it plays a key role in adipocyte differentiation and insulin sensitivity [30]. It exists as two isoforms, PPAR γ 1 and PPAR γ 2, generated by alternative splicing and differential promoter usage. PPAR γ 2 harbors a 30 amino acid extension at its N-terminus, and its expression is exclusively restricted to adipose tissue [31]. PPAR γ activation induces the differentiation of preadipocytes into adipocytes and favors lipid storage pathways through the transcription of several genes involved in glucose and lipid metabolism and energy balance, including genes that encode lipoprotein lipase, FA transporter protein, adipocyte FA-binding protein, fatty acyl-CoA synthase, malic enzyme, glucokinase, and GLUT4 glucose transporter [32]. TZDs, for example, rosiglitazone and pioglitazone, are full agonists of PPAR γ that stimulate glucose uptake in skeletal muscle and enhance the effects of circulating insulin (by decreasing insulin resistance) in adipose tissue, muscle, and liver and are prescribed to patients with T2D who present insulin resistance. In addition, TZDs also preserve pancreatic beta-cell function, reducing the incidence of T2D in high-risk people [33, 34]. Despite the well-characterized beneficial effects

Table 14.1 PPAR α - or PPAR γ -targeted drugs that are approved in Europe or the United States.

Compound CAS number (name)	Drug indication	Target
41859-67-0 (Bezafibrate) 	Treatment of primary hyperlipidemia types IIa, IIb, III, IV, and V	PPAR α ^{a)}
637-07-0 (Clofibrate) 	Treatment of primary dysbetalipoproteinemia (Type III hyperlipidemia) that does not adequately respond to diet	PPAR α
49562-28-9 (Fenofibrate) 	For use as an adjunct therapy to diet to reduce elevated LDL-C, total cholesterol, and triglycerides and Apo B levels and to increase HDL-C levels in adult patients with primary hypercholesterolemia or mixed dyslipidemia	PPAR α
25812-30-0 (Gemfibrozil) 	Treatment of adult patients with very high elevations of the serum triglyceride levels (types IV and V hyperlipidemia) who are at risk of developing pancreatitis and who do not adequately respond to a strict diet	PPAR α
111025-46-8 (Pioglitazone) 	Treatment of type II diabetes mellitus	PPAR γ
122320-73-4 (Rosiglitazone) 	Treatment of type II diabetes mellitus	PPAR γ

The data were extracted from DrugBank. Only targets with known pharmacological action were included.

a) Some studies suggest that this compound may have some activity toward PPAR γ and PPAR δ .
Source: <https://www.drugbank.ca/releases/latest#full>. Used under CC BY NC 4.0 <https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

of these compounds on treating insulin resistance, PPAR γ full agonists have several adverse effects, such as weight gain, fluid retention, bone fractures, and increased risk of ischemic cardiovascular events [35, 36]. For this reason, the Food and Drug Administration (FDA) announced in September 2010 that “it will significantly restrict the use of the diabetes drug Avandia (rosiglitazone) to patients with T2D who cannot control their diabetes on other medications. These new restrictions are in response to data that suggest an elevated risk of cardiovascular events, such as heart attack and stroke, in patients treated with Avandia.” Meanwhile, in Europe, the European Medicines Agency (EMA) recommended that the drug be suspended from the European market because its benefits no longer outweighed the risks [37]. Rosiglitazone was withdrawn from the market in the United Kingdom and India in 2010 and in New Zealand and South Africa in 2011. In 2013, the FDA “required removal of the prescribing and dispensing restrictions for rosiglitazone medicines after determining that data did not demonstrate an increased risk of heart attack with rosiglitazone medicines compared to the standard T2D medicines metformin and sulfonylurea” [38]. In December 2015, the FDA eliminated the risk evaluation and mitigation strategy for rosiglitazone-containing T2D medicines, concluding that “the risk evaluation and mitigation strategy is no longer necessary to ensure that the benefits of rosiglitazone medicines outweigh their risks” [39]. However, the use of medicines containing rosiglitazone is still currently suspended in the European Union.

Partial agonists of PPAR γ have been developed to overcome the adverse effects of PPAR γ full agonists. Full and partial PPAR γ agonists bind to the LBD of PPAR γ in different manners [40]. The LBD of PPAR γ has a Y-shaped form, consisting of a hydrophobic entrance (arm III) that branches off into two subsites: the polar arm I, which is extended toward helix H12, and the hydrophobic arm II, which is located between helix H3 and β -sheet [41] (see Figure 14.3). PPAR γ full agonists interact with arms I and II and establish a network of hydrogen bonds with the side chains of Ser289, His323, His449, and Tyr473. These interactions stabilize helix H12 and are responsible for the trans-activation activity of PPAR γ .

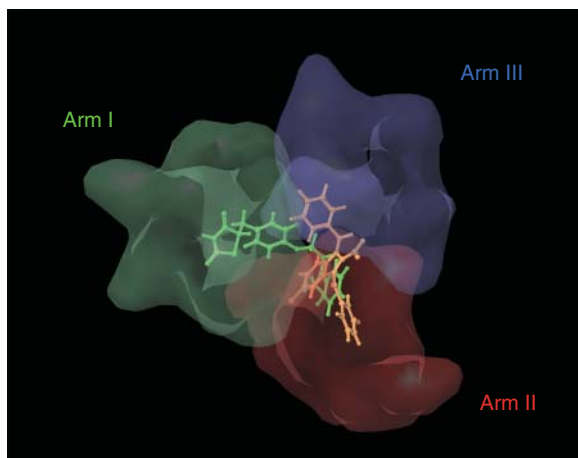


Figure 14.3 The arms of the LBDs of PPAR γ are represented as different colored surfaces (arm I in green, arm II in red, and arm III in blue). The full agonist rosiglitazone (green), from PDB file 1FM6, and the partial agonist SR145 (orange), from PDB file 2Q61 are shown.

[42]. Effective partial agonists mainly interact with the arm II of the PPAR γ LBD through a hydrophobic moiety and establish a hydrogen bond with Ser342 or an equivalent residue in arm III [40]. In addition, although interactions with arm I increase the binding affinity, this region should be avoided so that the trans-activation activity of potential PPAR γ ligands is not increased [40]. Thus, PPAR γ partial agonists do not interact with H12 and stabilize H3, affecting the recruitment of coactivators and decreasing the trans-activation activity of PPAR γ . The differences in binding and the lack of interactions between partial agonists and arm I of the LBD of PPAR γ (particularly interactions with H12) explain why partial agonists promote less PPAR γ trans-activation and do not show some of the adverse effects (such as weight gain and fluid retention) of TZDs and other full agonists [43]. Consistent with these observations, the Pro12Ala polymorphism in the human PPAR γ 2 gene causes a partial reduction of PPAR γ transcriptional activity that has been associated with reduced weight gain and improved insulin sensitivity [44]. Although several PPAR γ partial agonists have shown therapeutic effects on treating T2D and most of them have fewer side effects than rosiglitazone and pioglitazone, none of them have reached the marketplace because of their side effects or insufficient efficacy [45, 46].

However, the differences in binding between the full and partial agonists do not explain the antidiabetic properties of both types of compounds. The current knowledge suggests that the adverse effects of PPAR γ agonists are associated with the trans-activation activity of PPAR γ and the antidiabetic effects are partially associated with the capacity of PPAR γ ligands to inhibit cyclin-dependent kinase 5 (CDK5)-mediated phosphorylation of PPAR γ at Ser273 (Ser245 in isoform 1) [47], which is, interestingly, induced by obesity and other pro-inflammatory signals, explaining why most obese people also develop insulin resistance [47]. PPAR γ phosphorylation alters the PPAR γ -RXR complex, affecting the LBD-LBD dimerization interface and the AF-2 coactivator-binding region of PPAR γ [48] and leading to the dysregulation of a large number of genes whose expression is altered in obesity, including a reduction in adiponectin (an insulin-sensitizing adipokine) expression [47]. Thrap3 (thyroid hormone receptor-associated protein 3) plays a crucial role in this dysregulation. It can directly interact with PPAR γ when it is phosphorylated at Ser273, and this interaction controls the diabetic gene programming mediated by the phosphorylation of PPAR γ [49]. Thrap3 knockdown restores the expression of most of the genes that were dysregulated by CDK5 action on PPAR γ in cultured adipocytes [49].

The above data suggest that CDK5-mediated PPAR γ phosphorylation is involved in the pathogenesis of insulin resistance, indicating an opportunity for the development of an improved generation of antidiabetic drugs through PPAR γ [47]. This new antidiabetic mechanism of PPAR γ ligands is independent of the classical receptor activity of PPAR γ , and it is consistent with the observation that the rank order potency of compounds that activate PPAR γ is inconsistent with the rank order of their antidiabetic efficacy [45]. For example, the PPAR γ antagonist SR1664 binds to PPAR γ with high affinity and inhibits the CDK5-mediated phosphorylation of PPAR γ at Ser273, improving insulin resistance [50]. This inhibition maintains the transcription of several insulin-response

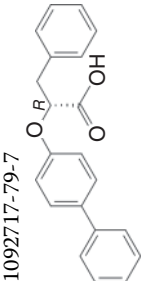
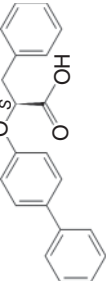
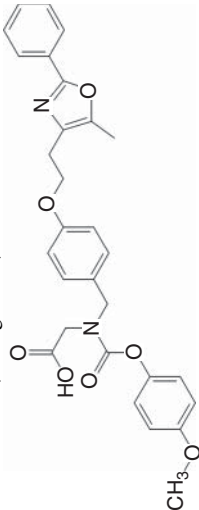
genes, such as adiponectin and adipsin [50]. Interestingly, SR1664 does not act as a transcriptional agonist, it does not cause fluid retention and weight gain (two of the serious side effects that have been described for TZDs and other PPAR γ ligands), and it does not interfere with bone formation [50]. In an effort to identify novel PPAR γ ligands with an improved pharmacological profile and fewer adverse effects, current research interest has been shifted toward the development of competitive PPAR γ antagonists that block the CDK5-mediated PPAR γ phosphorylation at Ser273 [29, 51–53]. As these compounds do not induce the trans-activation activity of PPAR γ , but can block the CDK5-mediated phosphorylation of PPAR γ at Ser273, they would have a good antidiabetic activity by avoiding the adverse effects associated with TZDs. In addition, several studies have identified non-PPAR protein targets of TZDs, such as the mitochondrial targets mitoNEET and mTOT, which may have a role in targeting insulin resistance and will be a focus of future investigations [46].

14.2 Dual and Pan PPAR Agonists

Because of the complementary beneficial effects of different PPAR subtypes, a combination strategy that simultaneously activates different PPAR subtypes has been developed. The explored approaches include the development of dual PPAR α/γ agonists, as well as PPAR α/δ , PPAR δ/γ , and PPAR $\alpha/\delta/\gamma$ (pan-agonist) agonists (see Table 14.2). The major challenge of this strategy is to identify molecules with a balanced potency at each receptor subtype, an overall efficacy, and an improved safety profile in the target tissues [29].

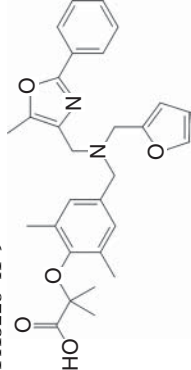
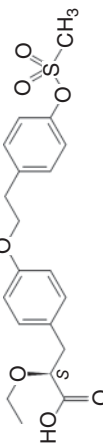
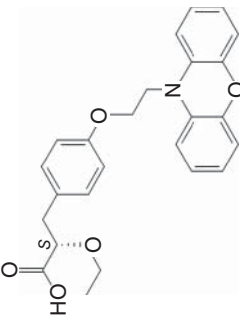
Patients with T2D can suffer from both hyperglycemia and dyslipidemia [2]. There is an abundance of evidence showing that individuals with metabolic syndrome or T2D are at increased risk of developing cardiovascular disease [46]. PPAR γ ligands, such as TZDs, suppress hyperglycemia but not dyslipidemia, and PPAR α ligands, such as fibrates, suppress dyslipidemia but do not ameliorate hyperglycemia. Based on these findings, it is expected that dual agonists of PPAR α and PPAR γ might provide broad beneficial effects on metabolism. In recent years, a great number of dual PPAR α/γ agonists, such as glitazars, have been developed, and several of them have shown a higher efficacy in improving lipid and glucose homeostasis compared with selective PPAR agonists [73]. Some of these dual agonists have even successfully progressed to phase III clinical trials, but several compound-specific safety concerns have emerged [46]. Several dual PPAR α/γ agonists, such as muraglitazar, naveglitazar, and ragaglitazar, but not aleglitazar, induce bladder tumor formation in rodents [46]. However, aleglitazar was withdrawn from phase III trial because of safety concerns about bone fractures, heart failure, and gastrointestinal bleeding [45]. Tesaglitazar was terminated in phases II and III trials because it was associated with renal problems [74]. The dual PPAR α/γ agonist saroglitazar and lobeglitazone have only recently been approved in India [75] and Korea [76], respectively. The adverse effects of dual PPAR α/γ agonists have been related with the PPAR γ agonistic activity. Therefore, any ligand that binds to PPAR γ in

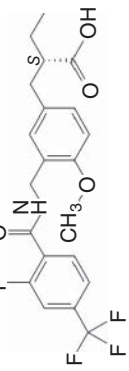
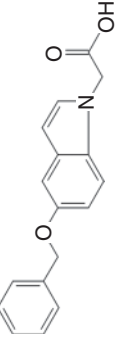
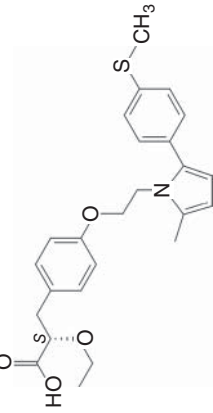
Table 14.2 Some compounds that simultaneously activate two or three PPAR subtypes.

Compound CAS number (name)	Type of agonist	PPAR α EC ₅₀ trans-activation activity (μ M)	PPAR δ EC ₅₀ trans-activation activity (μ M)	PPAR γ EC ₅₀ trans-activation activity (μ M)	References
1092717-79-7 	Dual PPAR α / γ agonist	0.22 $\pm$ 0.002 (100%) ^{a)}		0.48 $\pm$ 0.08 (35%) ^{f)}	[54]
862901-87-9 	PPAR α agonist and PPAR γ partial agonist	0.22 $\pm$ 0.02 (100%) ^{a)}		0.48 $\pm$ 0.08 (35%) ^{f)}	[54]
331741-94-7 (muraglitazar) 	Dual PPAR α / γ agonist	0.28 $\pm$ 0.03	>30	0.16 $\pm$ 0.04	[55]

(continued overleaf)

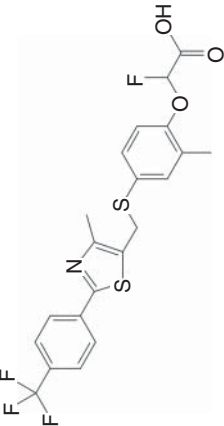
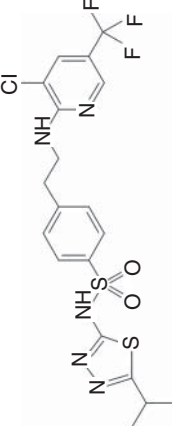
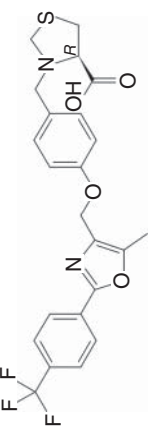
Table 14.2 (Continued)

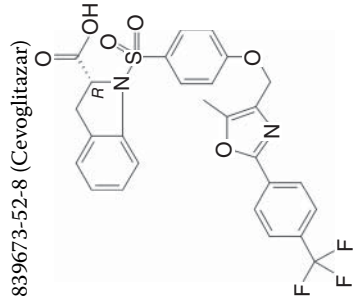
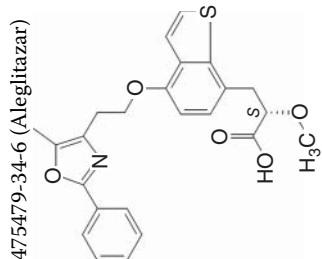
Compound CAS number (name)	Type of agonist	PPAR α EC ₅₀ trans-activation activity (μ M)	PPAR δ EC ₅₀ trans-activation activity (μ M)	PPAR γ EC ₅₀ trans-activation activity (μ M)	References
1415228-41-9 	Dual PPAR α/γ agonist	0.0017		0.0047	[56]
251565-85-2 (tesaglitazar) 	Dual PPAR α/γ agonist	1.2		1.3	[57]
476436-68-7 (naveglitazar) 	Dual PPAR α/γ agonist	2.8 $\pm$ 0.035 (43%)		0.36 $\pm$ 0.035 (77%) ^{f)}	[58]
222834-30-2 (ragaglitazar) 	Dual PPAR α/γ agonist	3.2 $\pm$ 1.1 (96.8%) ^{a)}		0.57 $\pm$ 0.2 (117%) ^{f)}	[59]

876296-16-1		Pan-agonist	0.010	0.012	1.9	[60]
374818-88-9 ([5-(benzyloxy)-1 <i>H</i> -indol-1-yl]acetic acid)		Dual PPARδ/γ agonist		134.2	18.1	[61]
495399-09-2 (Saroglitazar)		Dual PPARα/γ agonist	0.65×10^{-6}		0.003	[62]

(continued overleaf)

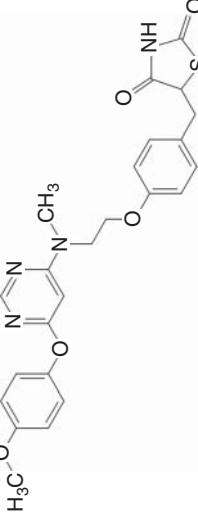
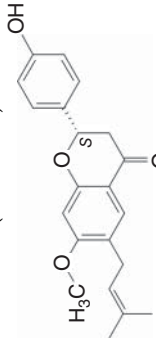
Table 14.2 (Continued)

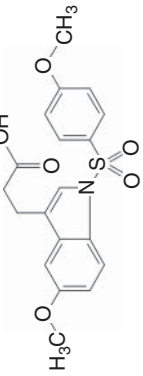
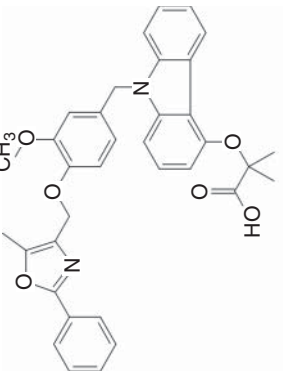
Compound CAS number (name)	Type of agonist	PPAR α EC ₅₀ trans-activation activity (μ M)	PPAR δ EC ₅₀ trans-activation activity (μ M)	PPAR γ EC ₅₀ trans-activation activity (μ M)	References
1350915-98-8 	Dual PPAR α/δ agonist	0.56 $\pm$ 0.025	0.055 $\pm$ 0.004		[63]
1303519-43-8 	Dual PPAR δ/γ agonist		0.016 (26%) ^d	0.336 (26%) ^f	[64]
1065332-51-5 (CG301269) 	Dual PPAR α/γ agonist	0.029		5.3	[65]

839673-52-8 (Cevoglitazar)		Pan-agonist	0.0013	0.12	0.0028	[66]
475479-34-6 (Aleglitazar)		Dual PPAR α/γ agonist	0.050 (156%) ^{b)}		0.021 (67%) ^{g)}	[67]

(continued overleaf)

Table 14.2 (Continued)

Compound CAS number (name)	Type of agonist	PPAR α EC ₅₀ trans-activation activity (μ M)	PPAR δ EC ₅₀ trans-activation activity (μ M)	PPAR γ EC ₅₀ trans-activation activity (μ M)	References
607723-33-1 (Lobeglitazone) 	PPAR α partial agonist and PPAR γ agonist	0.02		0.018	[68]
19879-30-2 (Bavachinin) 	Pan-agonist	4.0	8.1	0.74	[69]
1415609-13-0 (GL479) 	PPAR α agonist and PPAR γ partial agonist	0.6 $\pm$ 0.01 (158%) ^(c)		1.4 $\pm$ 0.02 (21%) ^(b)	[70]

835619-41-5 (Indeglitazar)		Pan-agonist	0.51 (100%) ^{a)}	2.7 (67%) ^{e)}	0.37 (45%) ^{f)}	[71]
885478-11-5		PPAR α partial agonist and PPAR γ antagonist	1.22 (60%) ^{a)}		IC ₅₀ = 0.481	[72]

-
- a) The % of maximal activation compared to the PPAR α full agonist Wy-14643 is shown in parentheses.
b) The % of maximal activation compared to the PPAR α full agonist farglitazar is shown in parentheses.
c) The % of maximal activation compared to the PPAR α full agonist clofibric acid is shown in parentheses.
d) The % of maximal activation compared to the PPAR δ full agonist GW501516 is shown in parentheses.
e) The % of maximal activation compared to the PPAR δ full agonist L-165041 is shown in parentheses.
f) The % of maximal activation compared to the PPAR γ full agonist rosiglitazone is shown in parentheses.
g) The % of maximal activation compared to the PPAR γ full agonist edaglitazone is shown in parentheses.
h) The % of maximal activation compared to the PPAR γ full agonist pioglitazone is shown in parentheses.

a similar way as the PPAR γ full agonists is expected to have the adverse effects of these compounds. Figure 14.4 shows the structures of PPAR α and PPAR γ complexed with the dual PPAR α/γ agonist aleglitazar. In both cases, the four typical hydrogen bonds between the ligand carboxylate and the Ser, His, and Tyr residues of PPAR α and PPAR γ , which are observed for most PPARs full agonists, can be identified (see Figures 14.4c,d) [67]. Note that the PPAR α residues Ser280, Tyr314, His440, and Tyr464 and the PPAR γ residues Ser289, His323, His449 and Tyr473 occupy equivalent positions in the alignment of both receptors (see Figure 14.1). Therefore, aleglitazar forms equivalent interactions with PPAR α and PPAR γ . The same interactions are shown for other dual PPAR ligands. Figure 14.5 shows the structures of PPAR α and PPAR γ complexed with GL479, a PPAR α agonist and PPAR γ partial agonist [70]. GL479 binding to PPAR α is stabilized through a combination of hydrogen bonds between its carboxylic group and the Ser280, Tyr314, His440, and Tyr464 residues (see Figure 14.5c)

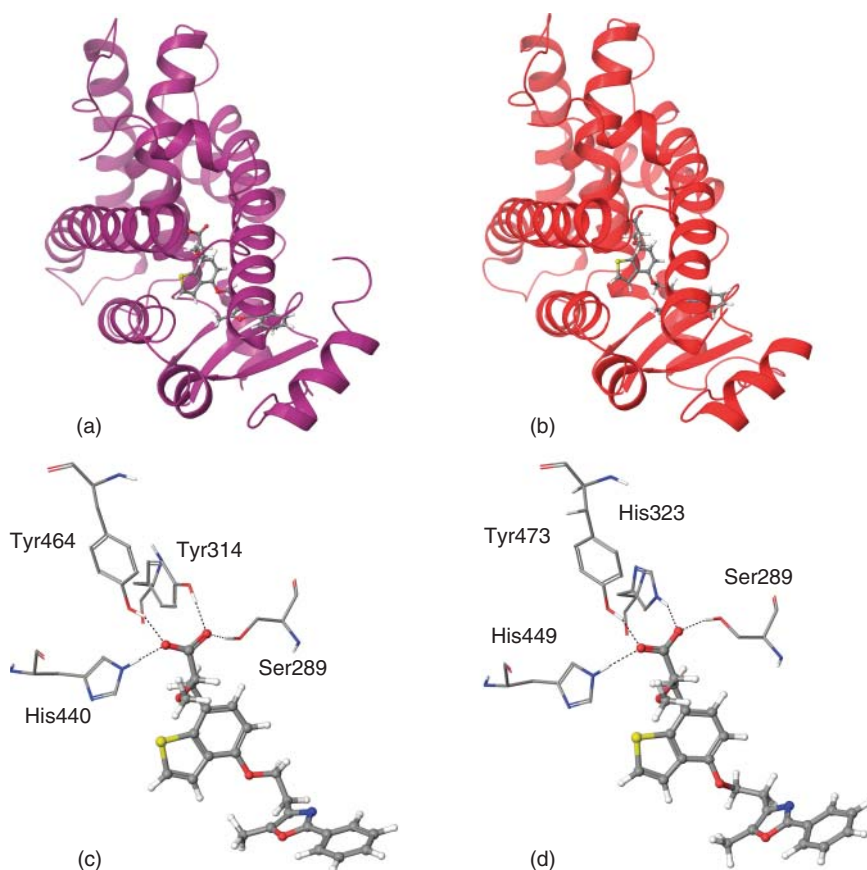


Figure 14.4 Structures of aleglitazar complexed with the LBDs of (a) PPAR α (from PDB file 3G8I) and (b) PPAR γ (from PDB file 3G9E). (c) and (d) show the ligand interaction diagrams of aleglitazar with PPAR α and PPAR γ , respectively. Hydrogen bonds and electrostatic interactions are shown as dashed black lines.

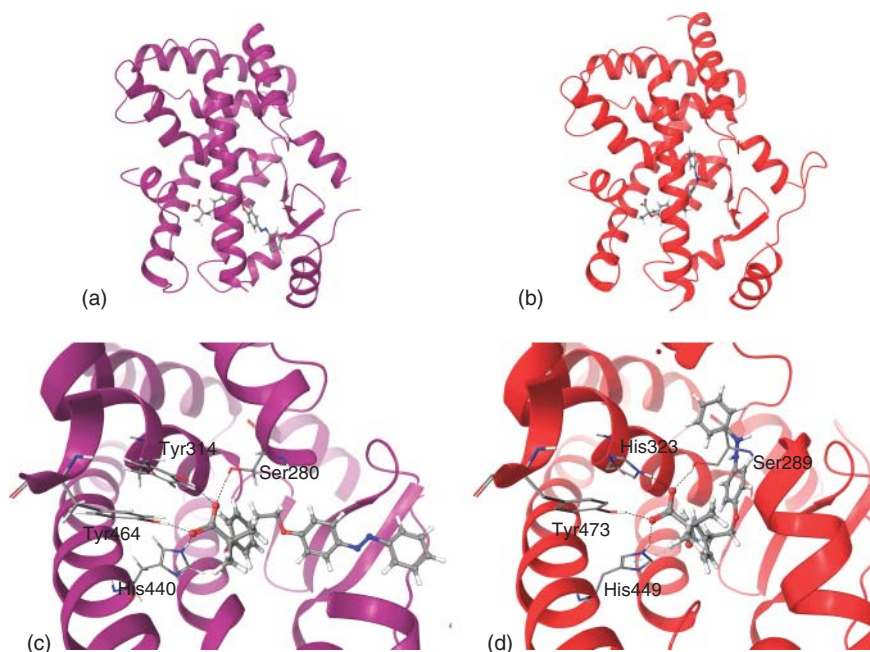


Figure 14.5 Structures of GL479 complexed with the LBDs of (a) PPAR α (from PDB file 4CI4) and (b) PPAR γ (from PDB file 4CI5). (c) and (d) show the ligand interaction diagrams of GL479 with PPAR α and PPAR γ , respectively. Hydrogen bonds and electrostatic interactions are shown as dashed black lines.

[77]. In addition, many hydrophobic interactions are formed between the ligand tail and arm III of the LBD of PPAR α [77]. Similar to the PPAR α and GL479 complex, GL479 presents the typical pattern of hydrogen bonds clustered with residues from arm I of the LBD of PPAR γ , involving residues Ser289, His323, His449, and Tyr473 (see Figure 14.5d) [77]. Again, the carboxylic group of GL479 forms the same interactions with PPAR α and PPAR γ . However, the hydrophobic tail of GL479 bends upward into arm II of the LBD of PPAR γ [77].

A pan-agonist that is capable of simultaneously stimulating the three PPAR subtypes would be expected to be particularly useful in the treatment of T2D [71]. However, most pan-agonists, including indeglitazar, GW677954, DRL-11605, GW625019, and netoglitazone, have been discontinued because of safety problems [69]. Again, the same interactions with arm I of the LBD of PPAR γ could be responsible for the PPAR γ trans-activation activity of these compounds and the reason for their adverse effects. Figure 14.6 shows the structures of PPAR α , PPAR δ , and PPAR γ complexed with the pan-agonist indeglitazar. Although this compound shows partial responses for PPAR γ , again, the same equivalent network of hydrogen bonds is observed.

The PPAR γ trans-activation activity must be reduced to overcome the adverse effects of multiple PPAR ligands. Reducing this activity and improving the mechanisms, such as the inhibition of PPAR γ phosphorylation at Ser273, which is

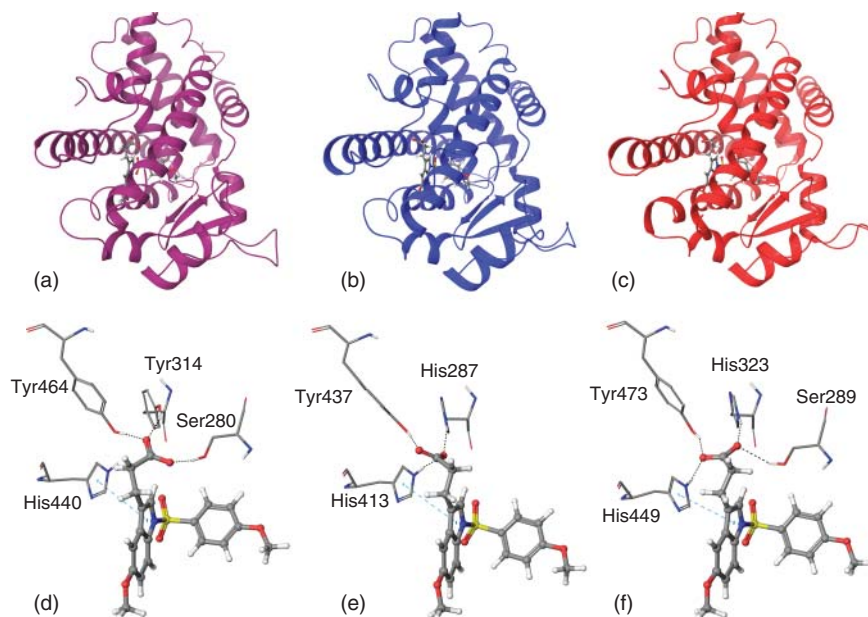


Figure 14.6 Structures of indeglitazar complexed with the LBDs of (a) PPAR α (from PDB file 3ET1), (b) PPAR δ (from PDB file 3ET2), and (c) PPAR γ (from PDB file 3ET3). (d–f) show the ligand interaction diagrams of indeglitazar with PPAR α , PPAR δ , and PPAR γ , respectively. Hydrogen bonds and electrostatic interactions are shown as dashed black lines, and π – π stacking interactions are shown as dashed light blue lines.

responsible for the antidiabetic effects of PPAR γ ligands, would allow the development of new drugs with improved efficacy and fewer adverse effects [21]. In this sense, a combination of the PPAR γ antagonistic activity with other activities might be clinically useful for preventing or treating obesity and obesity-related metabolic disorders [72]. In this sense, the Z-551 and imatinib compounds show that the combination of PPAR γ antagonistic activity with other activities is possible. Z-551 is a PPAR α agonist and a PPAR γ antagonist [72]. In mice fed with a high-fat diet, Z-551 significantly suppressed body weight gain and ameliorated insulin resistance and abnormal glucose and lipid metabolisms [72]. Imatinib, which is marketed by Novartis as Gleevec or Glivec, is a tyrosine-kinase inhibitor used to treat multiple cancers [78]. Using a high-throughput phosphorylation screen, it was found that Gleevec blocks the CDK5-mediated phosphorylation of PPAR γ , but is devoid of classical PPAR γ agonistic activity [79]. In high fat-diet-fed mice, Gleevec improves insulin sensitivity without the adverse effects associated with other PPAR γ -targeting drugs [79]. Furthermore, Gleevec reduces lipogenic and gluconeogenic gene expression in the liver, ameliorates inflammation in adipose tissues, and increases the browning of white adipose tissue, showing that Gleevec could be a novel therapeutic agent for use in treating insulin resistance and T2D [79].

14.3 Other Multiple Ligands that Act through PPARs

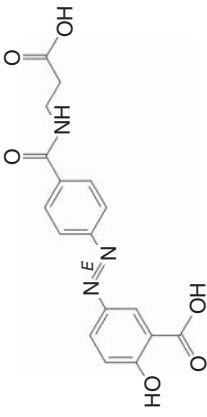
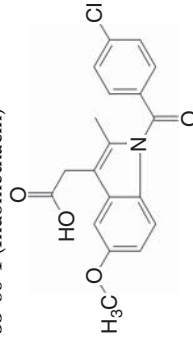
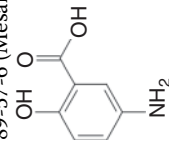
The multimodal drug concept of dual and pan PPAR agonists can also be extended to combinations of PPARs and other enzymes or receptors. Table 14.3 shows a list of approved drugs that are also PPAR ligands. Table 14.4 shows some PPAR ligands that also present additional activities through other targets. Below, we summarize the main characteristics of some of these compounds.

14.3.1 Angiotensin II Receptor Blockers/PPAR γ Agonists

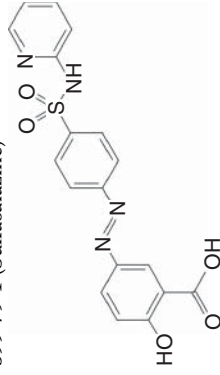
Sartans are angiotensin II receptor blockers (ARBs) that inhibit the activation of angiotensin II type 1 receptors and reduce blood pressure [94]. These compounds are used as antihypertensive drugs, but they are also indicated for the treatment of many cardiovascular morbidity syndromes. Clinical studies and meta-analyses suggest that ARBs are equivalent to angiotensin-converting enzyme (ACE) inhibitors, but with a better tolerability profile [95]. In addition, some ARBs, such as telmisartan, have an active role in cardiovascular protection in patients with vascular disease or high-risk diabetes [96]. Furthermore, the renin/angiotensin system (RAS) plays critical roles in the pathogenesis of atherosclerosis. Clinical studies have shown that pharmacological blockade of RAS with ARBs is effective in the treatment of patients with cardiovascular disease [97]. Although it was suggested that ARBs could increase the risk of certain cancers, the FDA concluded in 2011 that “treatment with an ARB medication does not increase a patient’s risk of developing cancer.” Recently, the European Society of Hypertension guideline stated that no clear-cut correlation can be demonstrated between ARB therapy and neoplastic diseases [98].

There are multiple lines of evidence showing the therapeutic effects of telmisartan. Telmisartan decreases lipid deposition and increases the collagen contents in plaques in ApoE^{-/-} mice, inhibits the progression of atherosclerosis in ApoE^{-/-} AT1aR^{-/-} mice, and decreases macrophage accumulation and lipid deposition, indicating plaque stabilization [97]. These results suggest that telmisartan has protective effects on the development of atherosclerosis and metabolic disorders in addition to its role in blocking the angiotensin II type 1 receptor in ApoE-deficient mice. Telmisartan functions as a PPAR γ partial agonist, with an EC₅₀ of 4.5 μ M, activating the receptor to 30% of the maximum level achieved by the full agonist rosiglitazone [99]. The binding mode of telmisartan to the LBD of PPAR γ differs greatly from that observed from full agonists, consisting of a hydrogen-bonding network around helix 12 [100]. Telmisartan binding results in a less stable H12 that in turn leads to attenuated coactivator binding, thus explaining the mechanism of partial activation [100]. As a selective modulator of PPAR γ , telmisartan does not cause the side effects of fluid retention and weight gain that are associated with the conventional TZD ligands of PPAR γ . Combined blockage of the angiotensin II type 1 receptor and selective PPAR γ modulation by molecules such as telmisartan could provide greater protection against new-onset diabetes and cardiovascular disease than drugs that target either the RAS or PPAR γ alone [101]. Moreover, given that blockade of the RAS can inhibit renal sodium reabsorption and attenuate the fluid retention and edema

Table 14.3 Approved drugs that are also PPAR ligands.

Compound CAS number (name)	Drug indication	Targets
80573-04-2 (Balsalazide) 	Anti-inflammatory agent indicated for the treatment of mildly to moderately active ulcerative colitis	Arachidonate 5-lipoxygenase, cyclooxygenases 1 and 2 and PPAR γ
53-86-1 (Indomethacin) 	Anti-inflammatory agent for moderate-to-severe rheumatoid arthritis including acute flares of chronic disease, ankylosing spondylitis, osteoarthritis, acute painful shoulder (bursitis and/or tendinitis) and acute gouty arthritis	Cyclooxygenases 1 and 2, phospholipase 2 and PPAR γ
89-57-6 (Mesalazine) 	Anti-inflammatory agent indicated for the treatment of active ulcerative proctitis	Arachidonate 5-lipoxygenase, cyclooxygenases 1 and 2 and PPAR γ

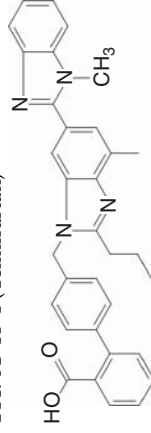
599-79-1 (Sulfasalazine)



Anti-inflammatory agent indicated for the treatment of Crohn's disease and rheumatoid arthritis as a second-line agent

Arachidonate 5-lipoxygenase, cyclooxygenases 1 and 2 and PPAR γ

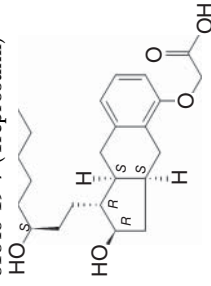
144701-48-4 (Telmisartan)



Used to treat hypertension, as well as diabetic nephropathy in hypertensive patients with type 2 diabetes mellitus, and patients with congestive heart failure (only in patients who cannot tolerate ACE inhibitors)

Angiotensin II receptor type 1 and PPAR γ

81846-19-7 (Treprostinil)



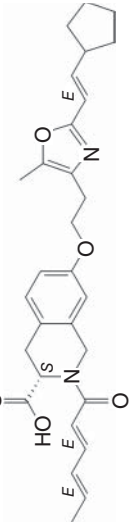
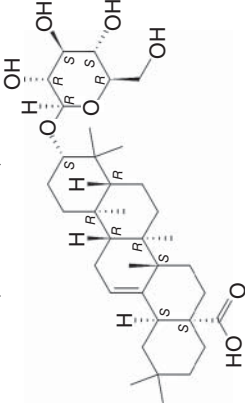
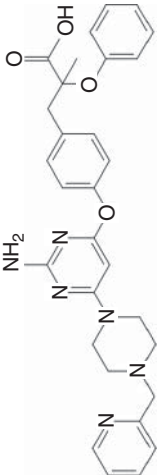
Used to treat pulmonary arterial hypertension in patients with NYHA classes II–IV symptoms to diminish symptoms associated with exercise

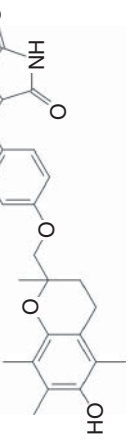
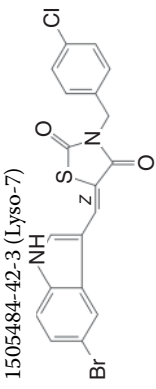
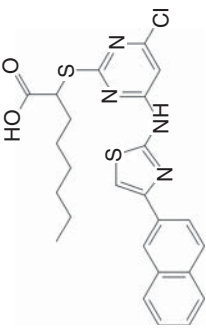
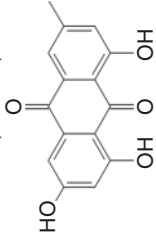
Prostacyclin receptor, P2Y purinoceptor 12 and PPAR δ

The data were extracted from DrugBank. Only targets with known pharmacological action were included.

Source: <https://www.drugbank.ca/releases/latest#full>. Used under CC BY NC 4.0 <https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

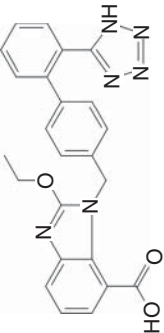
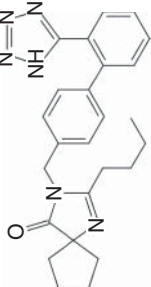
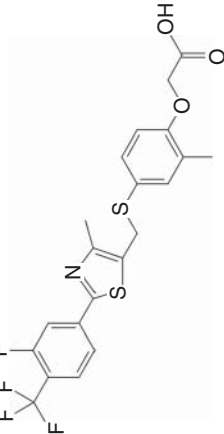
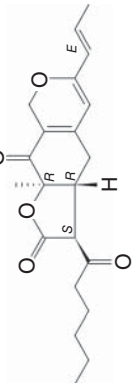
Table 14.4 Some ligands of PPARs that also present additional activities through other targets.

Name	PPAR-related activity	Other activities	References
477532-62-0 	Dual PPAR α / γ agonist	PTP1B inhibitor	[80]
TZD derivatives 3391-80-8 (Vitalboside A) 	PPAR γ agonist PPAR γ partial agonist	PTP1B inhibitor PTP1B inhibitor	[81, 82] [83]
Imidazo[4,5-b]pyridines derivatives 1141923-50-3 	PPAR γ agonist PPAR γ agonist	Angiotensin II receptor type 1 inhibitor 11 β -HSD1 inhibitor	[84] [85]

97322-87-7 (Troglitazone)		PPAR γ agonist	MGL inhibitor	[86]
1505484-42-3 (Lyso-7)		Pan-agonist	COX inhibitor	[87]
1492060-44-2		Pan-agonist	5-LO/mPGES1 inhibitor	[88]
518-82-1 (Emodin)		PPAR γ agonist	AMPK activator	[89]

(continued overleaf)

Table 14.4 (Continued)

Name	PPAR-related activity	Other activities	References
139481-59-7 (Candesartan) 	PPAR γ agonist	Angiotensin II receptor type 1 inhibitor	[90]
138402-11-6 (Irbesartan) 	PPAR γ agonist	Angiotensin II receptor type 1 inhibitor	[91]
317318-84-6 (GW0742) 	Pan-agonist	Vitamin D receptor and androgen receptor inhibitor	[92]
21516-68-7 (Monascin) 	PPAR γ agonist	Activates nuclear factor-erythroid 2-related factor 2	[93]

associated with peripheral vasodilators, these molecules could also lead to the development of new antidiabetic PPAR γ ligands with improved safety profiles [99]. The dual pharmacology of telmisartan and telmisartan analogs has been reported in animal models, showing efficacy in reducing blood pressure and the blood glucose levels [84, 102]. Additional variables, such as the capacity for inhibiting the Cdk5-mediated phosphorylation of PPAR γ at Ser273, for directly measuring the antidiabetic action of telmisartan and derivative compounds in structure–activity analyses are needed.

In addition to its hypotensive and hypoglycemic properties, telmisartan has been shown to exert antidyslipidemic effects by activating PPAR α [103]. Telmisartan significantly decreases the serum triglyceride level to approximately 75% of the baseline in hypertensive patients with T2D [104]. Additional studies of hypertensive patients with or without diabetes have confirmed that telmisartan significantly reduced the plasma triglyceride levels [105, 106]. Telmisartan induces hepatic PPAR α target gene expression and reduces the serum triglycerides levels in diet-induced obese mice [107]. However the *in vitro* activity of telmisartan as a PPAR α agonist is observed in the higher micromolar range, with 22.5% of the maximum response induced by the reference PPAR α agonist Wy-14643 [107]. Thus, the activity of telmisartan as a PPAR α agonist has much room for improvement.

14.3.2 COX Inhibitors/PPAR γ Agonists

Some PPAR γ activators have broad anti-inflammatory effects, but the mechanisms of these effects are not fully understood [46]. SUMOylation of PPAR γ 2 at Lys395 represses the transcription of inflammatory genes in macrophages and is believed to inhibit transcription by preventing the clearance of corepressor complexes [108]. Rosiglitazone and other PPAR γ ligands promote the SUMOylation of PPAR γ , maintaining a repressed state of NF κ B inflammatory target genes [109]. Recently, a novel molecular mechanism of PPAR γ that regulates adipocyte inflammation has been identified [110]. This mechanism consists of PPAR γ phosphorylation at Tyr78 that suppresses the expression of pro-inflammatory genes [110]. In addition to the role of PPAR γ ligands in promoting the SUMOylation or phosphorylation of PPAR γ , it appears that some anti-inflammatory effects of PPAR γ ligands may be PPAR γ independent [46].

Nonsteroidal anti-inflammatory drugs (NSAIDs) display anti-inflammatory, analgesic, and antipyretic activities by inhibiting cyclooxygenases (COX-1 and COX-2) and blocking prostaglandin production [111]. Several studies have suggested that some NSAIDs also modulate PPAR γ activity at pharmacologically relevant concentrations, raising the possibility that such off-target effects contribute to the spectrum of clinically relevant NSAID actions [112]. Diclofenac, indomethacin, sulindac, and ibuprofen are NSAIDs that act as PPAR γ partial agonists and display adipogenic activities [112].

Figure 14.7 shows the 3-D structure of indomethacin complexed with PPAR γ , phospholipase A2, and COX-2. Note that the orientation of the chlorobenzene

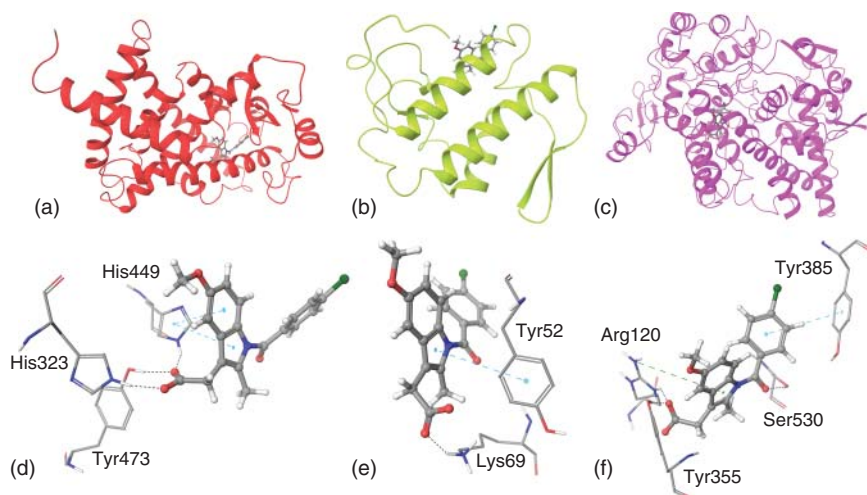


Figure 14.7 Structures of indomethacin complexed with (a) the LBDs of PPAR γ (from PDB file 3ADX), (b) phospholipase A2 (from PDB file 3H1X), and (c) cyclooxygenase-2 (from PDB file 4COX). (d–f) show the ligand interaction diagrams of indomethacin with PPAR γ , phospholipase A2, and cyclooxygenase-2, respectively. Hydrogen bonds and electrostatic interactions are shown as dashed black lines, π - π stacking interactions are shown as dashed light blue lines and cation- π interactions are shown as dashed green lines.

moiety and the rotation of the carboxylic group of indomethacin are different between the three structures. The indole ring and the carboxyl group function as the indomethacin core moiety for binding to the AF-2 pocket of PPAR γ [113]. Tyr473, His323, and His449 directly interact with the carboxyl group of indomethacin through hydrogen bonds (see Figure 14.7d). The interaction with Tyr473 seems to be very important, as the PPAR γ Y473F mutant (where Tyr473 is mutated to a phenylalanine) was totally insensitive to indomethacin [113]. A π - π stacking interaction between the indomethacin indole ring and His449 is also observed. The indole ring and the carboxyl group of indomethacin also interact with the residues that are important for the catalytic activity and the anticoagulant action of phospholipase A2 [114]. One of the oxygen atoms of the carboxylic group of indomethacin interacts with Asp49 and His48 through a catalytically important water molecule (interactions not shown in Figure 14.7e), whereas the second carboxylic oxygen atom forms an ionic interaction with the side chain of Lys69 (see Figure 14.7e) [114]. In addition, a π - π stacking interaction between the indomethacin indole ring and Tyr52 is also observed. These interactions with phospholipase A2 make indomethacin the first compound that simultaneously exhibits anticoagulation and anti-inflammatory effects [114]. Again, the indole ring and carboxyl group of indomethacin interact with the COX-2 enzyme. The carboxyl group forms a salt bridge with Arg120 and Tyr355 (see Figure 14.7f), and the indole ring interacts with Val349 and Ser353 [115]. In addition, the benzoyl oxygen of indomethacin interacts with the side chain hydroxyl of Ser530 [115], and π - π stacking between the chlorobenzene ring and Tyr385 is also observed (see Figure 14.7f).

14.3.3 Protein Tyrosine Phosphatase 1B Inhibitors/PPAR Agonists

Protein tyrosine phosphatase 1B (PTP1B) is a cytosolic protein tyrosine phosphatase that is expressed in various cells, including the liver, muscle, and fat [116]. It is a negative regulator of the insulin- and leptin-signaling cascades. Therefore, PTP1B inhibitors have been proposed as potential therapeutics for the treatment of T2D, obesity, and other related metabolic diseases [117, 118]. Compounds containing scaffolds similar to TZDs exhibit inhibitory effects against PTP1B, suggesting that dual PTP1B inhibitors/PPAR γ agonists are possible. Several compounds show IC₅₀ values in the μ M range against PTP1B and are able to activate the transcription of PPAR γ , showing *in vivo* efficacy as an anti-obesity and hypoglycemic agent in a high-fat diet-induced mouse model system [81, 82]. The compound with CAS number 477532-62-0 is a dual PPAR α/γ agonist with inhibitory activity (IC₅₀ = 1.85 μ M) against PTP1B and shows stronger hypoglycemic and hypotriglyceridemic effects on male KK-A γ mice [80]. However, the PPAR γ trans-activation activity of these compounds, which is comparable to that of rosiglitazone and other full agonists, suggests that they might have comparable adverse effects as other PPAR γ ligands. Recently, the compound vitalboside A isolated from *Syzygium cumini* has been characterized [83]. Vitalboside A acts synergistically as a PTP1B inhibitor and PPAR γ partial agonist [83]. Combining a PTP1B inhibitor with a PPAR γ antagonist that inhibits the CDK5-mediated phosphorylation of PPAR γ could be a promising therapeutic agent in the management of obesity and diabetes.

14.3.4 11 β -Hydroxysteroid Dehydrogenase Type 1 Inhibitors/PPAR Agonists

11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is a microsomal enzyme that is highly expressed in the liver, the adipose tissue, and the central nervous system and catalyzes the conversion of cortisol and other 11 β -hydroxysteroid to active metabolites (cortisone in the case of cortisol). These metabolites activate glucocorticoid receptors, thus amplifying local glucocorticoid action and exploiting the substantial circulating levels of inert 11-ketosteroids [119]. Inhibition of 11 β -HSD1 represents a potential target for the therapy of disorders that might be ameliorated by a local reduction of glucocorticoid action, including T2D, obesity, and age-related cognitive dysfunction [119, 120]. As PPAR γ full agonists induce adipogenesis, which causes the side effect of weight gain, and 11 β -HSD1 inhibitors prevent adipogenesis, it might be beneficial to combine both activities for the treatment of obesity in T2D patients [85]. In 2009, Ye *et al.* [85] designed and synthesized a series of α -aryloxy- α -methylhydrocinnamic acids as dual-function agents that simultaneously activate PPAR γ and inhibit 11 β -HSD1. The most promising compound (the compound with CAS number 1141923-50-3) showed dual effects on PPAR γ activation and 11 β -HSD1 inhibition, with an EC₅₀ = 6.76 μ M and IC₅₀ = 0.76 μ M, respectively [85]. In addition, this compound did not promote adipogenesis and showed excellent efficacy for reducing the glucose levels, triglycerides levels, body fat, and body weight in well-established mouse and rat models of diabetes and obesity [85]. Again, combining an 11 β -HSD1 inhibitor with a PPAR γ antagonist that inhibits the CDK5-mediated phosphorylation of PPAR γ could be more interesting.

14.4 Conclusions

Although extensive research has been performed on multiple ligands of PPARs for the last few decades, few of these compounds have reached the market. Taking into account that the antidiabetic (and probably other effects, such as the anti-inflammatory effect) action of PPAR γ ligands is not related to the trans-activation activity of this receptor, this activity must be reduced or even suppressed to overcome the adverse effects associated with PPAR γ ligands. In addition, other mechanisms, such as the inhibition of PPAR γ phosphorylation at Ser273, must be taken into account. The Z-551 compound [72] and Gleevec [79] have shown how the combination of PPAR γ antagonistic activity with other activities may be clinically useful for preventing or treating obesity and obesity-related metabolic disorders.

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List of Abbreviations

11 β -HSD1	11 β -hydroxysteroid dehydrogenase type 1
AF	transcriptional activation function
COX	cyclooxygenase
DBD	DNA-binding domain
FA	fatty acids
HDL	high density lipoprotein
LBD	ligand-binding domain
NSAIDs	nonsteroidal anti-inflammatory drugs
PPARs	peroxisome proliferator-activated receptors
PTP1B	protein tyrosine phosphatase 1B
RXR	retinoid X receptor
T2D	type 2 diabetes
TZDs	thiazolidinediones

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15

Antibiotics*Jean-Philippe Surivet and Philippe Panchaud*

Idorsia Pharmaceuticals Limited, Drug Discovery Chemistry, Hegenheimermattweg 91, CH-4123 Allschwil, Switzerland

Since the mid-twentieth century, antibacterial chemotherapies have revolutionized medical practices and have contributed to the reduction of mortality and morbidity of man [1]. During the “Golden Age” of antibacterial drug discovery, new chemical classes of antibiotics were introduced into clinical practice at a continuous pace: β -lactams (1941), aminoglycosides (1946), tetracyclines (1948), macrolides (1952), glycopeptides (1958), cephalosporins (1964), fluoroquinolones (FQs) (1984), and carbapenems (1985). Unfortunately, their clinical use was accompanied with the inexorable development and dissemination of resistant pathogenic bacteria. At first, the early identification of penicillin- and methicillin-resistant *Staphylococcus aureus* (MRSA) strains had little consequences on medical practices as novel classes of effective antibiotics active against those strains were continuously discovered and developed. However, the antibacterial drug discovery engine stalled, and multidrug-resistant pathogenic bacteria started to thrive. At the dawn of the twenty-first century, the situation reached an alarming level and cases of untreatable infections because pan-resistant bacteria were no longer rare [2, 3]. One explanation for the scientific failure to discover novel effective therapies can be found in part in the one-target–one-drug paradigm that culminated in 1995 with the advent of the genomic era. Hundreds of single gene-encoded targets with defined attributes (essentiality, conservation across bacterial species, selectivity vs human counterpart) were identified and believed to be rapidly addressed with modern screening techniques like high-throughput screening (HTS) followed by medicinal chemistry optimization [4]. Even though single-target agents with promising *in vitro* and *in vivo* antibacterial activity were reported, only a few could successfully be clinically developed as broad-spectrum antibacterial drug [5]. Conversely, the history of antibacterial research showed that monotherapeutic agents successful in the treatment of bacterial infections rely on mechanisms much more complex than single-enzyme inhibition. Multiplicity of action [6] is a well-established concept for successful monotherapeutic antibiotics since β -lactams, FQs, macrolides, or aminoglycosides were found to exert their biological activities by blocking more than one molecular target or by addressing targets encoded

by multiple genes. Designing such multitarget-directed ligands is therefore of paramount importance and constitutes today a tremendous challenge with the requisite of achieving appropriate affinities on more than one target while preserving adequate physicochemical and absorption, distribution, metabolism, and excretion (ADME) properties. One key benefit associated with multiplicity of action is the expected low level target-based resistance due to single genetic change in the host. Since single amino acid mutations appear at frequencies ranging from 10^{-6} to 10^{-9} on an individual target, resistance to ligands interfering with multiple targets should develop at a much lower rate. Benefits associated to multitarget-directed ligands are covered in more detail in Chapter 7.

The present chapter, which covers literature up to February 2014, is not an exhaustive review of all design examples reported but rather a selection of those with the objective to illustrate the main two strategies that have been employed to inhibit multiple targets with a single chemical entity: (i) single-pharmacophore molecules designed to address multitarget inhibition and (ii) design of hybrid molecules as single chemical entities obtained by fusing or linking two antibacterial pharmacophores. Combination therapies, such as the β -lactam/ β -lactamase inhibitor combination, which are essential components of the antibacterial armamentarium, are not covered in this chapter.

15.1 Design of Single-Pharmacophore Molecules Acting on Multiple Targets

Designing and synthesizing a novel multitarget-directed ligand, in the sense that it addresses several individual targets, is a primitive concept in the field of antibacterial drug discovery. Thousands of discrete β -lactams [7] or FQs [8], two main families of antibiotics, were produced before the true promiscuous nature of these drugs was established. In any given bacterium, a β -lactam antibiotic inhibits several transpeptidases (penicillin-binding proteins) PBPs and any FQ potentially blocks two DNA topoisomerases. While intensive efforts are still dedicated to the discovery of novel and effective β -lactams or FQs, cross-resistance due to specific resistance mechanisms nonetheless mandates researchers to expand their activities toward the discovery of novel chemical classes of inhibitors. Among all potential antibacterial drug targets [9], a few are suitable for a multitarget inhibition approach. As illustrated hereafter, the most promising novel classes address “old” but validated targets such as DNA topoisomerases, for which different “druggable” binding sites have been identified. In the second part of this section, examples of designed multitarget-directed ligands addressing other targets like Mur ligases and type II fatty acids synthases will be shortly presented. Finally, the exploitation of novel but clinically nonvalidated targets constitutes an alternative approach to circumvent the cross-resistance issue. The challenges faced with such a strategy will be highlighted at the end of the chapter.

15.1.1 Dual Inhibitors of Bacterial DNA Gyrase and Topoisomerase IV

Bacterial DNA replication plays an essential role in the bacterial cell survival. Several “druggable” enzymes involved in the bacterial replication machinery, such as DNA polymerases and DNA ligase, have been recently under the

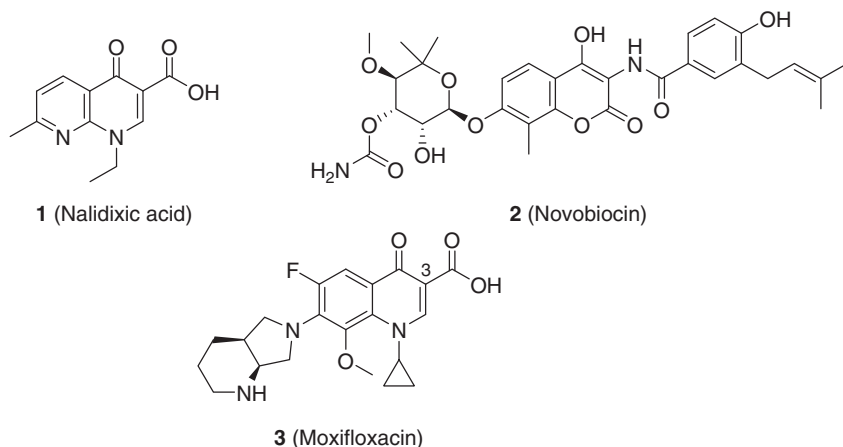


Figure 15.1 Representative bacterial topoisomerases inhibitors.

spotlights [10]. Nonetheless, DNA gyrase and DNA topoisomerase IV (topoIV) are still the most popular choices for the design of multitarget-directed ligands. DNA gyrase, a heterotetramer of two GyrA and two GyrB subunits, regulates the supercoiling of the circular bacterial chromosome and unwinds DNA at the replication fork, whereas topoIV, a heterotetramer of two ParC and two ParE subunits, mediates chromosome partitioning by decatenating sister chromatids. These two type II topoisomerases are clinically validated antibacterial drug targets ever since two unrelated antibacterial drug classes, the quinolones (later the FQs) and the coumarins, were found to block their essential function. Quinolones such as nalidixic acid **1** (Figure 15.1) bind at the interface between the GyrA or ParC subunits and cleaved DNA, whereas coumarins such as novobiocin **2** interact with the ATP binding site located in the GyrB and ParE subunits. These known binding sites, together with recently identified additional ones, provide opportunities for the design of novel dual DNA gyrase–topoIV inhibitors.

15.1.1.1 Dual Inhibitors Targeting the Binding Site of Fluoroquinolones

Following the discovery of nalidixic acid **1** in the early 1960s, incremental structural modifications of the 4-quinolone nucleus have led to the discovery of a large family of drugs with broader antibacterial spectrum and improved clinical utility: the FQs [11] such as moxifloxacin **3** (Figure 15.1). Initial investigations performed on *Escherichia coli* identified DNA gyrase as the target of FQs. Their promiscuous nature could only be demonstrated once *E. coli* topoIV was isolated and characterized [12, 13]. It was subsequently reported that in *S. aureus* most of the FQs primarily target topoIV rather than DNA gyrase [14]. The key objective in designing novel DNA topoisomerase inhibitors is to restore the antibacterial activity against bacteria that have developed resistance to FQs. Among several resistance mechanisms [15], a single amino acid mutation in the quinolone resistance-determining region (QRDR) of the primary topoisomerase target, either GyrA (Ser84Trp) or GrlA (Ser80Phe)¹ [16, 17] in *S. aureus*, was shown to reduce dramatically FQs binding affinity thereby reducing their

¹ GrlA is the ParC subunit of *S. aureus*.

inhibitory effect [18]. Dual and balanced inhibitors that affect both targets with similar potency may elicit lower resistance frequencies and restore antibacterial activities against existing mutants [19]. As of 2009, successive X-ray structures have revealed the binding mode of FQs to *Streptococcus pneumoniae* [20, 21] or *Acinetobacter baumannii* [22] topoIV. As shown in Figure 15.2, the flat quinolone system stacks between base pairs at the DNA cleavage site and binds to ParC and DNA through a divalent magnesium ion and conserved water molecules.

The availability of these invaluable crystal structures should enable structure-based design of multitarget-directed ligands; however to date no report on such efforts has appeared. Prior to these structural insights, structure–activity relationships (SARs) of FQs were thoroughly established and many working models were built to rationalize the experimental evidences [23, 24]. Even though these models are now obsolete, their use in designing novel topoisomerase inhibitors showed some successes [25]. The design of ligands devoid of carboxylic acid function in 3-position was unsuccessful until isothiazoloquinolones (ITQs) such as A-62824 **4** were synthesized and characterized at Abbott (Figure 15.3). Compound **4** showed a 10-fold improved inhibitory potency of *E. coli* DNA gyrase as compared with ciprofloxacin **6**, its closest FQ analog [26]. Exquisite antibacterial activities against a panel of Gram-positive and Gram-negative organisms were also observed. It is likely that under physiological conditions the nitrogen of the isothiazolo ring system is deprotonated, resulting in a tautomeric form able to coordinate the magnesium ion as in the FQ case.

Optimization efforts were pursued at Achillion and yielded derivatives such as compound **5**, which displayed strong antibacterial activity against MRSA [27] and various quinolone-resistant pathogens. The lower minimal inhibitory concentration (MIC) shift observed for ITQ **5** compared with **3** on a FQ-resistant *S. aureus* was explained with the enzyme inhibition data on

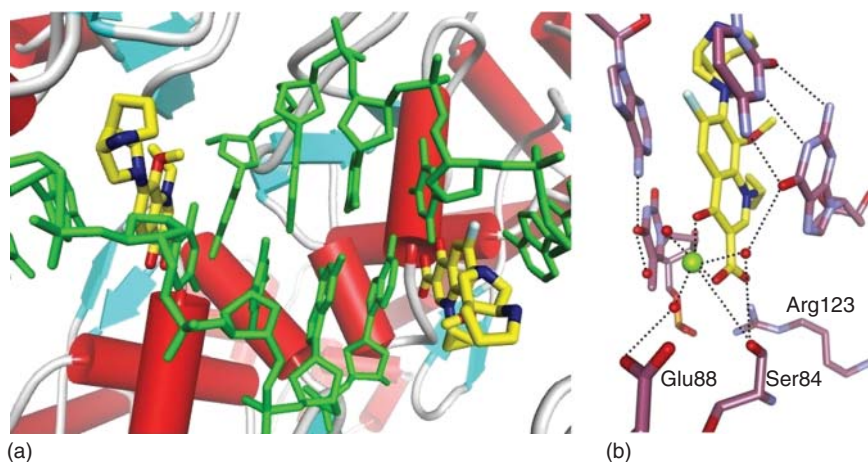


Figure 15.2 (a) Moxifloxacin **3** (yellow) bound to *A. baumannii* ParC and cleaved DNA (green). The complementary strand is omitted for clarity. (b) Close view of **3** stacked between DNA base pairs and bound to Mg^{2+} (green). Hydrogen bonds as black dotted lines (PDB code: 2XKK).

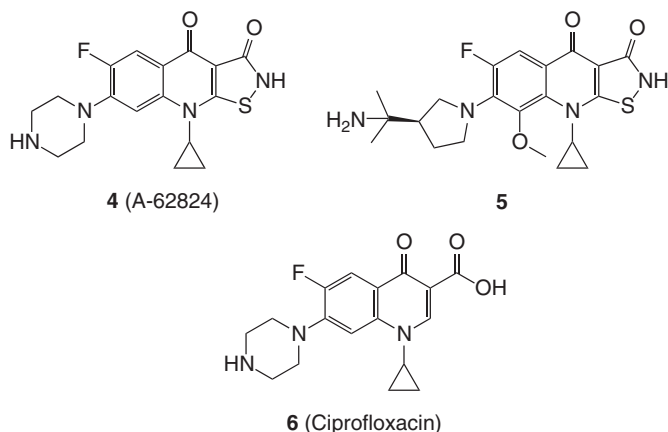


Figure 15.3 Structures of two representative isothiazoloquinolones and ciprofloxacin.

Table 15.1 Comparative *in vitro* activities of moxifloxacin **3** and ITQ **5**.

Compounds	IC ₅₀ ^{a)} (μM)				MIC ^{b)} (μg/mL)	
	<i>S. aureus</i> topoIV wt	<i>S. aureus</i> ^R topoIV ^{c)}	<i>S. aureus</i> gyrase wt	<i>S. aureus</i> ^R gyrase ^{d)}	<i>S. aureus</i> wt ^{e)}	<i>S. aureus</i> ^{Rf)}
3	1	11.9	7.7	>200	0.06	2
5	0.12	0.6	0.68	4.2	0.004	0.08

a) Inhibition of wild-type (wt) and mutant *S. aureus* topoIV (decatenation assay) and gyrase (supercoiling assay).

b) Minimum inhibitory concentration.

c) Ser80Phe mutant.

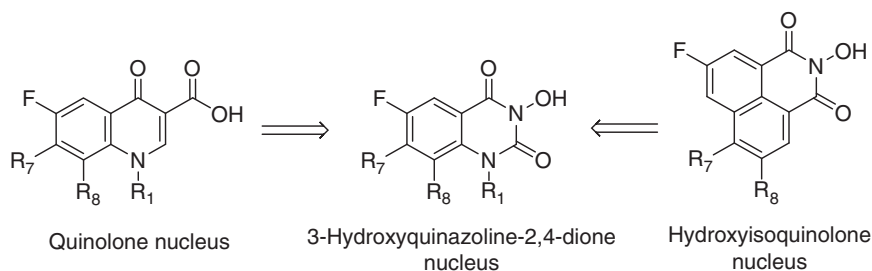
d) Ser84Leu mutant.

e) *S. aureus* strain ATCC 29213.

f) *S. aureus* strain BK2384 mutant.^{c), d)}

S. aureus topoisomerases shown in Table 15.1. Indeed, moxifloxacin **3** showed markedly higher IC₅₀'s on mutated DNA-topoIV and DNA gyrase (>10-fold and >30-fold increase, respectively) than on wild-type enzymes. ITQ **5** exhibited more potent and balanced inhibitory potencies against wild-type and mutated topoisomerase enzymes (~fivefold increase of IC₅₀'s only) and retained potent antibacterial activity against the mutant *S. aureus* strain. The spontaneous mutant selection measured for **5** against wild-type *S. aureus* occurred at low frequency (9×10^{-11} at 4× MIC), approximately one log-unit lower than that measured for ciprofloxacin **6** (1×10^{-9} at 4× MIC) [28].

A few years later, Pfizer identified hydroxyisoquinolone as a promising antibacterial scaffold. As shown in Scheme 15.1, the combination of its structural features with the quinolone nucleus gave rise to the 3-hydroxyquinazoline-2,4-dione scaffold. The SAR was investigated according to the knowledge accumulated around FQs. Whereas the *N*-hydroxy substituted molecules showed activities comparable to FQs in a gyrase–drug induced cleavage assay [29], the modest



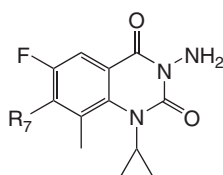
Scheme 15.1 Design of the 3-hydroxyquinazoline-2,4-dione scaffold.

antibacterial activities on wild-type pathogens likely resulted from a poor cellular accumulation. The biological activity of this series was assumed to be linked to the acidic nature of the 3-hydroxyl group (pK_a 6.3–7.0) and to its coordination to the magnesium ion. The lack of *in vivo* efficacy for the best derivatives of this series was attributed to poor pharmacokinetic properties.

The search for 3-hydroxy replacement with improved drug metabolism and pharmacokinetics (DMPK) properties culminated in the discovery of 3-aminoquinazolinediones (3-AQD) **7**, which have neither marked basic nor acidic character (Figure 15.4). 3-AQDs were identified as potent *E. coli* gyrase inhibitors [30] and SAR on the substitution at the C-7 position was investigated (Table 15.2).

The optimal heterocycle was found to be a pyrrolidine ring (comparing compounds **7a** and **7b**). The introduction of an amine (**7c**) or an aminoalkyl group on the pyrrolidine ring (**7d**, **7e**) resulted in improved inhibitory activities of *E. coli* gyrase and enhanced antibacterial activities on Gram-positive and Gram-negative bacteria. The X-ray structure of PD 0305970 **7e**, the best compound identified in this series, bound to *S. pneumoniae* topoIV [20] was solved. As shown in Figure 15.5, the 3-AQD scaffold stacked similarly as FQs between DNA base pairs (see Figure 15.2). Although 3-AQDs and FQs share a similar binding site and interact with the same amino acids in *E. coli* DNA gyrase, no cross-resistance was observed. The explanation was found in the different mechanisms by which each class interferes with the DNA resealing process.

No enzyme inhibition data were provided to support the dual inhibitory activity of **7e**, but mutation experiments conducted with a *Neisseria gonorrhoeae* strain showed that 3-AQDs exert their activities through specific dual inhibition of DNA gyrase and topoIV [30]. The resistance frequency measured on a *S. aureus* strain was in a similar range than the one measured for levofloxacin, a dual and balanced FQ ($<6 \times 10^{-8}$ for **7e** and 8.9×10^{-9} for levofloxacin at $4\times$ MIC) [14].



7 (3-AQD scaffold)

Figure 15.4 3-Aminoquinazolinediones **7** core scaffold.

Table 15.2 Structure–activity relationship at C-7 position of 3-AQD scaffold.

Compounds R ₇		IC ₅₀ (μM)	MIC ^{a)} (μg/mL)				
		<i>E. coli</i> gyrase ^{b)}	<i>S. aureus</i> 29213	<i>S. pyogenes</i> C-203	<i>E. faecalis</i> MGH-2	<i>E. coli</i> ^{c)} MC4100	<i>E. coli</i> ^{d)} TolC
	7a	1.9	1	1	2	16	0.13
	7b	6.3	4	4	4	>64	0.25
	7c	0.2	8	1	8	1	0.25
	7d	0.8	0.25	0.03	0.13	2	0.03
	7e	0.2	0.13	0.015	0.06	0.5	0.03

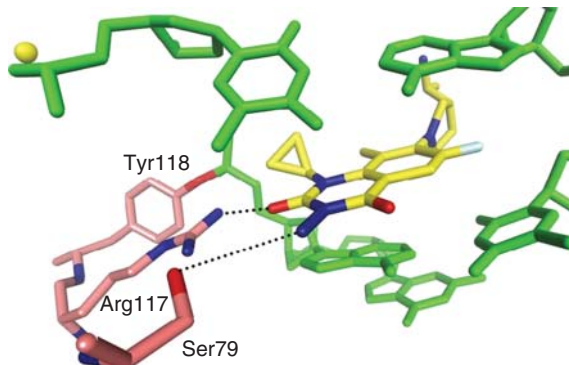
a) Minimum inhibitory concentration.

b) Supercoiling inhibition assay.

c) Wild type.

d) Efflux knockout mutant.

Figure 15.5 Structure of **7e** (yellow), bound to *S. pneumoniae* ParC, stacked between cleaved DNA base pairs (green). Key hydrogen bonds as black dotted lines (PDB code 3LTN) (PDB code: 2Y1O).



Despite interesting antibacterial properties and the marked lack of cross-resistance with FQs, neither ITQ nor 3-AQD antibiotics have moved into advanced stages of clinical development.

15.1.1.2 (Non-fluoroquinolone) Dual Inhibitors of DNA Topoisomerases

In 1999, GlaxoSmithKline (GSK) disclosed a novel family of antibacterial drugs based on the quinolyl propyl piperidine (QPP) scaffold obtained by degradation of quinine [31]. Even though the mode of action of this new series was undisclosed, the lack of cross-resistance with quinolone antibiotics made the new chemotype attractive to the scientific community. A couple of years later, Aventis disclosed related molecules such as AVE-6971 **8** (Figure 15.6) featuring an extra

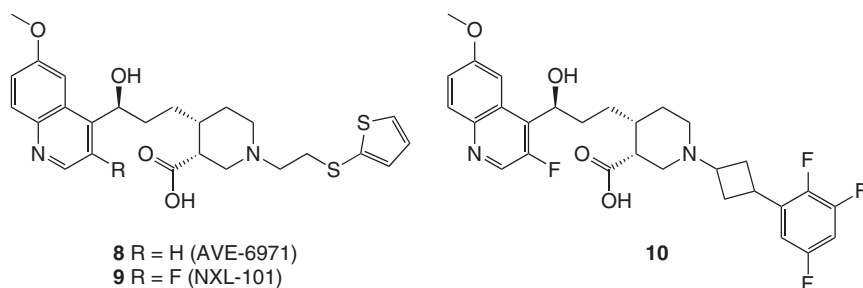


Figure 15.6 Bacterial topoisomerase inhibitors featuring a quinolyl propyl piperidine scaffold.

carboxylic acid function [32]. Lead optimization was carried out mainly through modification of both extremities of the lead molecule and NXL-101 **9** emerged as a clinical candidate. The antistaphylococcal activities of **9** were associated with potent inhibition of DNA gyrase rather than topoIV (which is opposite to the target bias usually observed with FQs). Furthermore, while cleaved-complex formation against *S. aureus* topoisomerases is a characteristic feature for the poisoning activity of FQs, no evidence of cleaved-complex formation was found using high concentration of **9** against these topoisomerases [33]. The profound difference in the mode of action between FQs and NXL-101 **9** was finally proven when key mutations conferring high resistance level to **9** were detected in QRDR of GyrA at Asp83 (to Asn) and Met121 (to Lys), which are amino acid mutations never observed with FQs [33].

To slow down the potential emergence of resistant mutants, a Pfizer research team embarked on designing dual and balanced DNA gyrase–topoIV inhibitors exploiting gaps in Aventis patents. Constrained side chains devoid of a thiophene moiety were introduced on the QPP scaffold [34]. Novel inhibitors were profiled using topoIV decatenation and DNA gyrase supercoiling assays. As shown in Table 15.3, compound **10** had improved topoIV inhibitory activity while retaining the same potency on DNA gyrase and exhibited good antibacterial activity on wild-type and quinolone-resistant *S. aureus* strains. The ~fourfold improved topoIV inhibition measured for **10**, comparatively to **9**, accounts for

Table 15.3 Inhibitory and antibacterial activities of molecules **9** and **10**.

Compounds	IC ₅₀ ^{a)} (μM)		MIC ^{b)} (μg/mL)		
	<i>S. aureus</i> topoIV	<i>S. aureus</i> gyrase	<i>S. aureus</i> ^{c)} wt	<i>S. aureus</i> ^{d)} QR	<i>S. aureus</i> ^{e)} D83N
9	30.4	1.5	0.125	0.125	32
10	8.4	1.5	≤0.063	0.125	8

a) Inhibition of wild-type (wt) *S. aureus* topoIV (decatenation assay) and gyrase (supercoiling assay).

b) Minimum inhibitory concentration.

c) Wild-type *S. aureus* strain 1146.

d) FQ-resistant *S. aureus* strain.

e) DNA gyrase D83N *S. aureus* mutant.

its lower MIC measured on a D83N *S. aureus* mutant. However, it remained unclear whether the inhibitory profile of **10** was dual enough to minimize the spontaneous emergence of mutants.

Beyond the resistance development consideration, the clinical development of NXL-101 **9** was halted because an unacceptable prolongation of the QT interval was detected. The phenomenon was linked to a significant hERG K⁺ channel inhibition (**9**: IC₅₀ = 23 μM). Compound **10** exhibited a similar potency with an IC₅₀ = 30 μM.

The QPP scaffold was rapidly abandoned by GSK researchers, who designed novel bacterial topoisomerase inhibitors (NBTIs) using a central saturated six-membered ring, on which two 1,4-oriented side chains of defined lengths were appended. An impressive number of aminopiperidine-based [35] and cyclohexyl-amide [36] inhibitors, such as **11–12** and **13–14**, respectively, was produced (Figure 15.7). Nearly exhaustive SAR investigations were conducted exploring both left-hand sides (LHS) and right-hand sides (RHS) as well as the substitution pattern of the central ring.

As shown in Table 15.4, all NBTIs exhibited excellent antibacterial activity against Gram-positive *S. aureus* and *S. pneumoniae* strains. The antibacterial activity of **11–12** against the fastidious Gram-negative *Haemophilus influenzae* strain matched well the *H. influenzae* DNA gyrase inhibitory activity. Neither inhibitory activity on topoIV enzymes nor resistance frequencies on key pathogens were reported. As inhibition of the hERG K⁺ channel was also of concern, the parameter was scrutinized during the optimization program. NBTIs **11–13** showed strong inhibition of hERG K⁺ channel. No correlation between hERG inhibition and lipophilicity could be drawn as minute structural changes resulted in dramatic variations. For instance, structurally related compounds

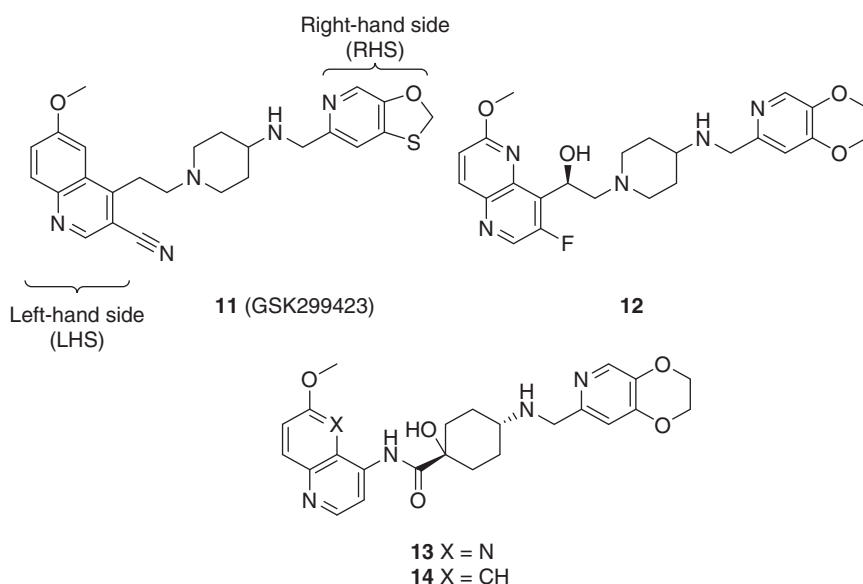


Figure 15.7 Novel bacterial topoisomerase inhibitors designed at GSK.

Table 15.4 Inhibitory and antibacterial activities of representative GSK NBTIs **11–14**.

Compounds	IC ₅₀ ^{a)} (μg/mL)	MIC ^{b)} (μg/mL)			IC ₅₀ ^{c)} (μM)
	DNA gyrase	<i>H. influenzae</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>	hERG inhibition
11	0.06 ^{d)}	0.25	0.01	0.01	2
12	0.078 ^{d)}	0.5	0.03	0.06	5
13	0.013 ^{e)}	0.5	0.01	0.125	24
14	0.01 ^{e)}	1	0.125	0.125	200
17^{f)}	0.035 ^{d)}	1	0.125	0.125	310

a) DNA replication inhibition scintillation proximity assay developed at GSK.

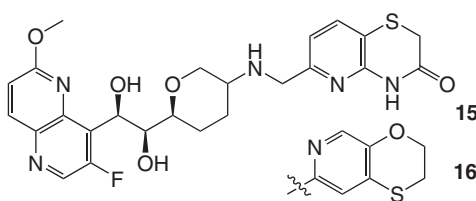
b) Minimum inhibitory concentration.

c) Measured using a whole-cell patch clamp assay.

d) Measured on *H. influenzae* enzyme.

e) Measured on *E. coli* enzyme.

f) See Figure 15.10.

**Figure 15.8** Novel bacterial topoisomerase inhibitors designed at Actelion.

13 and **14**, with equivalent $c \log P$ (~ 2.8), showed dramatic difference of hERG inhibition (IC₅₀s leaped from 24 to 200 μM).

Actelion has reported on a series of tetrahydropyran (THP)-based dual topoisomerase inhibitors such as compounds **15** and **16** (Figure 15.8) [37]. The design of these molecules was driven by the idea that hydroxyl groups introduced in the linker that unifies the central THP core to the LHS would improve water solubility. In this context, the *syn*-diol linker was found to be the most promising one. The nature of the RHS played as well a critical role in defining the properties of the inhibitors.

As shown in Table 15.5, compound **15** with a pyridothiazinone RHS is a dual DNA gyrase–topoIV inhibitor, whereas compound **16** featuring an oxathiinopyridine RHS is a rather DNA gyrase-biased inhibitor. The antibacterial activity against Gram-positive bacteria for both compounds was excellent, but the spontaneous resistance frequency (at 16× MIC) established on *S. aureus* for compound **15** was 10-fold lower than the one measured for compound **16** (data not shown), thereby confirming the importance of the dual mode of action. hERG inhibition was influenced by the nature of the RHS. Oxathiinopyridine-containing compounds seemed less prone to block the hERG K⁺ channel than pyridothiazinone-based ones as indicated by the lower inhibition values recorded for **16** as compared with **15**.

The 2.1 Å crystal structure of GSK299423 **11** in complex with DNA and *S. aureus* DNA gyrase shed light on the binding mode of NBTIs [38] (Figure 15.9).

Table 15.5 Inhibitory antibacterial activities and hERG block of two Actelion NBTIs.

Compounds	IC ₅₀ ^{a)} (μ M)		MIC ^{b)} (μ g/mL)			hERG inhibition (%) ^{c)}
	<i>S. aureus</i> gyrase	<i>S. aureus</i> topoIV	<i>S. aureus</i> ^{d)}	<i>S. pneumoniae</i> ^{e)}	<i>E. coli</i> ^{f)}	
15	0.03	0.5	≤ 0.03	≤ 0.03	1	54
16	0.03	8	≤ 0.03	≤ 0.03	8	19

a) Inhibition of wild-type (wt) and mutant *S. aureus* gyrase (supercoiling assay) and topoIV (decatenation assay).

b) Minimum inhibitory concentration.

c) Percent block of hERG K⁺ channel measured at 10 μ M using Qpatch.

d) *S. aureus* strain ATCC 29213.

e) *S. pneumoniae* strain ATCC 49619.

f) *E. coli* strain ATCC 25922.

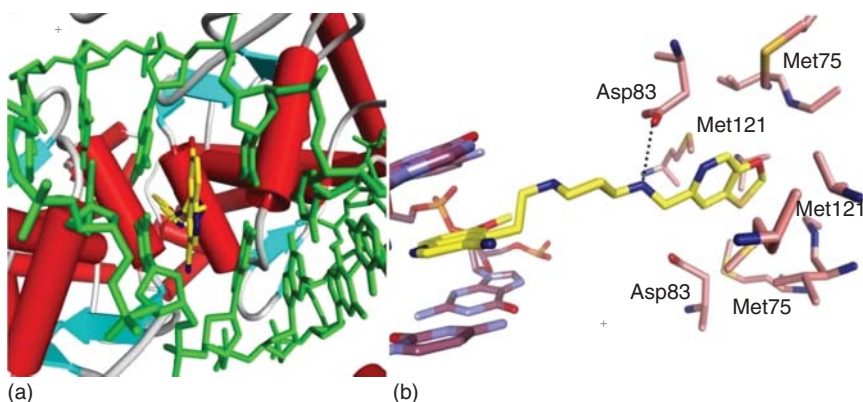


Figure 15.9 (a) Structure of **11** (yellow) bound at the interface between uncleaved DNA (green) and *S. aureus* GyrA subunits. (b) Close view of **11**. Only key GyrA amino acids are indicated. Key hydrogen bond as a black dotted line (PDB code: 2XCS).

Whereas two FQs bind to the DNA–topoIV cleaved complex (Figure 15.2), only one NBTI binds to a pre-cleavage complex midway between the two GyrA active sites on the twofold symmetry axis. The substituted bicyclic aromatic LHS intercalates between base pairs. A non-catalytic pocket appears upon complex formation and allows the heteroaromatic bicyclic RHS to bind via van der Waals interactions. The amino acid residues that form the RHS binding pocket are relatively well conserved in both DNA gyrase and topoIV enzymes across Gram-positive and Gram-negative bacteria (except for the Met-121 residue present in *S. aureus* exchanged to an Ile in Gram-negatives), thereby enabling the design of multitarget broad-spectrum antibacterials. However, Asp83, which forms a crucial interaction with the basic nitrogen of **11**, is nonessential and can easily mutate, leading to a loss of target inhibition and antibacterial activity.

The twofold symmetry of the binding site allows two equivalent binding modes for **11**. This observation probably triggered the design of NBTIs featuring a pseudosymmetric tricyclic LHS (Figure 15.10). Compound **17** displayed a

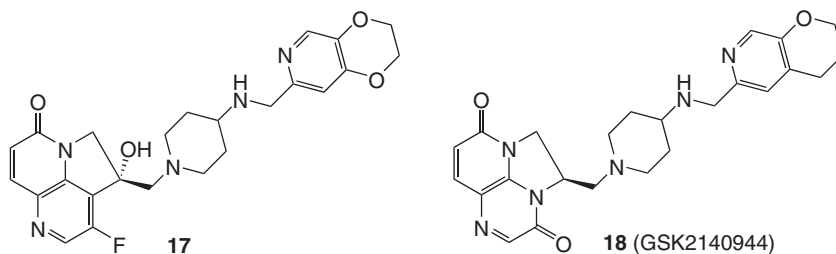


Figure 15.10 NBTIs featuring a tricyclic left-hand side.

strong inhibition of *H. influenzae* DNA gyrase enzyme, a potent antibacterial activity, and a remarkably lowered inhibition of hERG K⁺ channel (Table 15.4) [39]. Recently, GSK2140944 **18**, a structurally related compound, entered clinical development for the treatment of infections caused by biothreat and conventional pathogens [40].

There were intensive efforts dedicated to the design of topoisomerase inhibitors wherein the LHS was N-linked to an aminopiperidine central core. The aim remained to lower the hERG K⁺ channel inhibition while keeping a clinically relevant antibacterial profile. AstraZeneca [41] and Toyama [42] were the first to release almost simultaneously N-linked aminopiperidine inhibitors such as **20** (Figure 15.11). GSK reported similar derivatives shortly thereafter [43].

As shown in Table 15.6, derivative **19** displayed inhibition of *E. coli* topoIV ATPase activity, which correlated with good MICs on wild-type pathogens. However, hERG K⁺ channel inhibition had to be improved as prolongation of

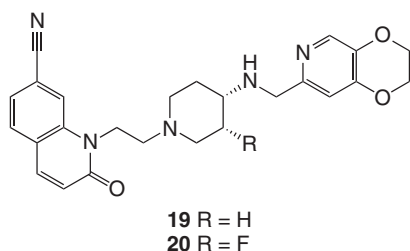


Figure 15.11 Representative NBTIs featuring a LHS N-linked to the central core.

Table 15.6 Characterization of N-linked aminopiperidines **19** and **20**.

Compounds	IC ₅₀ ^{a)} (μM)	MIC ^{b)} (μg/mL)			IC ₅₀ ^{c)} (μM)
	<i>E. coli</i> topoIV	<i>E. coli</i> ^{d)}	<i>S. aureus</i> ^{e)}	<i>S. pneumoniae</i> ^{f)}	hERG inhibition
19	3.2	0.5	0.03	0.08	44
20	48	4	0.06	0.13	233

a) ATPase activity inhibition assay.

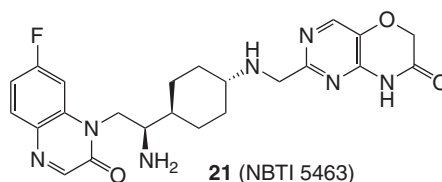
b) Minimum inhibitory concentration.

c) hERG Ionworks IC₅₀s.

d) *E. coli* W3110.

e) Methicillin-susceptible *S. aureus*.

f) *S. pneumoniae* D39 (penicillin susceptible).

Figure 15.12 NBTI featuring a cyclohexyl central core.**Table 15.7** Comparative biological activities of NBTI 5463 **21** and ciprofloxacin **6**.

Compounds	IC ₅₀ (μM)		MIC ^{a)} (μM)
	<i>E. coli</i> topoIV ^{b)}	<i>E. coli</i> gyrase ^{c)}	<i>E. coli</i> wt
21	0.006	0.02	0.06
6 (ciprofloxacin)	6	0.3	0.5

a) Minimum inhibitory concentration.

b) Relaxation inhibition assay.

c) Supercoiling inhibition assay.

the QT interval was still observed in preclinical animal models [44]. The basicity of the secondary amine that interacts with Asp83 was tuned accordingly by introducing a fluorine atom. The pK_a of the basic amine was decreased from 8.27 for **19** to 7.03 for **20**. The result was a sixfold reduction of the hERG inhibition for compound **20**. This improvement was made at the expense of *E. coli* topoIV inhibition that led to an elevation of the MIC against *E. coli*. Nevertheless, **20** still showed a potent antibacterial activity against Gram-positive organisms.

Subsequently, AstraZeneca reported N-linked cyclohexyl derivatives such as compound **21** (Figure 15.12) [45]. The basic nitrogen was shifted from the central core to the linking part, introducing a primary amine.

As shown in Table 15.7, NBTI5463 **21** featuring an unprecedented RHS targeted both *E. coli* DNA gyrase and topoIV and displayed good antibacterial activities against wild-type *E. coli* strain. The spontaneous mutation frequency measured on a *P. aeruginosa* strain was low (10^{-9} at $16\times$ MIC) [46].

As exemplified with compound **21**, oxazinone-containing RHSs seem to have a beneficial impact on antibacterial activity against Gram-negative strains [47]. As the Met-121 residue (Figure 15.9) present in *S. aureus* is replaced by a smaller Ile residue in Gram-negative topoisomerases, the hydrophobic pocket that accommodates the RHS appears slightly larger in Gram-negative bacteria. X-ray structures establishing the binding mode of the oxazinone moiety within this pocket would provide key information and ease further design efforts for new RHSs.

15.1.1.3 Designed Multitarget-Directed Ligands Addressing the ATP-Binding Pocket

Inhibitors of ATPase activity have the advantage to provide another novel mechanism of action against the bacterial type II topoisomerases and hence would be expected to overcome not only the existing resistance to FQs but also the resistance that will emerge for new GyrA/ParC antibacterials (vide supra).

Novobiocin **2** (Figure 15.1) was approved for clinical use in the 1960s and although toxicity has limited its use, it was administered in combination with rifampin to treat MRSA in the 1990s [48, 49]. Such a combination was necessary, not only because resistance to methicillin was developing and new treatment options were needed but also because novobiocin was needed to be protected from the resistance arising readily due to mutations in the *gyrB* gene [50, 51]. Studies on the mechanism of high-level novobiocin resistance acquisition in *S. aureus* showed that sequential specific point mutations occur first in the *gyrB* gene before the first point mutation could be observed in the *parE* gene [52]. At the enzyme level, novobiocin **2** was a more potent inhibitor of DNA gyrase ATPase activity (K_i GyrB = 19 nM) than of topoIV (K_i ParE = 900 nM), confirming that DNA gyrase was the primary and topoIV the secondary target in *S. aureus* [53]. Consequently, spontaneous resistance frequency of 1.2×10^{-8} was measured at a concentration of $8 \times \text{MIC}$ in accordance with values expected for a single-target agent. Inhibition of DNA gyrase and topoIV ATPase activity has been the subject of an intense research effort in the last two decades [54]. The ATP binding sites of gyrases and topoIVs share a great deal of homology, and the availability of X-ray structures allowed different approaches seeking to optimize the inhibition of these two enzymes in order to lower spontaneous resistance frequencies. A few recent contributions have been selected and are detailed in this section to illustrate the use of crystallographic data for the design of multitarget-directed ligands.

The pioneering work on GyrB/ParE multitarget inhibitors was performed by Vertex Pharmaceuticals [55]. Following an HTS looking for ATPase inhibition of *E. coli* gyrase using a focused library of approximately 30,000 compounds, the benzimidazole carbamate **22** (Figure 15.13) was identified as a modest inhibitor of both *S. aureus* and *E. coli* gyrases (Table 15.8). It did not show inhibitory activity of *E. coli* topoIV up to 60 μM and was inactive microbiologically.

Using both publicly available and proprietary structural information of the *E. coli* gyrase GyrB subunit [56], Charifson *et al.* designed two types of structural

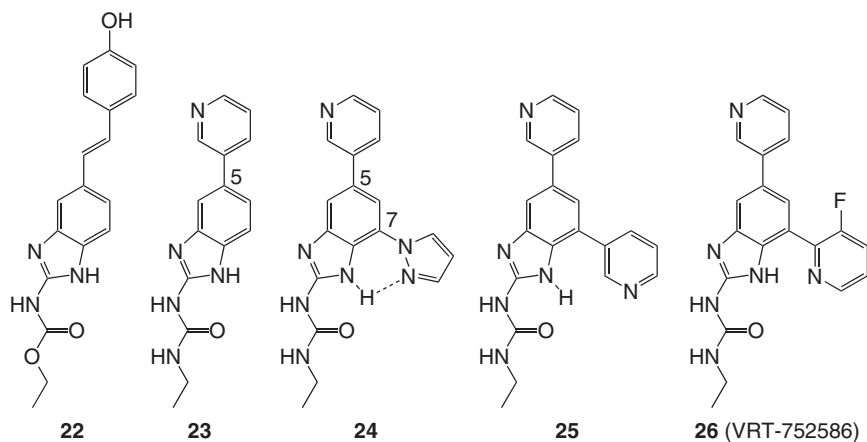


Figure 15.13 Novel GyrB/ParE inhibitors designed at Vertex.

Table 15.8 Gyrase/topoIV ATPase inhibition and antibacterial activity.

Compounds	K_i^a (μ M)			MIC^b (μ g/mL)
	<i>S. aureus</i> gyrase	<i>E. coli</i> gyrase	<i>E. coli</i> topoIV	<i>S. aureus</i> ^{c)}
Novobiocin 2	0.01	0.014	0.11	0.125
22	2	20	>60	>16
23	0.13	0.081	2.3	16
24	0.015	<0.004	0.046	0.063
25	0.017	0.006	1.3	4
26	0.014	<0.004	0.023	0.031

a) ATPase inhibition assay.

b) Minimum inhibitory concentration.

c) *S. aureus* strain ATCC 29213.

modifications that were implemented into **23** and allowed improving the binding to GyrB.

Firstly, the transformation of the carbamate moiety into the corresponding urea led to the replacement of a seemingly repulsive interaction of the oxygen with the Asp73 residue (*E. coli* numbering) by an attractive one (Figure 15.14). Asp73 is a residue conserved in both gyrase and topoIV of all bacterial species, and this hydrogen-bond network involving Asp73, the ligand, and a highly conserved water molecule is also observed upon binding of ATP and novobiocin. Secondly, the introduction of a heterocyclic hydrogen-bond acceptor on the C-5 position of the benzimidazole core mimicked a hydrogen-bond interaction existing between the coumarin ring of novobiocin and Arg136 [56].

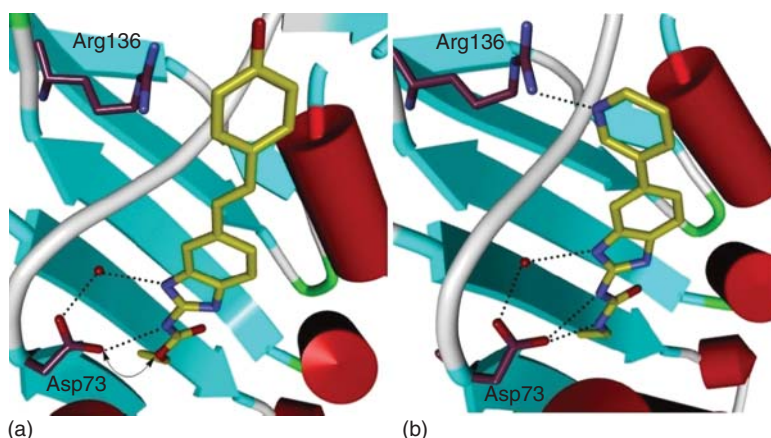


Figure 15.14 (a) Model of compound **22** (yellow) docked into the ATP-binding site, highlighting the interactions of the benzimidazole carbamate with water and Asp73. Potential repulsion between the carbamate oxygen and Asp73 is shown as a double arrow, with key hydrogen bonds as black dotted lines. (b) Model of compound **23** (yellow) docked into the ATP-binding site, highlighting the designed extended hydrogen-bond network involving **23**, Asp73, and the water, as well as the interaction with Arg136 (PDB code: 3FV5).

These first modifications resulted in a 250-fold and a 15-fold improvement in K_i against *E. coli* and *S. aureus* gyrase, respectively (Table 15.8). Interestingly, a >25-fold improvement in K_i against *E. coli* topoIV was also observed. Compound **23** started to show modest antibacterial activity with an MIC = 16 µg/mL against *S. aureus*.

In the absence of structural information for the ParE subunit, SAR studies were used to improve the activity against topoIV. Coplanarity of the C-7 substituent with the benzimidazole core appeared to be required for potent inhibition of topoIV as exemplified by the 30-fold loss in K_i for **25** relative to **24**. Indeed, the pyrazole ring in **24** is predicted to stay in plane with the benzimidazole core, owing to an intramolecular hydrogen bond, whereas the pyridine ring in **25** was predicted to be out of plane by approximately 60°. Vertex prospectively used quantum mechanical rotational barrier calculations to design and prioritize synthetic candidates based on this substituent coplanarity. Later, X-ray structures of novobiocin and benzimidazole urea derivatives bound to the topoIV ParE subunit of *E. coli* were solved [57]. The portion of the binding site accommodating the C-7 heteroaryl substituent appeared indeed slightly narrower in topoIV ParE than in gyrase GyrB, thereby confirming the coplanarity requirement for dual target inhibition. Compound **26** (VRT-752586) [55, 58, 59] is the result of further optimization and its spontaneous mutation frequency was measured as $<5.7 \times 10^{-10}$ ($8 \times$ MIC) in *S. aureus*. This frequency is much lower than the one measured for novobiocin **2** (1.2×10^{-8} at $8 \times$ MIC) and is consistent with the inhibition of more than one essential target in *S. aureus* (K_i = 14 nM on gyrase and <6 nM on topoIV), acknowledging the efforts invested in the design for multitarget inhibition. Importantly, compound **26** was shown to be selective for bacterial topoisomerase with no inhibitory activity of human topoisomerase II up to 25 µM, highlighting the differences that exist between prokaryotic enzymes and their human homolog. In addition, it showed an excellent antibacterial potency against susceptible and resistant Gram-positive and respiratory tract infecting pathogens *in vitro* as well as in animal infection models. The current development status of **26** or other derivatives from this series remains nevertheless unclear.

Trius Therapeutics has reported on another detailed design of DNA gyrase and topoIV dual inhibition taking advantage of a proprietary accumulated wealth of crystallographic data [60]. First, a fragment-based crystallographic screening effort was performed to identify scaffolds with adjacent hydrogen-bond donor and acceptor moieties capable of interacting with the conserved Asp73 and the structural water present in the active site. The hit pyrrolopyrimidine **27** (Figure 15.15) was selected for its advantage to display substitution sites facing all highly conserved sub-pockets of the GyrB and ParE ATP binding sites.

After a thorough analysis of their crystallographic data (Table 15.9), Trius showed that the volume of the ATP binding site “interior pocket” accessed with substitution on positions 5 and 6 was the smallest in the ParE subunit of Gram-negative bacteria *E. coli* and *Francisella tularensis*. All GyrB subunits were of intermediate volume, while the largest pocket was found in the ParE subunit of Gram-positive bacteria *Enterococcus faecalis*.

This observation was essential to identify inhibitors with a dual and balanced mode of action in both Gram-positive and Gram-negative bacteria. The chlorine atom and the ethyl group at positions 5 and 6 in **28–30** (Figure 15.15) were

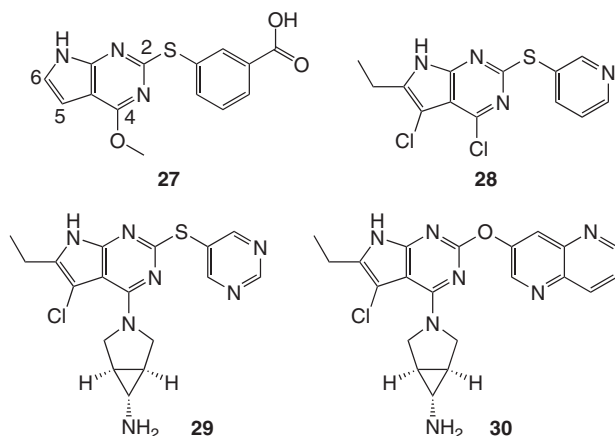


Figure 15.15 New pyrrolopyrimidines designed at Trius.

Table 15.9 Size of the ATP binding site interior pocket.

Enzymes	Volume ($\text{\AA}^3$)	Area ($\text{\AA}^2$)
<i>E. coli</i> ParE	116	121
<i>F. tularensis</i> ParE	129	126
<i>E. coli</i> GyrB	150	131
<i>E. faecalis</i> GyrB	141	134
<i>E. faecalis</i> ParE	196	165

able to fill optimally this interior pocket in all enzymes. Next, substitution at position 2 was designed to address the unique structural and electronic features of this active site region (Figure 15.16a). The heteroaryl substituents identified were interacting through π -cation interactions with a conserved salt bridge involving Arg76 and Glu50 (*E. coli* numbering). In addition, correctly positioned hydrogen-bond acceptors could also interact directly, or via a water molecule, with a conserved basic residue (typically a Lys or Arg) located on the outer rim of the salt-bridge pocket as previously observed with the 3-pyridyl group of **23–26** and the coumarin ring of novobiocin **2**.

Another characteristic important for the design of multitarget-directed ligand originated from the observation that a proline residue, located on the opposite side of the pocket as compared to the salt-bridge, was mobile and thereby narrowed the pocket in Gram-negative ParE. A sulfur atom was used to orient optimally the carefully optimized heteroaryl substituent over the salt bridge while providing sufficient flexibility to fulfill all interactions in every enzyme, including Gram-negative ParE. As shown for **28** (Table 15.10), an interesting balance in enzyme inhibition could be maintained in both Gram-positive (*E. faecalis*) and Gram-negative (*F. tularensis*) bacteria. Later, ether linkers appeared superior over sulfur, owing to lower conformational strain energy between the heteroaryl substituent and the pyrrolopyrimidine core in the bound conformation.

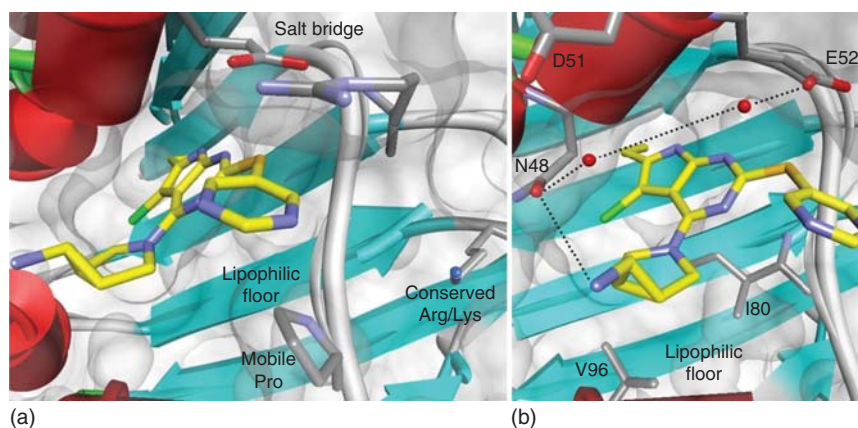


Figure 15.16 Structure of **29** (yellow) bound to *E. faecalis* GyrB. (a) Interaction with the salt bridge and the conserved Lys, and location of the mobile proline. (b) Interaction of the cyclic diamine with polar residues and solvent network. Key hydrogen bonds as black dotted lines (PDB code: 4GFN).

Table 15.10 Gyrase/topoIV ATPase inhibition and antibacterial activity.

Compounds	K_i^a (nM)						MIC ^{b)}
	<i>E. faecalis</i> gyrase	<i>E. faecalis</i> topoIV	<i>F. tularensis</i> gyrase	<i>F. tularensis</i> topoIV	<i>E. coli</i> gyrase	<i>E. coli</i> topoIV	
27	>100,000		>100,000				
28	400	3,500	500	2,000			
29	5	79	2	15		36	
30	<1	3	<1	<1	<1	<1	4
31	<0.3	<0.3	<0.3	<0.4	<0.05	<0.1	0.125
32	<0.3	<0.3	<0.3	0.8	0.3	0.9	0.125

a) ATPase inhibition assay.

b) Minimum inhibitory concentration ($\mu\text{g/mL}$).

c) *E. coli* strain ATCC 25922.

Finally, bicyclic diamines as in **29** and **30** proved to be the optimal substitution for position 4. They are coplanar with the central scaffold and occupy a region that is at the interface between the lipophilic interior pocket and the solvent (Figure 15.16b), such that the cycloheteroalkyl core faces a lipophilic environment on one side, while the amine with the appropriate stereochemistry is able to engage an ordered solvent network as well as polar residues in an anionic pocket on the other side. This clever design based on structural evidence resulted in exquisitely balanced inhibition of all enzymes tested (Table 15.10). Potent antibacterial activity was obtained against Gram-positive and respiratory tract-infecting pathogens (not shown), and significant activity was also observed for **30** against the more difficult-to-treat Gram-negative bacteria *E. coli* [61].

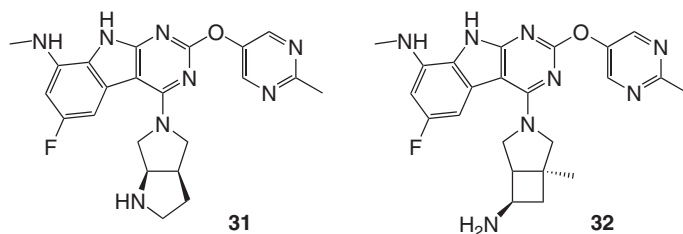


Figure 15.17 New pyrimidoindoles designed at Trius.

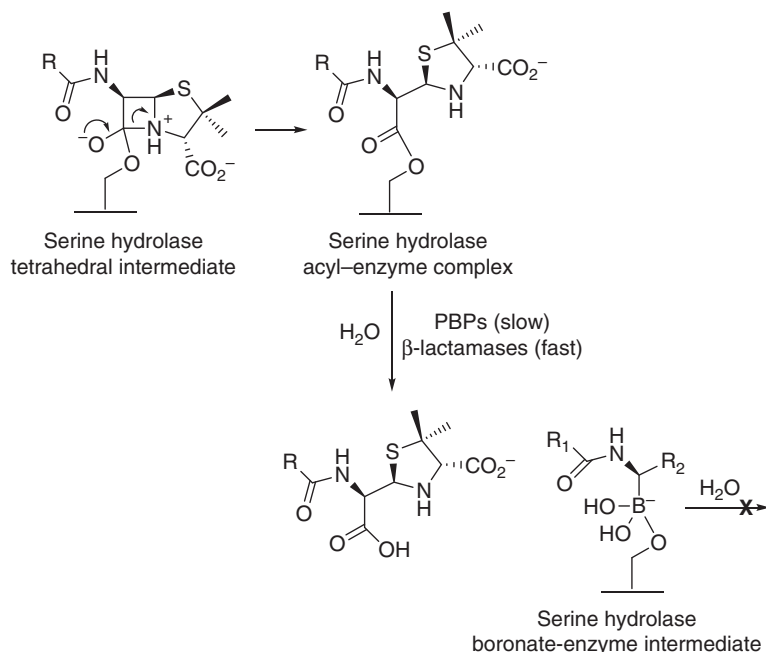
Recently, scientists at Trius went further in their design with new tricyclic pyrimidoindole inhibitors such as **31** and **32** (Figure 15.17) [62]. The additional aromatic ring, appropriately substituted with a fluorine atom allowed for an optimal and improved filling of the lipophilic interior pocket, while the aminomethyl group provided a second hydrogen-bond donor to interact with the conserved Asp73 residue (*E. coli* numbering), thereby increasing the binding affinity. The substitution at positions 2 and 4 are similar as in their first series. In *E. coli*, inhibitor **32** showed $K_i = 0.3$ nM for gyrase and 0.9 nM for topoIV inhibition (Table 15.10). This well-balanced dual activity resulted in very low spontaneous resistance frequencies of $<1.9 \times 10^{-11}$ at 4× MIC. In addition to this stability toward resistance development, compounds from this series showed exquisite antimicrobial activity against Gram-negative pathogens *in vitro* and *in vivo* [62, 63]. Best compounds in this series are currently undergoing further investigations as well as preclinical evaluation.

15.1.2 Multitarget Inhibitors of Peptidoglycan Biosynthesis

Peptidoglycan is an essential component of the cell envelope of Gram-positive and Gram-negative bacteria. It maintains cell rigidity, flexibility, and strength in growing bacteria while withstanding the high internal osmotic pressure and protecting them against the hostile environment. A large number of clinically used antibacterial drugs act on the peptidoglycan biosynthesis. For instance, β -lactams and glycopeptides inhibit the late-stage cross-linking of peptidoglycan by covalently acylating the PBPs or by blocking the function of transglycosidases, respectively.

In recent years, reversible inhibitors of PBPs that are not susceptible to the hydrolytic activity of β -lactamases have been identified. Catalytic activities of serine hydrolases (PBPs and serine β -lactamases) involve tetrahedral intermediates that evolve to acyl-enzyme complexes. Several warheads were identified as mimics of such transition states using publicly available PBPs structures (Scheme 15.2).

Among them, boron derivatives such as compound **33a** displayed significant inhibitory activity ($IC_{50} = 33$ μ M) against a class C PBP isolated from *Actinomyces* sp. (Figure 15.18). Further structure-guided hit-to-lead optimization led to more potent inhibitors such as compound **33b** ($IC_{50} = 1.8$ μ M) [64]. Derivative **34** was later reported to inhibit a class A PBP (PBP1b from *S. pneumoniae*, $IC_{50} = 26$ μ M), a class B PBP (PBP2xR6 from *S. pneumoniae*, $IC_{50} = 138$ μ M), and



Scheme 15.2 Reaction intermediates of β -lactams with PBPs and β -lactamases. Boronate enzyme intermediate.

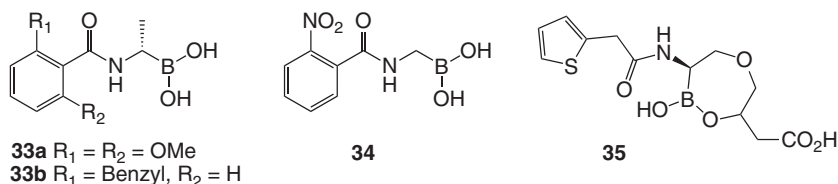


Figure 15.18 Boronic acid-based penicillin-binding-protein inhibitors.

a class C PBP (R39 from *Actinomadura* sp., $\text{IC}_{50} = 0.6 \mu\text{M}$) [65]. Heterocyclic boronate derivatives such as compound **35** have been recently claimed to target both classes C and D PBPs [66].

Mur ligases represent another family of attractive antibacterial drug targets that intervene during the peptidoglycan biosynthesis. MurC, MurD, MurE, and MurF are essential enzymes involved in the cytoplasmic biosynthesis of UDP-*N*-acetylmuramoyl (UDP-MurNAc)-pentapeptide (Park's nucleotide) using UDP-MurNAc as the starting substrate. MurC–F ligases catalyze the formation of an amide bond between the terminal carboxylic acid group of the elongating UDP-peptidic chain and a specific condensing amino acid, respectively, *L*-Ala (MurC), *D*-Glu (MurD), meso-diaminopimelic acid or *L*-Lys (MurE), and *D*-Ala-*D*-Ala dipeptide (MurF). They use ATP to form an acyl phosphate intermediate that further reacts with the condensing amino acid to afford the elongated peptide after elimination of inorganic phosphate. Crystal structures

of Mur ligases isolated from various bacterial strains show that these enzymes share a similar topology consisting of three domains. The C-terminal domain, which binds the respective condensing amino acids or dipeptide residues, and the central domain that contains the ATP-binding site are well conserved across Mur ligases. The N-terminal domain varies more significantly as its topology depends on the length of the UDP substrate it accommodates. Blocking the UDP substrate binding site of two ligases involved in consecutive steps of Park's nucleotide biosynthesis with a single entity is an appealing strategy since the designed inhibitors could potentially resemble either the product or the substrate of these enzymes. The initial investigations focused on synthesizing *N*-acylhydrazone derivatives. Some compounds showed inhibitory activities in the micromolar range against both MurC and MurD isolated from *E. coli*, but the antibacterial activities were rather disappointing [67]. Attempts to identify dual MurD–MurE [68] or dual MurD–MurF [69] inhibitors using a virtual screening approach did not deliver hit compounds with compelling chemical structures or satisfactory biochemical properties. In parallel, glutamic acid-based MurD inhibitors were designed using 2,4-diaminoquinazoline, quinazoline-2,4-dione or thiazolidine-2,4-dione ring systems to anchor the inhibitor to the target protein. Among those, compounds featuring the thiazolidinone ring were the most promising ones. Subsequent investigations led to compounds such as **36** (Figure 15.19), which displayed inhibitory activities around $\sim 500\ \mu\text{M}$ against *E. coli* MurD [70, 71] (Table 15.11).

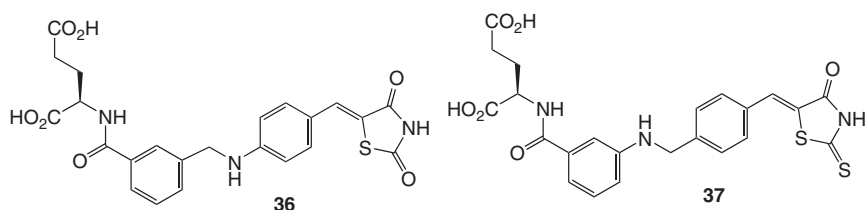


Figure 15.19 Chemical structures of Mur ligase inhibitors.

Table 15.11 Inhibitory activity of **36** and **37** against MurD and MurE ligases.

Enzymes	Residual activity ^a (%)	IC ₅₀ ^b (μM)
	36	37
<i>E. coli</i> MurD ligase	35	8.2
<i>S. aureus</i> MurD ligase	ND	6.4
<i>E. coli</i> MurE ligase	64	180
<i>S. aureus</i> MurE ligase	ND	17

ND, not determined.

a) Measured at $500\ \mu\text{M}$ compound.

b) PO_4^{3-} generated during the ligation reaction was measured by a colorimetric malachite green-based assay.

Slight modifications of the linker connecting the two phenyl rings resulted in improved inhibitory potency and 2-thioxothiazolidin-4-one **37** inhibited MurD enzymes isolated from *E. coli* and *S. aureus* with similar potency (8.2 and 6.4 μM , respectively). In addition, it showed some inhibitory activity against MurE enzymes and displayed weak antibacterial activity against *S. aureus* (MIC = 8 $\mu\text{g/mL}$) [72]. As shown in Figure 15.20, X-ray structure of **37** bound to *E. coli* MurD provides key information on its binding mode. Using the carboxylic acid of the Glu side chain and the thiazolidin-4-one ring as anchor points, more potent inhibitors with improved *in vitro* antibacterial activity may be designed. Nevertheless, *in vivo* efficacy has to be demonstrated to comfort Mur ligases as validated antibacterial drug targets.

15.1.3 Multitarget Inhibitors of Type II Fatty Acid Synthases

Type II fatty acid biosynthesis is another essential process that ensures bacterial cell viability. As shown in Scheme 15.3, FabH, FabG, FabA/Z, FabI/K/L, and FabB/F are enzymes involved in the synthesis of β -ketoacyl-acyl carrier protein (ACP), and several chemically unrelated inhibitors of this pathway are known. For example, both triclosan **38**, an antiseptic present in soaps, and isoniazid **39**, used in the treatment of tuberculosis, target FabI. One key issue associated with FabI inhibitors is their lack of broad-spectrum activity as *S. pneumoniae* expresses a different enoyl-ACP reductase (FabK). Cerulenin **40** is an irreversible and selective FabF/B inhibitor.

In the context of multitarget-directed ligands, two natural products are of particular interest: platencin **41** [73] and thiolactomycin **42** [74] isolated from *Streptomyces platensis* and a soil *Nocardia* species, respectively (Figure 15.21). Both entities have been reported to inhibit the elongation β -ketoacyl-ACP synthases I/II (FabB/F) as well as the initiation β -ketoacyl-ACP synthase III (FabH). Platencin **41** and its analog platensimycin **43** display broad anti-Gram-positive activity, whereas thiolactomycin **42** is only weakly active

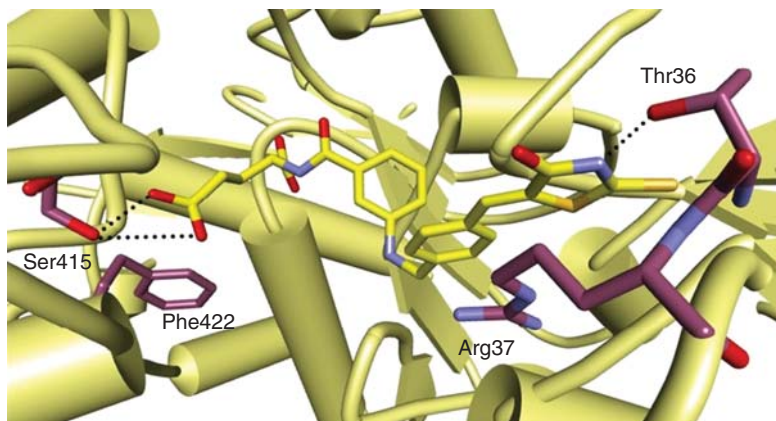
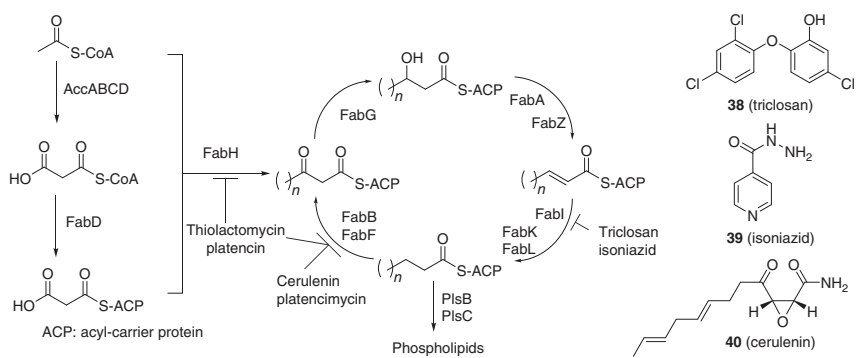


Figure 15.20 Structure of compound **37** (yellow) bound to *E. coli* MurD. Key direct hydrogen bonds as black dotted lines (PDB code: 2Y1O).



Scheme 15.3 Fatty acid biosynthesis and known sites of inhibition.

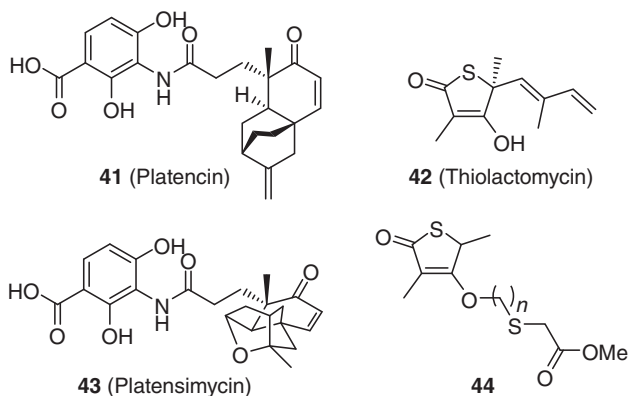


Figure 15.21 Chemical structures of fatty acid synthases inhibitors.

against some Gram-negative pathogens. Both antibacterial classes showed poor *in vivo* efficacy in animal models due to suboptimal pharmacokinetic properties. Recently, thiolactomycin **42** was shown to act as a dual inhibitor of KasA and KasB, two β -ketoacyl-ACP synthases found in *Mycobacterium tuberculosis* and involved in the synthesis of mycolic acid with IC_{50} s = 20 and 90 μ M, respectively [75]. Efforts to optimize the antimycobacterial activity consisted, for instance, in introducing alkyl substituents on the enol function, leading to compound **44** ($n = 8$). The latter displayed MIC values ranging from 1 to 4 μ g/mL against drug-sensitive and drug-resistant strains of *M. tuberculosis* [76, 77]. Platensimycin **43** has also been reported to be a dual *M. tuberculosis* KasA and KasB inhibitor with IC_{50} s = 2 and 4.2 μ g/mL, respectively [78]. In light of their intriguing dual inhibitory mode of action, thiolactomycin **42** and platensimycin **43** might be valuable entry points to design new antituberculosis drugs.

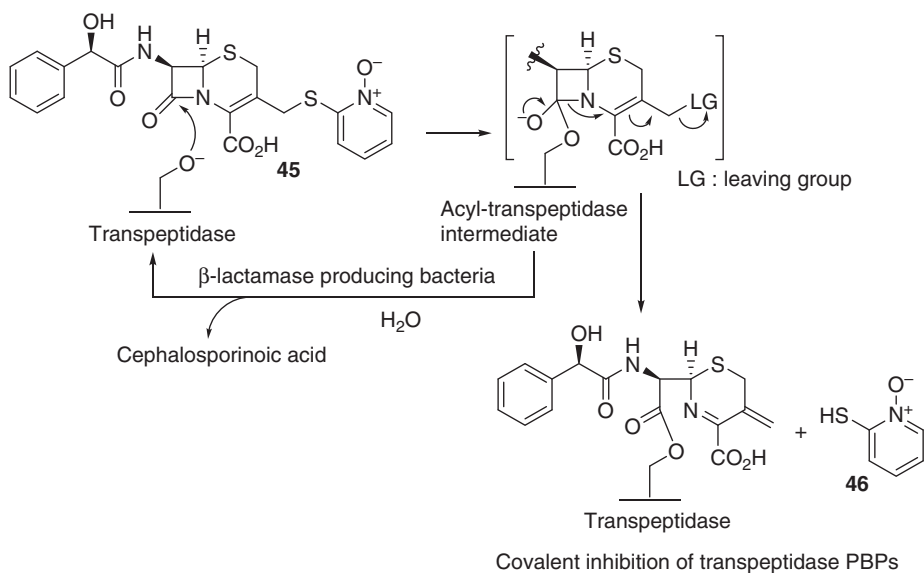
15.2 Design of Hybrid Molecules: Dual Pharmacophores Acting on Multiple Targets

Creating a novel antibacterial agent fusing together two known drugs acting on different cellular targets is an attractive concept to achieve multitarget inhibition. The design of such hybrid molecules can make use of a multitude of approved drugs. The expected advantage associated with hybrid molecules is an extended spectrum of activity, with one agent bridging the gaps of the second one, and vice versa. However, hybrid molecules must offer clear advantages over a combination of the two individual agents, not only in terms of antibacterial profile (spectrum and stability toward resistance development) but also in terms of pharmacokinetics and pharmacodynamics. The reported designs of hybrid molecules concentrated mostly on the choice of the partners and their linkage, tailored to preserve their respective inhibitory potency. As illustrated hereafter, some design contributions also involved a chemical or an enzymatic transformation in order for both pharmacophores to be operative.

15.2.1 Cephalosporin-Based Hybrid Molecules

β -Lactam antibiotics exhibit a broad antibacterial spectrum, covering Gram-positive and Gram-negative bacteria. However, bacteria such as *S. aureus*, *Klebsiella pneumoniae*, and *E. coli* have become rapidly resistant by expressing enzymes called β -lactamases that hydrolyze the β -lactam ring. As shown in Scheme 15.4, cephalosporins exert their antibacterial activity by acylating the active site serine residue present in the transpeptidase domain of PBPs, essential proteins involved in the cross-linking of peptidoglycan (see Section 15.1.2). The cleavage of the β -lactam ring results in the irreversible inactivation of the catalytic serine residue, thereby releasing a small entity called the leaving group (LG). The design of first cleavable conjugates was realized in the mid-1970s based on the idea that LGs of cephalosporins could act as antibacterial drugs and kill β -lactamase-expressing bacteria [79]. In this context, one of the first derivative made, **45**, utilized 2-mercaptopyridine *N*-oxide **46**. Unfortunately, the toxicity of **46** prevented any further use of cephalosporin **45**.

Researchers at Roche have thoroughly explored this concept, designing and producing novel cephalosporin-FQs hybrid molecules (Figure 15.22). The quinolone nucleus offered two handles suitable for constructing a linkage. Hence, first-generation molecules were built by alkylating the carboxylic acid of the FQ nucleus with a third-generation cephalosporin nucleus (e.g., the cefotaxime nucleus). Among those, Ro 23-9424 **47** demonstrated impressive *in vitro* whole-cell activity with MIC <2 μ g/mL against cephalosporin-resistant *S. aureus* strains [80]. The dual mode of action was postulated based on *in vitro* inhibition of PBPs. It was also shown that **47** was more active against some bacterial strains than the 1 : 1 combination of its components (deacetylcefotaxime



Scheme 15.4 Mechanism of transpeptidase-induced release of **46**.

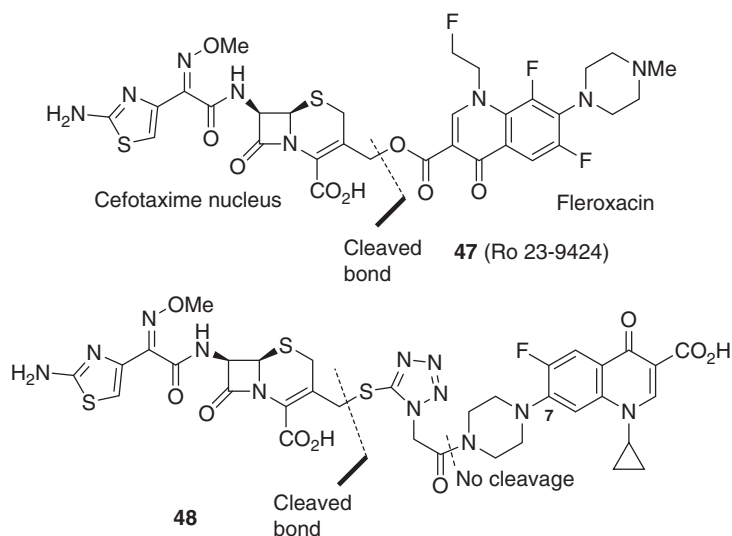


Figure 15.22 β -Lactam–fluoroquinolone hybrid molecules and their cleavage sites.

and fleroxacin). However, the enzyme-mediated mode of action could be not ascertained as some hydrolytic instability of **47** was observed in human plasma.

Several generations of β -lactam–FQ hybrid molecules were subsequently designed using an attachment point on the FQ C-7 side ring. This ring was either directly connected to the cephalosporin [81] or tethered to it via an extra spacer as in compound **48** [82]. In the latter case, further cleavage of the amide bond was anticipated in order to release ciprofloxacin **6**, but this did not occur. The LG containing the FQ exhibited weak intrinsic antibacterial activity only. As a result, the *in vitro* spectrum of **48** mirrored merely that of a third-generation cephalosporin and was devoid of quinolone-like activity.

A promising family of glycopeptide– β -lactam hybrid molecules (called heterodimers) has been designed, synthesized, and characterized at Theravance. Glycopeptide antibiotics, such as vancomycin, form a tight complex with D-Ala–D-Ala motifs present in peptidoglycan biosynthetic intermediates and lead to immature peptidoglycan strands that rupture, resulting in bacterial death. Unfortunately, resistance to glycopeptides is nowadays well established among Gram-positive pathogens. Vancomycin offers many handles suitable for attaching a cephalosporin nucleus (Figure 15.23). The second end of the linker can be attached either on the LG or on the C-7 side chain of the β -lactam component. The first series of hybrid molecules built from the vancosamine sugar of vancomycin [83] was reported and showed antibacterial activity against methicillin-resistant strains. Further optimization eventually led to the heterodimer TD-1792 **49**, obtained by linking vancomycin through its carboxylic acid moiety to THRX-169797, the cephalosporin component. As shown in Table 15.12, hybrid **49** showed identical antibacterial activity against staphylococci strains resistant to either vancomycin and/or THRX-169797. It has also been shown that hybrid **49** has restored activity against staphylococci strains

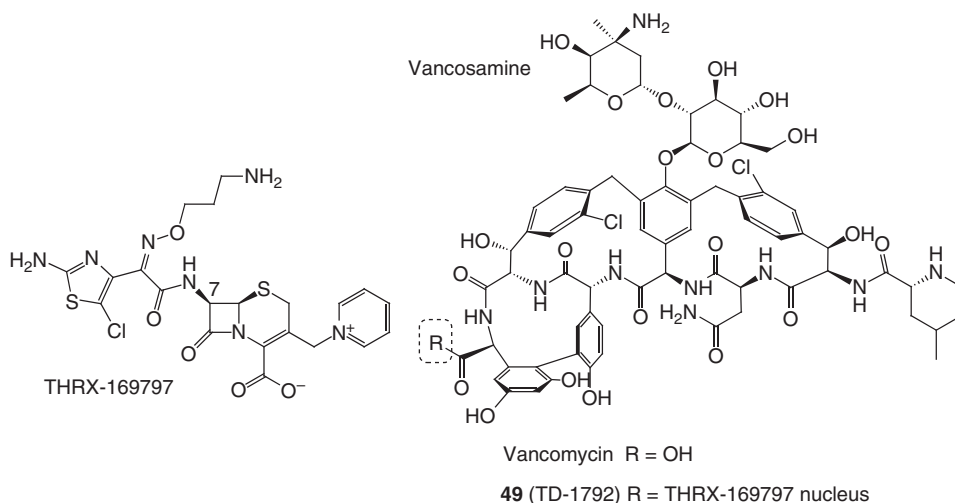


Figure 15.23 Structure of heterodimer **49** (TD-1792) and its two components.

Table 15.12 Antibacterial activity of hybrid **49** and its components against *S. aureus* strains.

	MIC ^{a)}			
	Methicillin-sensitive <i>Staphylococcus aureus</i> (MSSA) ^{b)}	MRSA ^{c)}	Vancomycin-intermediate <i>Staphylococcus aureus</i> (VISA) ^{d)}	Vancomycin-resistant <i>Staphylococcus aureus</i> (VRSA) ^{e)}
Vancomycin	0.78	0.78	6.25	8
THR-169797	1.56	25	25	ND
49 (TD-1792)	≤0.05	≤0.05	≤0.05	0.06

ND, not determined.

a) Minimum inhibitory concentration (μg/mL).

b) Methicillin-susceptible *S. aureus* ATCC 13709.

c) Methicillin-resistant *S. aureus* ATCC 33591.

d) Vancomycin-intermediate *S. aureus* HIP 5836.

e) Vancomycin-resistant *S. aureus* NJ992.

classes A, B, C, and D producing β-lactamases [84]. This peculiar antibacterial profile supported a dual mechanism of action. Furthermore, compound **49** (TD-1792) was efficacious *in vivo* against *S. aureus* in a murine thigh infection model, with ED₅₀ much lower than those usually observed for vancomycin [85]. The heterodimer **49** has recently entered Phase II clinical trial.

15.2.2 Fluoroquinolone–Oxazolidinone Hybrid Drugs

There are an impressive number of hybrid drugs that have been designed using the FQ nucleus as pharmacophore. In addition to β-lactam-based hybrids described in Section 15.2.1, nearly all classes of available antibacterial drugs have been covalently linked to FQs: oxazolidinones [86–88], anilinoaracils [89], rifamycins [90], macrolides [91], tetracyclines [92], aminoglycosides [93], and

trimethoprim [94]. The quinolone nucleus possesses several advantages that make it an excellent partner: broad antibacterial spectrum, easiness of chemical modifications, well-established SARs, known safety/toxicity profile, and low cost of goods. Oxazolidinone antibiotics inhibit bacterial protein synthesis by binding at the A-site of the ribosomal 50S subunit [95]. While oxazolidinones are ubiquitous protein synthesis inhibitors, they display antibacterial activity against Gram-positive pathogens only. Their inability to accumulate in Gram-negative bacteria prevents any activity against these organisms. To increase the cytoplasmic accumulation in Gram-negatives of oxazolidinones, several research teams had the idea to link them to a FQ nucleus. The first hybrid molecules were obtained by merging the 6-membered rings present in linezolid **50** and ciprofloxacin **6** (Figure 15.24).

The initial reports showed that the linker markedly influences the antibacterial profile of the hybrid compound. As shown in Table 15.13, when the linker was a rigid piperazine ring, compound **51** behaved as an oxazolidinone (weak inhibition of topoisomerase enzymes and low potency against a linezolid-resistant *S. aureus* strain). For compounds **52–54** with more flexible linkers, both modes of action operated and MICs improved. Compound **54**, articulated on a hydroxymethyl pyrrolidine spacer, displayed strong inhibitions of protein synthesis and DNA gyrase *in vitro* and was equally potent on wild-type and on linezolid- and quinolone-resistant *S. aureus* strains [86]. It also showed antibacterial activity on *H. influenzae* and wild-type *E. coli* stains, however with MIC ~ 30-fold higher than those of ciprofloxacin (data not shown).

Cadazolid **55** (Figure 15.25), which features oxazolidinone and FQ structural motives, has entered phase III of clinical development for the treatment of *Clostridium difficile*-associated diarrhea (CDAD). Cadazolid **55** shows potent *in vitro* activity against a wide range of *C. difficile* strains including strains resistant to linezolid and FQs [96]. Acting as a strong protein synthesis inhibitor, it blocks

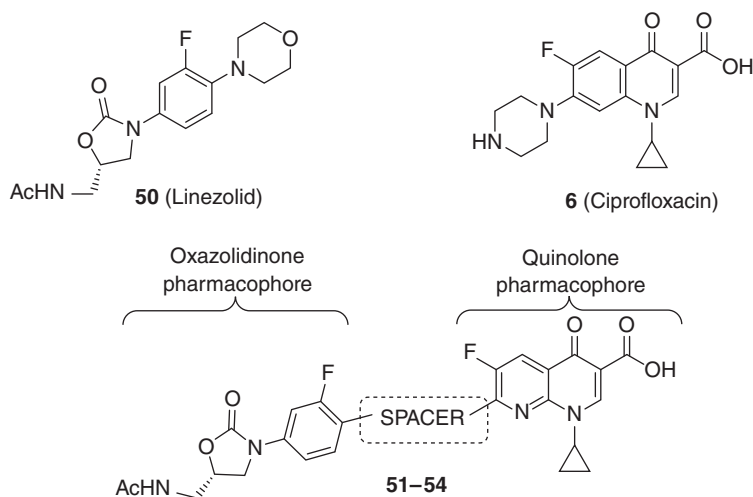


Figure 15.24 Structures of hybrid molecules based on linezolid and ciprofloxacin.

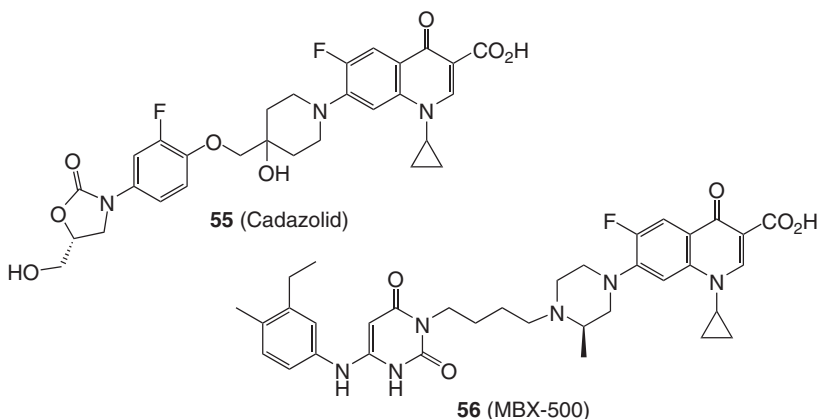
Table 15.13 Characterization of oxazolidinone–fluoroquinolone hybrid molecules.

Spacer	Compounds	IC ₅₀ (μM)			MIC (μg/mL)		
		<i>E. coli</i> gyrase ^{a)}	<i>E. coli</i> topoIV ^{b)}	<i>E. coli</i> protein synthesis ^{c)}	<i>S. aureus</i> wt	<i>S. aureus</i> CIP ^R	<i>S. aureus</i> LZD ^R
	51	50	10	2.8	0.25	0.25	8
	52	10	3	6.7	≤0.06	0.25	≤0.06
	53	50	5	5.2	0.25	0.25	1
	54	2	2	1.5	0.125	0.125	≤0.06
	50	NT	NT	4.1	0.5	1	64
	6	0.5	2.5	>20	0.5	>32	0.5

NT, not tested.

a) Supercoiling inhibition assay.

b) Relaxation inhibition assay.

c) *In vitro* transcription/translation assay with *E. coli* S30 Extract System.**Figure 15.25** Hybrid molecules with *in vitro* and *in vivo* anti-*Clostridium difficile* activities.

at relatively low concentration ($1 \times \text{MIC}$) *de novo* production of toxins A and B, considered to be responsible for most of the clinical complications (diarrhea and colitis).

15.2.3 Fluoroquinolone–Aminouracil Hybrid Drugs

6-Anilinoouracils (6-AUs) are nonclinically validated antibacterial drugs that selectively bind and inhibit the bacterial DNA polymerase III (DNA Pol III), an

essential enzyme involved in the bacterial DNA replication machinery. The spectrum of activity of the 6-AU molecules is restricted to Gram-positive pathogens, as DNA Pol III_C has little homology with its Gram-negative DNA polymerase III counterpart. Hybrid molecules, combining the FQ nucleus and the 6-AU pharmacophore, were initially synthesized to cover antibiotic-resistant Gram-positive organisms. MBX-500 **56** (Figure 15.25) inhibited DNA topoisomerases to a lesser extent (~ 10 -fold) than ciprofloxacin *in vitro* [97]. On the other hand, **56** was significantly more potent against DNA Pol III_C than its AU component alone, suggesting that the FQ nucleus contributed markedly to the binding. Compound **56** exhibited excellent antibacterial activity against wild-type and resistant Gram-positive pathogens. Its dual mode of action was further confirmed *in vitro* against strains that were resistant to FQs or 6-AUs, and, remarkably, **56** was more active than the *ad hoc* combination of its two components.

15.2.4 Fluoroquinolizine–Rifamycin Hybrid Drugs

Rifamycins are macrocyclic antibiotics that suppress RNA synthesis by inhibiting DNA-dependent RNA polymerase. Rifamycins are clinically utilized for the treatment of tuberculosis as well as infections caused by Gram-positive pathogens. However, resistance due to rapid target mutation has restricted their use to combination therapies. To improve the stability toward resistance development and to broaden the antibacterial spectrum, hybrid molecules designed on rifampin (a rifamycin antibiotic containing a hydrazone side chain) have been prepared. Researchers at Cumbre chose to covalently link rifamycin SV (Figure 15.26) to a 4*H*-4-oxo-quinolizine pharmacophore [98], an FQ bioisostere with proven activity against ciprofloxacin-resistant strains (Figure 15.26) [90]. CBR-2092 **57** displayed rifampin-like potency against wild-type *S. aureus* RNA polymerase with an $IC_{50} = 0.034 \mu M$ (rifampin $IC_{50} = 0.015 \mu M$) and balanced inhibition of both *S. aureus* DNA gyrase and topoIV with $IC_{50}s \sim 1.5 \mu M$. Acting as a multitarget-directed ligand, compound **57** exhibited antistaphylococcal activity against a wide range of sensitive and resistant strains. After completing a phase I program, the clinical development of CBR-2092 **57** has likely been discontinued.

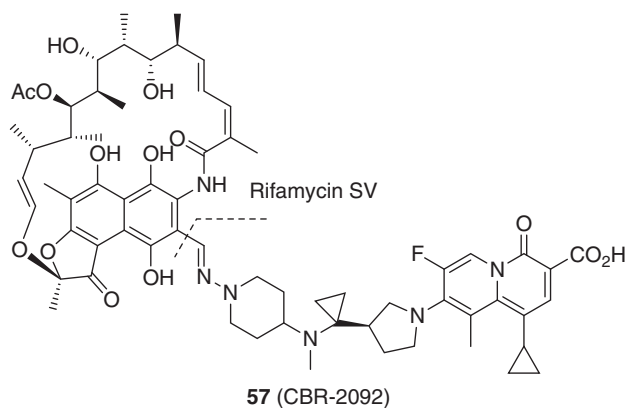


Figure 15.26 Structure of rifamycin and its fluoroquinolizine hybrid molecule **57**.

15.2.5 Hybrid Molecules: Limitations and Perspectives

To date, no hybrid molecule designed by linking two antibacterial pharmacophores has moved to advanced development phases for the oral treatment of systemic bacterial infections. As illustrated with the abovementioned examples, hybrid molecules were largely designed to fulfill microbiological requirements (coverage of multiresistant pathogens, low spontaneous mutation frequencies), thereby paying less attention to physicochemical properties required for oral absorption [99]. Studies on the optimal physicochemical property space of antibiotics have shown that antibacterial drugs, and especially those addressing Gram-negative pathogens, are less lipophilic than other drugs [100]. In this case, the outer membrane of Gram-negative bacteria acts as a permeability barrier and only compounds with well-defined attributes (especially polarity) can penetrate. As shown in Figure 15.27, oxazolidinone–FQ hybrid molecule MCB-3681 **58a** built from linezolid **50** and ciprofloxacin **6** displayed Gram-positive antibacterial activity only [101]. The advantageous features of the FQ nucleus leading to cytoplasmic accumulation in Gram-negatives could not be maintained in hybrid **58a**. In addition, with a molecular weight over 600 Da **58a** is not orally active (while both parent drugs are) and was intended to be intravenously administered as its phosphate prodrug MCB-3837 **58b** [102]. The rifamycin–quinolone hybrid **57** depicted in Figure 15.26 suffered from similar drawbacks and failed at the early development stage.

In an attempt to circumvent issues associated with unfavorable physicochemical properties, a novel class of low-molecular weight compounds illustrated

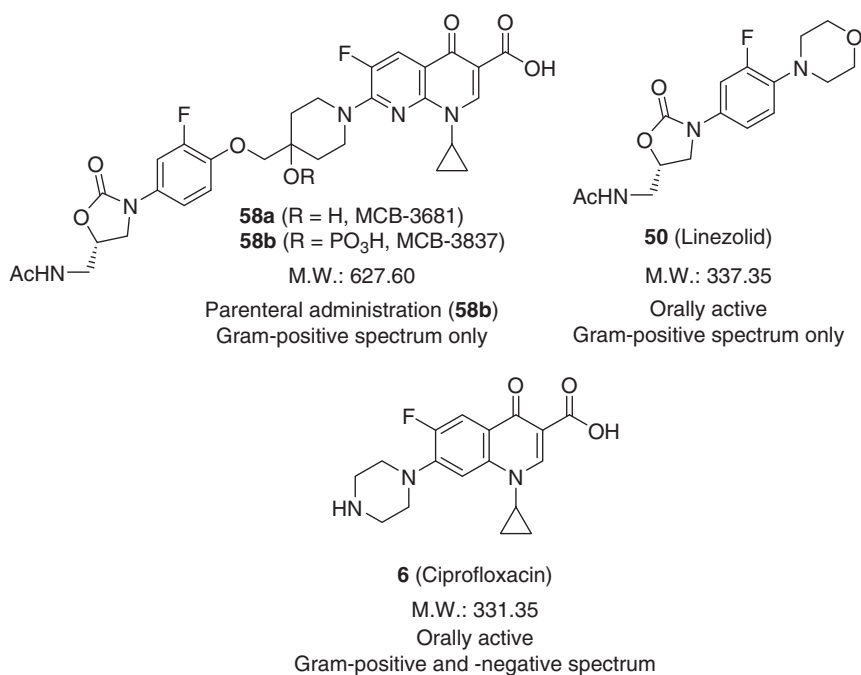


Figure 15.27 Comparative properties of compound **58a** (MCB-3681), linezolid, and ciprofloxacin.

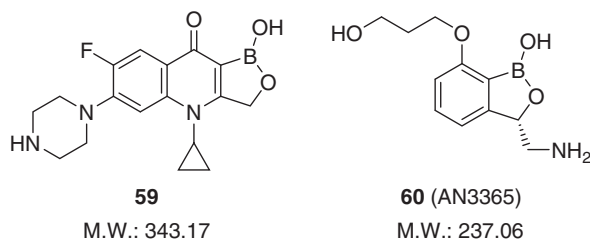


Figure 15.28 Novel oxaborole containing antibiotics.

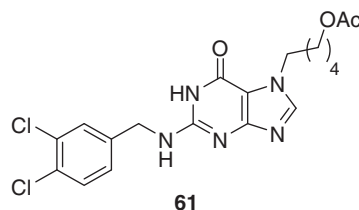
by compound **59** has been designed by merging ciprofloxacin **6** with the oxaborole-containing antibiotic **60** (Figure 15.28). This class of antibacterial compounds inhibits bacterial leucyl-tRNA synthetase by blocking its editing site that ensures the integrity of tRNA^{Leu}. As tRNA synthetases are essential enzymes that supply the building blocks for protein synthesis, interfering with their function leads eventually to bacterial death. It has been shown that boron-containing compound **60** displays exquisite antibacterial activity against *Enterobacteriaceae* and non-fermentative Gram-negative bacilli [103]. The design hypothesis was based on mimicking the 3-carboxylic acid of ciprofloxacin by an oxaborole-fused ring [104]. Unfortunately, compound **59** showed only modest antibacterial activity that was in part explained by the weak acidity of the oxaborole moiety.

15.3 Emerging Antibacterial Drugs Allowing Multitarget-Directed Ligand Design

The recent failure in phase II clinical trials of AN3365 **60** (Figure 15.28), a single-enzyme target inhibitor, due to rapid resistance development is a further testimony in favor of the multitarget-directed ligand approach. Even though the advantages of multitarget-directed ligands have been identified (lower probability of selecting for high-level target-based resistance), the initiation of a research program relies on the judicious choice of the biological targets. There is no doubt that well-established antibacterial targets, such as DNA topoisomerases or PBPs, and well-defined antibacterial drug classes will keep playing an essential role in the discovery of the next generation of multitarget-directed ligands. Unfortunately, this will not be sufficient to address the bacterial resistance issue on the long-term, and there is an urgent need to exploit novel antibacterial drug targets. Currently, a large number of bacterial genomes are publicly available and hundreds of potential gene targets have been deciphered. The appropriate use of bioinformatic methods and defined criteria (essentiality/redundancy, conservation, selectivity) should allow identifying novel “druggable” targets. In addition, a strong emphasis must be set on targets that enable antibacterial coverage of life-threatening Gram-negative bacteria (*P. aeruginosa*, *Enterobacteriaceae*, and *A. baumannii*), and especially strains expressing carbapenemases or extended spectrum β -lactamases.

In this context, DNA polymerases III, mentioned earlier in the context of hybrid molecules, may experience a renaissance. The design of dual inhibitors targeting DNA polymerase III (present only in Gram-positive bacteria) and

Figure 15.29 Novel *Bacillus subtilis* DNA Pol IIIC–Pol IIIE inhibitor.



DNA polymerase IIIE (present in both Gram-positives and Gram-negatives) has been reported [105, 106]. SAR studies were conducted using guanine as a template and showed that 7-substituted- N^2 -(3,4-dichlorobenzyl)guanines such as **61** (Figure 15.29) exhibited potent and competitive inhibition of DNA Pol IIIC and Pol IIIE of Gram-positive *Bacillus subtilis* (with K_i s ~ 50 nM) as well as some weaker competitive inhibition of *E. coli* DNA Pol IIIE ($K_i = 14$ μ M). Compound **61** showed antibacterial activity against a panel of Gram-positive strains *in vitro* (MICs ranging from 1 to 5 μ g/mL) and a dose-dependent increase in mice survival (intraperitoneal *S. aureus* infection mouse model). The lack of antibacterial activity on Gram-negative *E. coli* suggests that more potent inhibitors of DNA Pol IIIE are needed in such strains, provided they can accumulate sufficiently in the cytoplasm.

Multitarget inhibition of enzymes involved in the biosynthesis of lipid A, a unique and essential component of the outer membrane of Gram-negative bacteria is another appealing strategy. Until now, most of the efforts have been dedicated to the inhibition of LpxC, a zinc-dependent metalloamidase that catalyzes the first committed step in the biosynthesis of lipid A, and some successes have been reported [107]. A small peptide RJPXD33 has been shown to bind to LpxA and LpxD, two transacylases involved in lipid A biosynthesis [108]. The peptide competes for binding with LpxD substrate with a $K_d = 6$ μ M, whereas it inhibits LpxA to a slightly lower extent ($K_d = 20$ μ M). Intracellular production of RJPXD33 led to antibacterial activity that was suppressed by the overexpression of LpxD target. It remains to be demonstrated that a small-molecule entity can potentially inhibit LpxA and LpxD to a meaningful extent, thereby killing efficiently Gram-negative pathogens.

15.4 Conclusion

As illustrated in this chapter, the reported designs of multitarget-directed ligands addressing antibacterial drug targets and their optimization focused primarily on potency and antibacterial activity. In many instances, multiplicity of action was shown to favorably influence resistance development and to restore antibacterial activity on resistant pathogens. Over the past decades, efforts to create multitarget-directed ligands resorting to hybrid molecules had a limited outcome and did not bring a solution to the growing medical need associated with infections caused by Gram-negative pathogens. One lesson not to be forgotten is that potent enzyme inhibitors are not necessarily good antimicrobial agents. In case of cytoplasmic targets, inhibitors must enter and accumulate within the

cytoplasm at relevant concentration in order to exert their antibacterial activity, and hurdles to overcome are manifold: crossing bacterial cell membranes, avoiding efflux pumps, and escaping modifying or hydrolyzing enzymes. Once this conundrum is solved, transforming a potent *in vitro* antimicrobial into a drug faces the additional challenge of achieving safely high drug concentration at the site of infection. A successful multitarget drug program would therefore have physicochemical, pharmacokinetic, and toxicological properties integrated, monitored, and tuned accordingly as early as possible in the iterative ligand design process.

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16

Multiple Ligands in Neurodegenerative Diseases

Julien Lalut, Christophe Rochais, and Patrick Dallemagne

*Normandie Univ, UNICAEN, CERMN, Centre d'Etudes et de Recherche sur le Médicament de Normandie,
14000 Caen, France*

16.1 Introduction

Over the past decades, significant advances have been made in the comprehension of the molecular origin of neurodegenerative diseases. The prevalence of these diseases is still growing, and their economical impact on the society is also rising [1]. However, until now, the great progress in their understanding has not led to the discovery of agents able to prevent or treat them. If significant advances have been made in the management of neurodegenerative diseases with the discovery of cholinergic drugs for Alzheimer's disease (AD) and dopaminergic drugs for Parkinson's disease (PD), these treatments are all acting on the symptoms of the diseases rather than on their molecular origins. Between 2002 and 2012, such symptomatic agents still represented more than 36.6% of the clinical trials in AD [2]. In the meantime disease-modifying candidates, 53% of the trials, all failed to show a drug/placebo difference, leading to a success rate of 0.4% in the period, one of the lowest observed in any therapeutic area. The development of neuroprotective treatment for PD is comparable since no drugs engaged in clinical trials are able to show benefits for patients and no treatments are today approved by the FDA to impact the progression of the disease [3]. In order to explain this low success rate, several hypotheses have been proposed including a wrong design in the clinical trials, a heterogeneity between clinical presentation and biology, and difficulties in patient stratification [4]. Historically this high attrition rate is higher for central nervous system (CNS) drug development than for other pathologies, and several lines of hypothesis have been proposed to explain this problematic issue. First, if neurodegenerative diseases differ from their own molecular mechanism and clinical symptoms, they are presenting some similarities. It became notably clear that neurodegenerative diseases, including AD, PD, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), are sharing multifactorial pathogenic origins [5]. Among others, some generally dysregulated and pathogenic pathways could be found in these diseases including, among others, protein misfolding and aggregation [6], oxidative stress and mitochondrial dysfunction [7], metal dyshomeostasis, and abnormal phosphorylation

[8]. These dysfunctions generally occurred concurrently in all neurodegenerative diseases, and this multifactorial origin should be taken into consideration in the search for future disease-modifying agents [9].

Such pleiotropic intervention could be envisaged in different polypharmacological manner [10, 11]. First is the concomitant use of different drugs, known as drug cocktail or multiple-medication therapy. This strategy is of course in use in different neurodegenerative diseases with, for example, the association of memantine and donepezil in AD [12] and a combination of dextromethorphan and quinidine to treat the pseudobulbar affect of ALS [13]. However, if this strategy is still popular, with, for example, the association of donepezil in most of the ongoing clinical trials against AD, it suffers from drug–drug interaction or eventual compliance problems, especially in the case of patients suffering from dementia. A second approach has also been explored by trying to incorporate two drugs in the same pill, according to the multiple-medication therapy concept. We could cite the recent approval of Namzaric™ by the FDA for moderate to severe AD, the first combination pill containing memantine and donepezil [14].

A more innovative strategy could also consist in the design from the start of compounds able to act at the same time toward multiple targets according to the multitarget-directed ligand (MTDL) concept [15]. The application of this concept to complex etiology of neurodegenerative diseases has been completely described by Cavalli *et al.* [8], and since then many examples have been described, and several of them reached clinical trials [16]. We will briefly in this chapter describe the molecular bases of the different diseases before to highlight the development of the most promising MTDL in this field.

16.2 Molecular Bases of ALZHEIMER'S Disease

AD is a complex pathology. Its histological diagnosis relies on the presence, in the CNS, of two types of lesions found in abnormally high quantities that are tissular markers of the illness: amyloid plaques (or senile plaques) and neurofibrillary tangles (NFTs) (Figure 16.1) [17, 18]. However, the mechanisms leading to these lesions and the development of the disease are far from being fully understood and solved.

16.2.1 Amyloid Plaques

Amyloid plaques mainly consist of extracellular accumulations of β -amyloid ($A\beta$) peptides [19, 20]. $A\beta$ peptides result from the cleavage, by different secretases, of amyloid precursor protein (APP), a ubiquitous transmembrane glycoprotein notably found in the neuronal synapses.

16.2.1.1 The Origin of $A\beta$ Peptide Formation

APP can be cleaved following two metabolic pathways: a non-amyloidogenic pathway and an amyloidogenic pathway (Figure 16.2) [21]. Although active throughout life, secretase levels vary according to age: the non-amyloidogenic pathway dominates in young individuals (majority of α -secretase), whereas

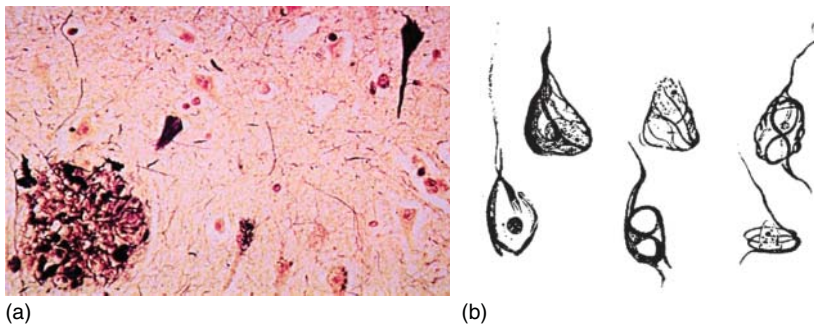


Figure 16.1 (a) Expansion of Alzheimer's disease lesions: amyloid plaques (bottom left) and neurofibrillary tangles (top right) [17]. (b) Drawing by Alois Alzheimer of the first neurofibrillary clusters observed during the autopsy of the brain of Auguste Deter. (Maurer *et al.* 1997 [18]. Reproduced with permission of Elsevier.)

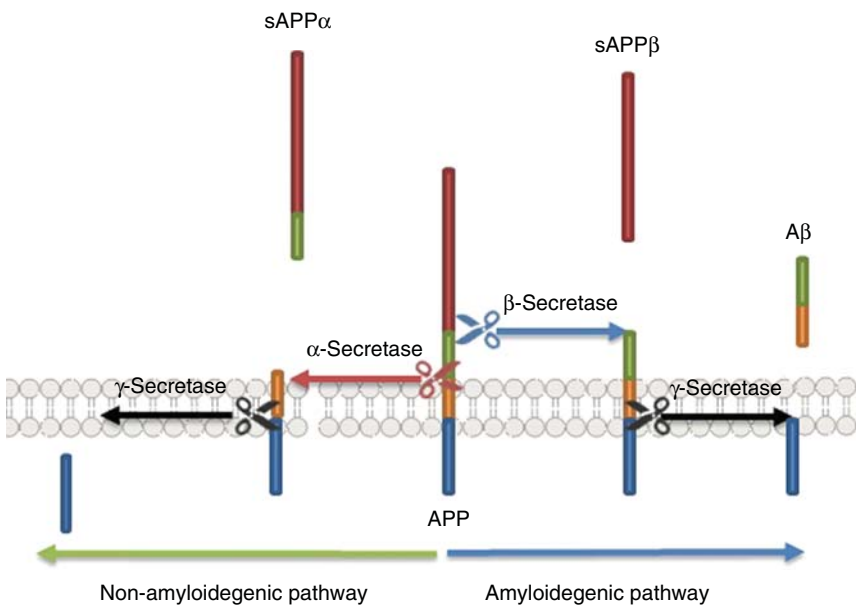


Figure 16.2 Cleavage of the amyloid precursor protein according to the non-amyloidogenic pathway and the amyloidogenic pathway.

the amyloidogenic pathway is most common in older subjects (majority of β -secretase).

In the non-amyloidogenic pathway, APP is cleaved by α -secretase into two fragments: an sAPP α fragment, a neurotrophic and neuroprotective soluble peptide released at the extracellular level that regulates cellular excitability and synaptic plasticity (improvement of long-term potentiation) [22], and a second fragment of 83 amino acids (C83).

In the amyloidogenic pathway, APP is cleaved by β -secretase (BACE1, β -site APP-cleaving enzyme 1), resulting in a fragment of 99 amino acids (C99), which

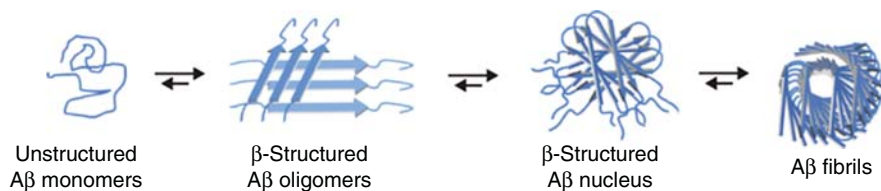


Figure 16.3 Process of forming amyloid fibrils [23].

is then successively cleaved by γ -secretase to produce an AICD fragment, and an A β peptide (4.1–4.3 kDa). The latter is insoluble and varies in length, from 39 to 43 amino acids, but has two main forms: A β_{40} peptide, mostly produced during the cleavage and made of 40 amino acids, and A β_{42} peptide, made of 42 amino acids and representing around 10% of A β peptides.

16.2.1.2 Amyloid Plaque Aggregation

The A β peptide aggregation process leading to amyloid plaques includes the formation of different intermediaries with distinct morphologies. A β oligomers (of up to 12 units), obtained through the transition from unstructured A β monomers to a β -sheet structure, tend to form protofibrils through interactions between the different β -sheets. The fibrillation process then allows these protofibrils to turn into amyloid fibrils, which tend to aggregate and form A β plaques (Figure 16.3) in the presence of other elements (metals, proteins, etc.) [23].

Hence senile plaques are compact, spherical deposits, mostly made of A β peptide aggregated in a β -pleated sheet conformation and mainly localized at the extracellular level. In the mild and moderate stages of AD, the diffuse plaques mostly contain the longer form, A β_{42} . A β_{42} peptide is more hydrophobic than A β_{40} peptide, so its solubility is lower and it can aggregate more easily in the extracellular environment. The two peptides are only found in combination in the senile plaques in the later stages of the disease.

16.2.2 The Amyloid Cascade Hypothesis

The “amyloid cascade” hypothesis has long been put forward to attempt to explain the disease mechanism [24]. This posits that an amyloidogenic pathway dysfunction leads to an overproduction of A β peptide (normally present in the organism and circulating in the CSF or in the blood) in the extracellular environment, forming fibrils, which in turn aggregate to form amyloid plaques. These plaques result in an alteration of cellular membranes, allowing a massive influx of calcium ions in the cell and causing neuronal death through necrosis and/or apoptosis. This neuronal loss may be intensified by a concomitant inflammatory reaction caused by oxidative stress (activation of the microglia, which secrete cytokines and neurotoxic free radicals) or also by an increase in the activation of the kinases, leading to hyperphosphorylation of tau.

The amyloid plaques have long been considered as the origin and the first link in AD pathogenesis. Nowadays, this hypothesis is widely debated. Amyloid toxicity may not be linked to the aggregation of A β peptide in plaques, but rather to A β peptide’s oligomeric forms, the soluble forms preceding the aggregation

steps, which appear correlated to the cognitive dysfunctions of AD [25, 26]. Nonetheless, plaques may act as a reservoir releasing oligomers at a later stage.

16.2.3 Neurofibrillary Tangles

Besides amyloid plaques, a second typical lesion has been observed in the brain of AD patients: intraneuronal NFTs, characterized by tangles of neurofibrils made of aggregates of abnormally hyperphosphorylated tau protein (tubule-associated unit). Not specific to AD, this type of lesion is found in various neurodegenerative disorders grouped under the name of “tauopathies” (Down syndrome, Pick's disease, FTDP-17, etc.).

Identified in 1975 by Weingarten *et al.* [27], tau protein, coded by the MAPT gene located on chromosome 17 and inducing six protein isoforms (352–441 residues), enables the assembly and stabilization of the neuronal cytoskeleton microtubules and ensures an efficient axonal transport.

Two forms of phosphorylation can be identified in AD patients: abnormal phosphorylation and hyperphosphorylation, two important phenomena in the process leading to intracellular aggregation of tau protein.

Abnormal phosphorylation consists in the detection and phosphorylation of new sites that are not normally concerned by phosphorylation under physiological conditions. Particularly dependent on the balance between kinases and phosphatases, tau protein is considered to be hyperphosphorylated when its multiple phosphorylation sites are fully, or nearly fully, saturated. Preventing tau protein from fulfilling its polymerization and stabilization role, hyperphosphorylation leads to the dissociation of the hyperphosphorylated tau protein from the microtubules, which deteriorate and cause an alteration of axonal transport, resulting in neurodegeneration. The freed hyperphosphorylated protein undergoes chemical transformations (conformational changes following oxidation) and aggregates in the intracellular environment in paired helical filaments (PHFs), thus creating neurofibrils following a process of polymerization with a nucleation phase. These NFTs then progressively invade the neurons and impair their function until their death (Figure 16.4) [28].

16.2.4 Oxidative Stress, Neuroinflammation, and Metal Toxicity

Defined as an imbalance between the oxidation systems and a cell's defense (antioxidant abilities), oxidative stress is, according to the amyloid cascade, one of the mechanisms linked to neuronal loss in AD. This mechanism is due to metal (Zn, Fe, Cu, Al) homeostasis dysfunctions, mitochondrial dysfunctions, or an excessive production of free radicals (reactive oxygen species (ROS)) by the A β peptides.

The damaging effects on brain aging, potentially caused by reactive oxygen intermediaries, are all the more reinforced by the neurons' high oxygen consumption and by the cerebral tissue's low antioxidant concentration, leaving it particularly vulnerable to oxidative stress. The formation of hydroxyl radicals ($\bullet$ OH) proceeds from the reaction of H_2O_2 with reduced metallic ions (Cu^+ , Fe^{2+}) present in the cell [29].

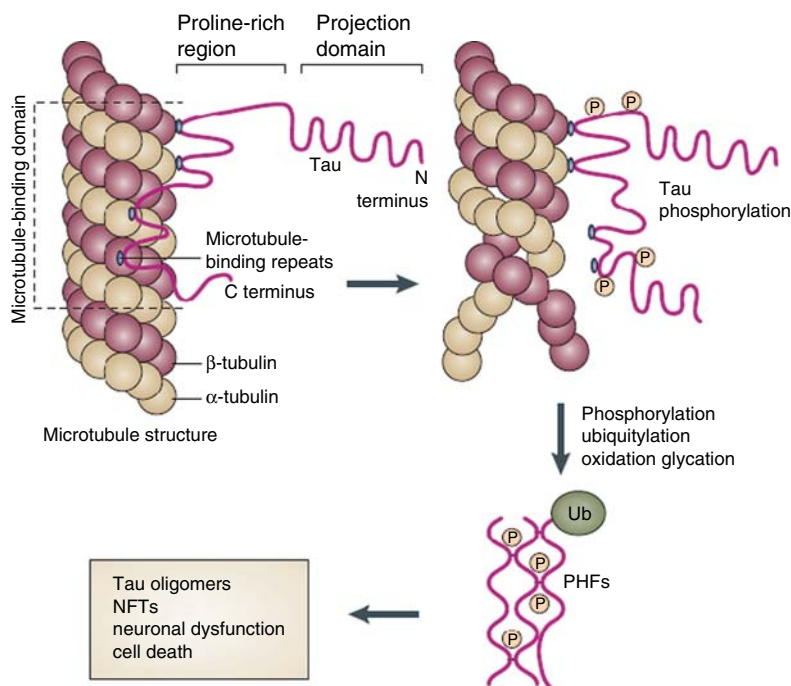


Figure 16.4 Hyperphosphorylation of tau and neurofilament formation [28].

The free radicals formed being unstable, they then seek to couple their free electron with a hydrogen atom from the neuronal membrane. Having become permeable, the denatured membrane lets through other free radicals, which attack the neuron's nuclear and mitochondrial DNA, causing a disruption of cellular functions.

In addition, the hydroxyl radicals generate the oxidation of many macromolecules and proteins, including APP, thus enabling the amyloidogenic pathway and the formation of A β peptides. An accumulation of iron in the cortex and the hippocampus can notably be observed in the brains of AD patients [30], and APP cleavage into A β peptide has been shown to be promoted by the presence of iron [31]. The A β aggregates can lead to the release of ROS; however ROS overproduction then accelerates the generation and accumulation of A β peptides, which facilitates AD's progression.

Among the many possible metabolic consequences of the accumulation and aggregation of A β peptides (incurring inside or outside the neurons), the modification of metal homeostasis and especially the entry of excessive amounts of calcium inside the neurons contribute to selective neuronal dysfunctions that lead to neuronal death.

Oxidative stress also leads to an inflammatory reaction. A β peptide accumulation induces an activation of the microglia and of the astrocytes, which then release inflammatory mediators (cytokines, chemokines, etc.) in response to these unrecognized elements, in turn speeding up the destruction of the neurons and the production of H₂O₂.

All these dysfunctions, caused by the accumulation and aggregation of A β peptide according to the amyloid cascade, are interconnected; however the link between the different phenomena and their chronology is probably not fully understood yet. Lee *et al.* put forward a summary of possible different molecular causes that may simultaneously lead to neuronal death and the development of AD [32].

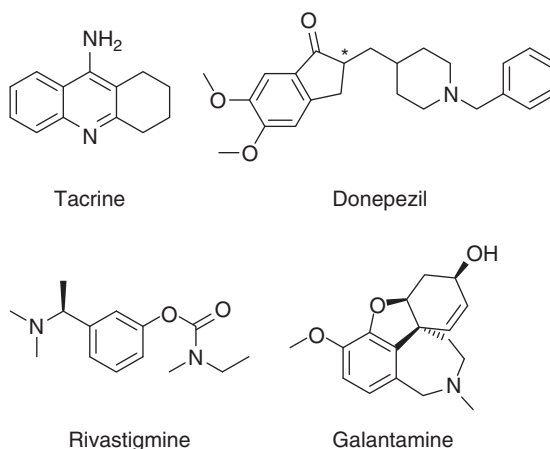
16.3 MTDLs Developed for the Treatment of Alzheimer's Disease

16.3.1 MTDLs Based on Acetylcholinesterase Inhibition

Acetylcholinesterase (AChE) has been studied extensively in order to uncover new drugs to treat AD, owing to the variety of substrates that can block its function and the range of interaction options. The only drugs to have been granted a marketing approval for the symptomatic treatment of the disease include reversible inhibitors allowing a non-covalent interaction with enzyme (tacrine, donepezil, and galantamine) and a pseudo-irreversible inhibitor allowing a transitory covalent interaction with enzyme (rivastigmine) (Figure 16.5).

In a context where developing MTDLs has become a key strategy, significant research efforts have been dedicated to the design of dual-binding site (DBS) inhibitors targeting the enzyme's two sites of therapeutic interest, namely, the catalytic active site (CAS) and the peripheral anionic site (PAS). Indeed, the latter appears implicated in a pathological chaperon role in AD, where it would interact with A β to promote the formation of AChE/A β aggregates. Many homodimer [33, 34] or heterodimer [35–38] inhibitors have been designed to simultaneously target the two AChE sites. These drugs have shown very good affinities with the enzyme and a capacity to reduce the aggregation of A β peptides induced by the AChE PAS. Few of them have been studied *in vivo*. This is however the case of compound NP-61, a DBS inhibitor of AChE, which has demonstrated an ability to reduce cognitive deficiencies as well as the number of A β plaques in the brain

Figure 16.5 Structures of cholinesterase inhibitors that have received marketing authorization.



of transgenic mice overexpressing human APP. From 2007, NP-61 was assessed in a phase I clinical trial in Europe and appears to have helped reduce the cognitive deficit of patients while preventing the formation of new amyloid plaques. Its structure has not yet been revealed; Garcia-Palomero *et al.* only reported a general hybrid chemical structure, (6-chloro)-tacrine-indole (Figure 16.6) [39].

The different DBS AChE inhibitors that have been designed constitute a genuine starting point for the development of MTDLs that, in addition to multi-site inhibition, present an affinity toward another pharmacological target while also frequently retaining a tacrine motif (Figure 16.7). Agis-Torres *et al.* recently published a very well-informed and referenced review of the latest developments relating to examples of DBS AChE-inhibiting MTDLs also performing many additional activities [40]. Highlighted among others were compounds that, as well as being multi-site inhibitors of the enzyme, are antioxidants, metal chelators, enzyme inhibitors (BACE1, monoamine oxidase B (MAO-B), etc.), or even receptor ligands (cholinergic, serotonergic, histamine, NMDA, $\alpha 1$, etc.).

The other examples of MTDLs presented in the following text have been selected to illustrate different additional activities granted to compounds that do not actually bear the tacrine motif in their structure.

Figure 16.6 General chemical structure of the series that include NP-61 [39].

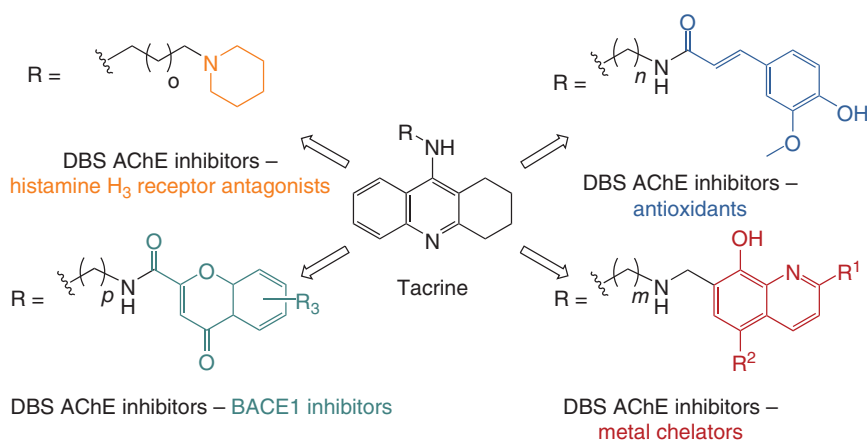
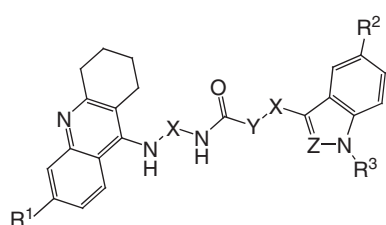


Figure 16.7 Examples of MTDL dual-binding site of AChE containing a tacrine and with other activities.

16.3.1.1 Multi-site AChE Inhibitors and Antioxidants

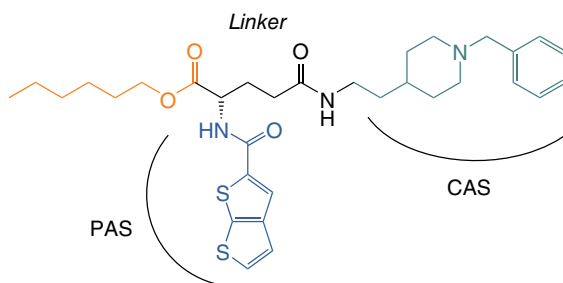
Arce *et al.* designed a new family of MTDL compounds by linking three relevant fragments via a glutamic acid analog linker: the *n*-benzylpiperidine fragment of donepezil, chosen to inhibit AChE through interaction with the CAS; an acetamide fragment, featuring different aromatic cores, able to interact with the PAS; and a lipophilic alkyl ester, facilitating the passage of the entire compound through the blood–brain barrier (BBB). Among several good obtained results, the compound presented below (Figure 16.8) displayed submicromolar affinity for human acetylcholinesterase (*h*AChE) and for human butyrylcholinesterase (*h*BChE), very good displacement of the propidium diiodide from the PAS of AChE, neuroprotective properties against mitochondrial free radicals, an ability to cross the BBB, and no toxicity [41].

Compounds in this new family of MTDLs were incubated at different concentrations with cells from the SH-SY5Y human neuroblastoma cell line before adding H₂O₂. They protected cells from H₂O₂ damage better than the positive control, TROLOX™, a vitamin E analog antioxidant, known to reduce oxidative stress and damage. Neuroprotection was then studied following two different protocols for the compounds' incubation with cells and a combination of toxins. Results suggested that the neuronal protection mechanism of this compounds' family is very clearly based on the expression of antioxidant proteins or on the activation of intracellular signaling pathways involved in cell survival.

16.3.1.2 Multi-site AChE Inhibitors and Metal Chelators

Zheng *et al.* developed HLA20A, a multifunctional prochelator offering promising prospects for the treatment of AD. This design strategy was inspired by the design of a prodrug, an inactive medicine that, after administration, requires *in vivo* metabolization prior to releasing an active substance. The HLA20A prochelator exhibits limited affinity for metal ions to avoid interfering with normal metal metabolism in healthy individuals until such time as it is activated by AChE linking and inhibition, when it simultaneously releases the active chelator HLA20. The latter chelates the excess metal ions (Cu, Fe, Zn) in the

Figure 16.8 Design strategy of a DBS AChE inhibitor/antioxidant [41].



$$IC_{50} \text{ hAChE} = 0.30 \pm 0.02 \mu\text{M} / IC_{50} \text{ BuChE} = 0.35 \pm 0.01 \mu\text{M}$$

Displacement of propidium diiodide from the PAS
42.2 ± 5.7 % (C = 0.3 μM)

Permeability BBB $P_e = 28.5 \pm 2.0 \cdot 10^{-6}$ cm/s

brain, protects neurons against oxidative stress, and modulates APP regulation and A β reduction (Figure 16.9) [42].

This prochelator design features structural elements of two AChE inhibitors, donepezil and rivastigmine; the chelator motif quinolin-8-ol present in two A β anti-aggregators (clioquinol, PBT-2), whose alcohol is protected by a carbamoyl grouping; and the propargylamine fragment, a motif reported by several studies to possess neuroprotective and neuronal regeneration properties (Figure 16.10).

16.3.1.3 Multi-site AChE and MAO Inhibitors

For many years, the Carotti group has been working on the design of ligands that either selectively and reversibly inhibit monoamine oxidases (MAOs) or inhibit cholinesterases. The experience acquired in this field naturally led them to look into the design of multitarget ligands exhibiting the dual MAO/AChE inhibition, a combination that seems to offer some synergies for the promising therapeutic effects in the treatment of AD.

Based on previous research studying the coumarin core as a relevant motif for the design of MAO/AChE inhibitors, Farina *et al.* recently developed a series of compounds using the polysubstituted coumarin core of the MC 1095 compound, a powerful and selective MAO-B inhibitor designed by Catto *et al.* [43], and the *N*-benzyl-aminomethyl moiety of donepezil (Figure 16.11). These compounds were pharmacomodulated in order to study the influence of the linker's flexibility, nature, and length and the influence of substituting the amine or the coumarin core. The most polar and hydrophilic compounds exhibited very efficient activities on the two pharmacological targets aimed at. The compound presented below, the most promising of the series, inhibits *h*AChE with a submicromolar affinity and is highly selective of MAO-B with a nanomolar affinity, thus avoiding unwanted side effects linked to an MAO-A inhibition. Other cellular tests highlighted the compound's absence of neurotoxicity, its ability to cross the BBB and to combat oxidative stress induced by H₂O₂ [44]. This compound also exhibits capacity to interact with the two AChE sites and to interfere with A β aggregation promoted by the enzyme's peripheral site.

16.3.2 Multi-site AChE Inhibitors and Serotonin 5-HT₄ Receptor Agonist

Donecopride was the first reported MTDL exhibiting both serotonin 5-HT₄ receptors (5-HT₄R) partial agonist and DBS AChE inhibition activities. This compound was conceived as a structural compromise between RS-67333, a 5-HT₄R partial agonist, and donepezil, one of the most used AChEI on the market (Figure 16.12) [45, 46].

Donecopride retains from its precursors a high affinity and selectivity toward 5-HT₄R versus numerous other receptors, a potent and selective AChE inhibitory effect versus BuChE, the ability to promote the release of sAPP α , a neurotrophic protein issued from the non-amyloidogenic cleavage of the amyloid peptide precursor, and finally the capacity to displace propidium iodide from the PAS of AChE (Table 16.1).

Since activating 5-HT₄R also promotes the release of ACh, donecopride is theoretically able to restore the cholinergic neurotransmission and to limit the amyloid aggregation and finally to display both a symptomatic and a disease-modifying interest in AD treatment (Figure 16.13).

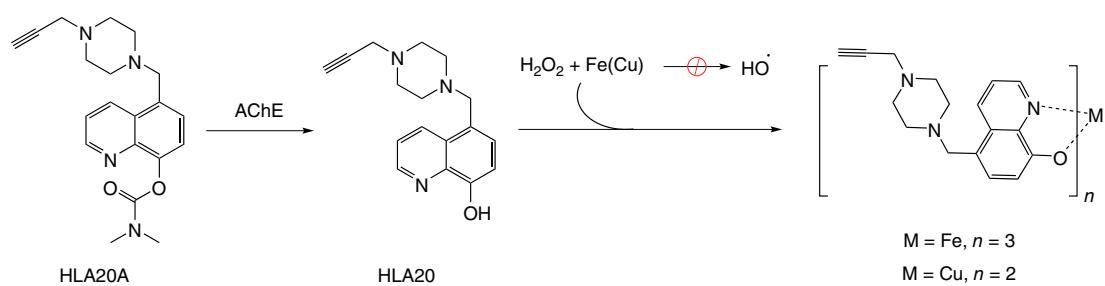


Figure 16.9 Release of the active chelator HLA20 from its pro-chelator and chelation of metal ions [42].

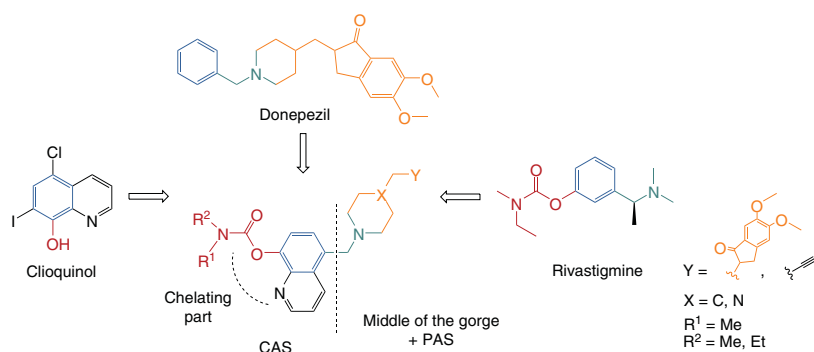


Figure 16.10 Design strategy of the prochelator HLA20A [42].

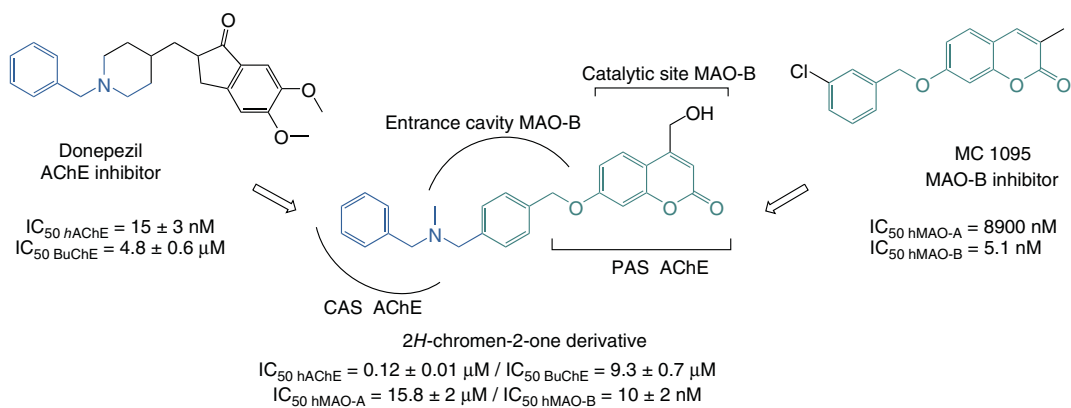


Figure 16.11 Design strategy of a DBS AChE/MAOs inhibitor containing a coumarin unit [44].

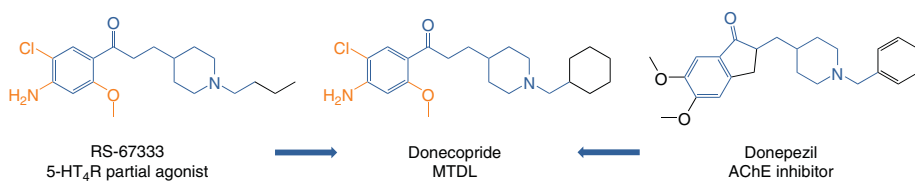
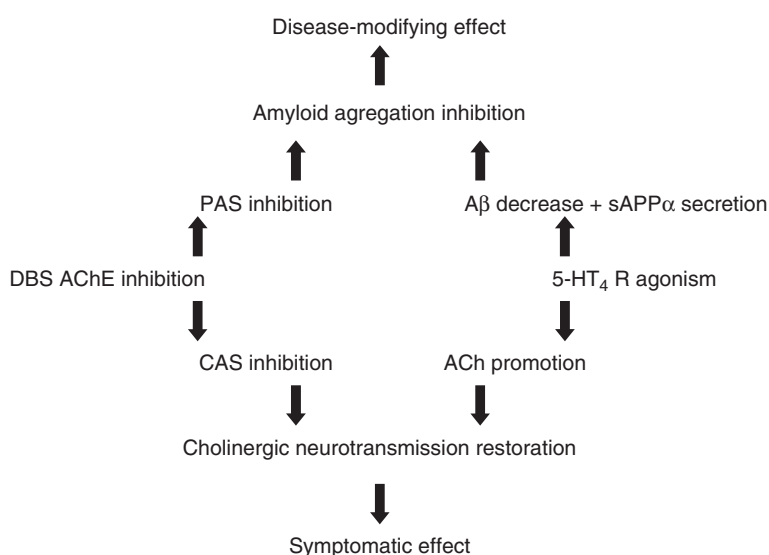


Figure 16.12 Design strategy of donecopride.

Table 16.1 Pharmacological activities for RS-67333, donepezil, and donecopride.

	RS-67333	Donepezil	Donecopride
IC ₅₀ <i>h</i> AChE (nM)	403 ± 86	6 ± 0.6	16 ± 5
IC ₅₀ <i>eq</i> BuChE (nM)	—	7000	3530 ± 50
K _i <i>h</i> 5-HT _{4e} R (nM)	13 ± 4	10% at 10 ⁻⁶ M	8.5 ± .3
Agonist response <i>h</i> 5-HT _{4e} R (%)	48.7 ± 5.2	—	48.3 ± 0.9
EC ₅₀ sAPPα (nM)	27.2 ± 10.6	—	11.3 ± 10.0
Displacement of propidium diiodide at 10 ⁻⁵ M (%)	—	21.5 ± 1.5	24 ± 2

**Figure 16.13** Synergistic effect of 5-HT₄R activation and AChE inhibition.

In vivo, donecopride exerted a procognitive effect in NMRI mice with an improvement in memory performances at 0.3 and 1 mg/kg on the object recognition test and an anti-amnesic effect observed at doses starting from 0.3 mg/kg on the scopolamine-induced spontaneous alternation deficit test (Figure 16.14a, b). Donecopride is also able to promote the release of sAPPα in C57BL/6 mice (Figure 16.14c).

16.3.2.1 Multi-site AChE and M2 Muscarinic Receptor Inhibitors

Melchiorre *et al.* based their design of *caproctamine* on *benextramine*, a polyamine compound originally intended as an irreversible α-adrenergic receptor antagonist that also exhibited a significant affinity for M2 muscarinic receptors and the ability to reversibly inhibit AChE (Figure 16.16). As a DBS AChE inhibitor and M2 muscarinic receptor antagonist, caproctamine therefore enables to safeguard the cholinergic transmission in the cerebral regions affected

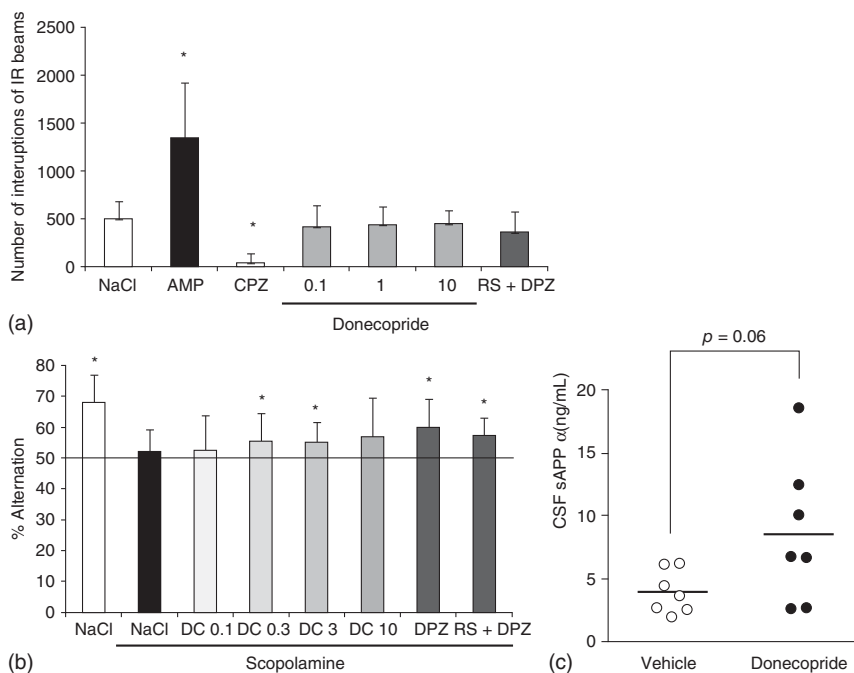


Figure 16.14 Evaluations *in vivo*: memory performances, antiamnesic effect, and release of sAPP α .

by the disease and to prevent A β aggregation induced by AChE through enzyme inhibition, while the M2 muscarinic receptor inactivation facilitates the release of acetylcholine in the synapse [47]. It has much better affinity for AChE and AChE/BuChE selectivity than benextramine. Moreover, it is a dim M1 and M3 receptor antagonist although it exhibits an affinity for M2 receptors equivalent to its affinity for AChE. Molecular dynamics simulations and docking studies showed caproctamine's ability to establish favorable interactions with throat residues and to bind simultaneously to the two AChE sites. This compound has since been pharmacomodulated (mainly changing the nature of the linker between the two amides), leading to compounds with enhanced activities (Figure 16.15).

16.3.2.2 AChE Inhibitors with a Complex Pharmacological Profile

In the manner of natural products (berberine, curcumin, epigallocatechin gallate, resveratrol, etc.) or analogs (phenserine, lipocrine, etc.) that have proved to be real MTDLs, other compounds designed as AChE inhibitors present complex pharmacological profiles due to the number of beneficial activities they exert in the treatment of AD. Dimebon, memoquin, ladostigil, and even M30 are good examples of such ligands, owing to their multiple action mechanisms. Two of these compounds are discussed later.

Caproctamine, presented earlier, was the real starting point for the design of memoquin. In fact, having designed caproctamine a few years earlier, Bolognesi

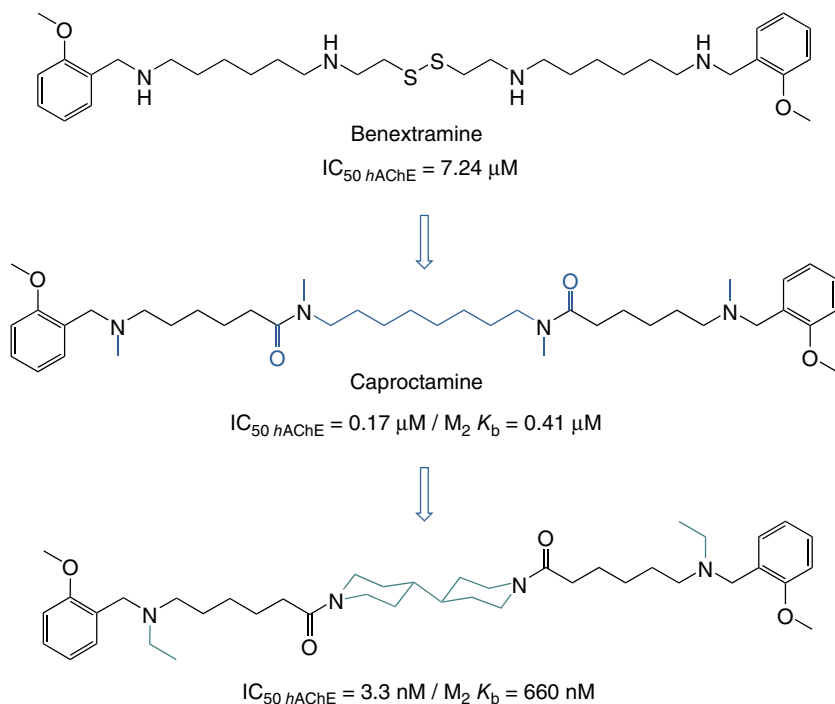


Figure 16.15 Structures of benextramine, caproctamine, and a rigidified analog.

et al. wished to exploit its pluriactivity and to pharmacomodulate it in order to bestow it with other beneficial activities for the treatment of AD, especially antioxidant properties [48].

To this end, memoquin was rationally designed by combining the extremities of caproctamine with the free radicals trapping motif 1,4-benzoquinone (**idebenone**), a synthetic analog of coenzyme Q10 (Figure 16.16). Idebenone is an oxidant that, in cellular cultures, has shown an ability to protect rat hippocampal neurons from toxicity induced by A β peptides and to induce an increase in the growth factor of nervous cells in rats. It also acts as a respiratory chain activator and safeguards ATP formation.

Idebenone was also assessed in several clinical trials. However, study results are not always in agreement. One of the double-blind trials reported a statistically significant improvement in the memory of patients suffering from mild to moderate AD, whereas another trial did not show any benefit from the compound. Idebenone is currently marketed in Italy for use at a low dosage in cognitive troubles and is under phase III clinical trial for the treatment of Friedreich's ataxia (oxidative lesions due to a misregulation of iron metabolism at the mitochondrial level).

In order to reveal the multiple action mechanisms of memoquin, a number of *in vitro* and *in vivo* assessments were conducted and allowed to highlight that memoquin inhibits AChE and BACE1, interferes with A β aggregation, and is a good substrate for NQO1 enzyme, a dehydrogenase enabling the transformation

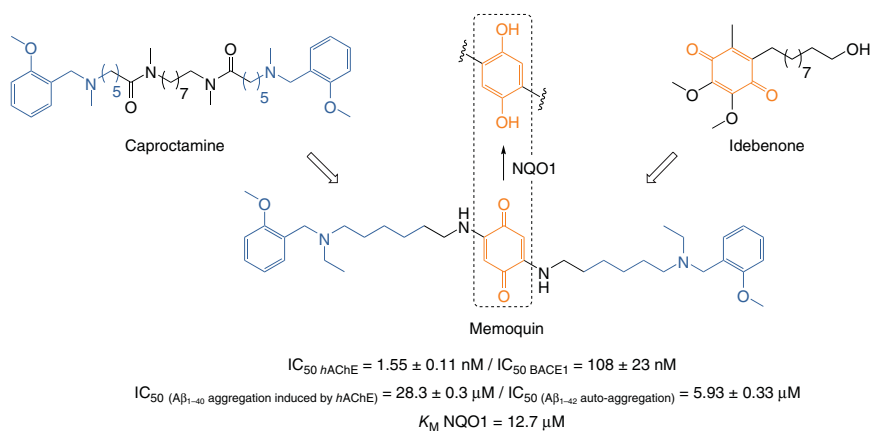


Figure 16.16 Strategy design of memoquin and transformation of quinone to hydroquinone.

of quinone into hydroquinone, whose quantity increases with AD. Whereas oxidized and reduced forms of memoquin therefore seem able to coexist *in vivo*, the reduced form has turned out to be the antioxidant species, in the same manner as 1,4-benzoquinone derivatives (coenzyme Q10, idebenone). This can explain that the antioxidant capacity of memoquin, assessed in its oxidized form, appears weaker than the reference (TROLOXTM). In transgenic mice (AD11, anti-nerve growth factor), memoquin led to a noticeable reduction in the formation of the two main markers of AD, A β plaques, and NFTs, as well as in behavioral deficits assessed through an object recognition test [49].

Several series of compounds derived from memoquin were designed either by introducing a ramification or by replacing one of the central benzoquinone motifs' lateral chains with lipoic acid, an antioxidant preventing neurodegeneration induced by the mitochondria. These compounds have been useful to study the role of oxidative paths in AD.

The design of MTDLs including both a motif pertaining to AChE inhibition and a specific MAO inhibition motif, such as the MAO-B/AChE inhibitor including a coumarin motif previously described, has shown promise for the treatment of AD. Hence, Sterling *et al.* developed *ladostigil* (TV-3,326) by joining together the structural elements of two active principles: rivastigmine's carbamate grouping, a reversible inhibitor of AChE and BuChE, with the antiparkinsonian rasagiline's indolamine (Figure 16.17), an irreversible and selective inhibitor of MAO-B [50].

Ladostigil inhibits both AChE and BuChE for a longer period of time than rivastigmine and, like the latter, helps to improve cognition, as shown by its ability to reverse deficits induced by scopolamine in spatial learning in rats. The compound only inhibits the cerebral MAOs, not the ones at the peripheral level, which makes it relevant for the treatment of dementia in subjects suffering from depression. In mice, it also prevents the onset of parkinsonian symptoms caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [51].

Initially designed as an MAO and cholinesterase (AChE and BuChE) inhibitor, ladostigil has revealed, in neuronal cellular cultures and at much lower concentrations than those for enzyme inhibition, other neuroprotective and antioxidant activities. Propargylamine was preserved in the course of the design of ladostigil. Research on this fragment's different components uncovered its antioxidant

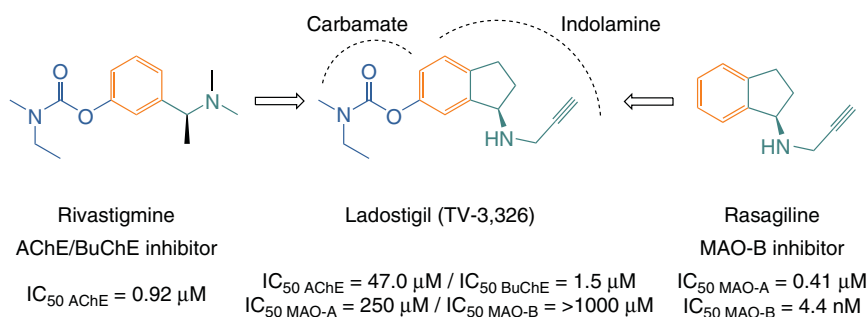


Figure 16.17 Strategy design of ladostigil by adjoining two active principles [50].

and neuroprotective properties. Different explanations have been put forward regarding its likely role in the stabilization of the mitochondrial membrane: the trapping of peroxynitrite ions through the triple bond or even an indirect antioxidant profile that requires the activation of different proteins (antiapoptotic proteins, kinase C protein, mitogen-activated protein kinase (MAPK)).

Ladostigil also improves the expression of neurotrophic factors (glial cell line-derived neurotrophic factor (GDNF); brain-derived neurotrophic factor (BDNF)). It reduces oxidative stress and microglial activation and inhibits the pro-inflammatory cytokines. Weinreb *et al.* even reported that the induction of these neurotrophic factors by ladostigil could eliminate apoptosis and lead to neurogenesis [52]. It also appears to be able to act on apoptosis through regulation of the Bcl-2 protein family (increase of antiapoptotic Bcl-2 proteins and decrease of proapoptotic Bax and Bad) and on APP regulation (stimulation of sAPP α release) [33].

A study on the effects of chronic treatment with ladostigil on 6–16-month-old rats uncovered the compound's ability to prevent the age-related increase in astrocytes and microglial activation in several areas of the hippocampus and of the white matter. It also prevented the age-related reduction in the activity of the cortical AChE and increase in the activity of BuChE in the hippocampus. The treatment revealed an improvement in the spatial memory of old rats; however it did not have any effect on the memory of young rats [53].

Early treatment with ladostigil would help to slow the progress of AD, in which oxidative stress and inflammatory processes are omnipresent. Ladostigil has already reached clinical phase testing for depressed patients or those with parkinsonian symptoms and is currently being assessed for its safety and efficiency at a low dose with patients suffering from MCI in a phase II clinical trial (clinical trial NCT01429623). Results are expected during the third quarter of 2016.

16.3.3 MTDLs Targeting Other Activities Relevant for the Treatment of AD

In addition to MTDLs based on AChE inhibition, others, albeit smaller in numbers, have been developed without actually targeting the enzyme by combining different rational therapeutic activities for the treatment of AD. Several examples of such MTDLs are presented in the following text.

16.3.3.1 MTDLs Modulating γ -Secretase and PPAR γ

Hieke *et al.* designed a range of MTDLs acting as modulators of γ -secretase and PPAR γ , thus, respectively, allowing to selectively block the generation of A β _{1–42} peptide by shifting the final APP cleavage stage with γ -secretase and by stimulating the nuclear receptors in order to reduce A β levels, inflammation, and cerebrovascular disorders and to regulate insulin sensitivity [54]. These MTDLs build on the strong pharmacological results of a series of similar multitarget ligands previously developed by Hieke *et al.* around a 2-(pyrimidin-2-ylthio)-hexanoic acid scaffold [55]. The pharmacomodulations performed on this first-generation scaffold allowed to study the structure–activity relationships (SAR) of replacing the central pyrimidine core with a benzene and the thioether function with

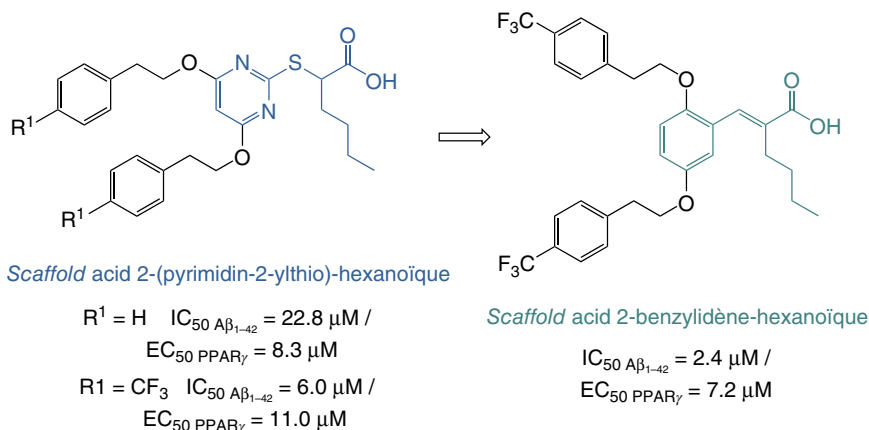


Figure 16.18 Pharmacomodulation of compounds of first generation to obtain more powerful MTDL γ -secretase/PPAR γ modulators.

different linkers in the first place. Different benzene motifs and substitution positions were then studied. The compound presented below (Figure 16.18), designed around a new 2-benzylidene-hexanoic acid scaffold disubstituted at positions 2 and 5, performed best in terms of modulation of γ -secretase and PPAR γ .

Because γ -secretase modulators are known for inhibiting cyclooxygenases through micromolar or even nanomolar activities, the compound was tested for COX-1 and COX-2 inhibition, which revealed that it did not inhibit COX-2 and that its COX-1 inhibition was very low. Regarding Notch, a substrate of γ -secretase whose inhibition causes many secondary effects, the compound did not significantly impact Notch-1 activity in a cellular test. It now needs to be assessed in animal models of AD to determine whether it constitutes a relevant MTDL for the treatment of AD.

16.3.3.2 MTDL BACE1 Inhibitors/Metal Chelators

Huang *et al.* designed a series of MTDLs simultaneously inhibiting β -secretase and possessing metal chelation properties [56]. BACE1 inhibition allows to interfere with the first APP cleavage according to the amyloidogenic pathway and thus to reduce A β peptide production, while metal chelation allows to decrease metal ion levels in the brain of AD patients.

Huang *et al.* previously developed a pharmacophore model for BACE1 inhibitors thanks to different modeling software and designed *N*-phenyl-1-arylamide and *N*-phenylbenzenesulfonamide derivatives based on the pharmacophore built [57]. Among the two chemical series, one of the *N*-phenyl-1-arylamide showed the best BACE1 inhibition activity ($IC_{50} = 18.33 \pm 2.80 \mu M$). Molecular docking studies sought to understand its interaction with the target using the crystallized enzyme structure together with a potent BACE1 inhibitor (OM99-2, code PDB 1W51). This led to the observation, notably, of hydrogen bonds between the phenylethylamine motif's amide or nitrogen and several enzyme residues and of hydrophobic interactions between an enzyme pocket and the compound's naphthyl motif. This compound's motif was

combined with the LR-90 metal chelator motif to design new MTDLs around a diphenylurea scaffold (Figure 16.19).

The most effective MTDL showed a β -secretase inhibition comparable to the activity of the BACE1 inhibitor used initially. It was tested for its iron and copper chelation properties (UV-visible spectroscopy) and proved to be a potent chelator of those metal ions through its urea motif. Docking studies helped to investigate the MTDL's interactions with the enzyme. They uncovered interactions that were sometimes different to those observed with the BACE1 inhibitor: urea established a network of hydrogen bonds with the enzyme, whereas the benzene ring substituted by bromine appeared to interact with the benzene core of a tyrosine residue from the enzyme via π -stacking interactions and via hydrophobic interactions with the same enzyme pocket previously implicated in an hydrophobic interaction with the BACE1 inhibitor's naphthyl. This family of compounds constitutes a new MTDL design strategy for the treatment of AD and can be used as a basis for future studies.

16.3.3.3 MTDLs Inhibiting Muscarinic and σ 1 Receptors

Over the last few years, Anavex Life Sciences Corp., a clinical-stage biopharmaceutical company specializing in the development of drug candidates for the treatment of AD and other CNS diseases, has been developing compounds simultaneously targeting σ 1 and muscarinic receptors to improve neuroprotection and cognition in AD (Figure 16.20). mAChR stimulation, and especially of the M1 subtype, has shown a beneficial effect on restoring cognition in AD patients and on decreasing pathological A β and tau proteins in different animal models. The role of σ 1 receptors is increasingly studied. Hayashi *et al.* suggest that σ 1 receptors act as chaperone proteins vis-à-vis the membrane at the interface of the endoplasmic reticulum and the mitochondria (mitochondria-associated ER membrane (MAM)), thus regulating calcium signaling and cellular survival [58]. An activation of the σ 1 receptors by selective agonists appears to have an effect on Ca²⁺ ion homeostasis and on signal transduction pathways, allowing an efficient neuroprotection against excitotoxicity, oxidative stress, and amyloid toxicity.

In vitro studies first showed the neuroprotective role of PRE-084 and (–)-MR-22, σ 1 receptor agonists, capable of lowering the expression of proapoptotic BAX protein induced by a prolonged exposure of A β _{25–35} fragments in rat cerebral cortex cultures and thus neuronal death. *In vivo* studies then uncovered that PRE-084 prevents oxidative stress induced by A β _{25–35} fragment and learning problems in mice, whereas (–)-MR-22 appeared capable of improving cognitive capacities and of exerting a protective role in rats presenting with cognitive impairments, cholinergic loss, and A β accumulation [59, 60].

A series of aminotetrahydrofuran compounds was developed, of which ANAVEX1-41 and ANAVEX2-73 proved to be promising MTDLs of σ 1 and muscarinic receptors. According to the authors, the design of such ligands lies on these MTDLs' ability to activate a neuroprotective pathway (such as the M1/PLC/DAG/PKC signaling pathway) and to amplify it thanks to a simultaneous activation of σ 1 receptors.

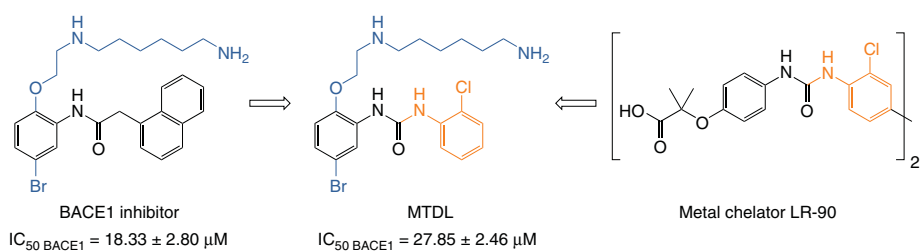


Figure 16.19 Strategy design of a MTDL BACE1 inhibitor/metal chelator.

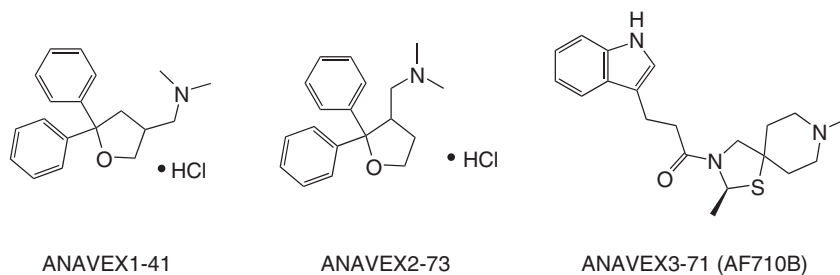


Figure 16.20 Structures of MTDL mAChRs/ σ 1 receptors inhibitors.

Evaluated *in vitro*, ANAVEX1-41 is a σ 1 receptor activator with very good affinity ($K_{i\sigma 1} = 44$ nM) and selectivity ($K_{i\sigma 2} = 4$ μ M), as well as *in vitro* nanomolar affinities ($K_i = 18$ –114 nM) for muscarinic receptors (M1 > M3, M4 > M2). As for ANAVEX2-73, it displays more moderate affinities for the two targets, submicromolar affinity for σ 1 receptors ($IC_{50} = 0.86$ μ M) and nanomolar affinities ($IC_{50} = 18$ –114 nM) for muscarinic receptors (M1 > M3, M4 > M2). The antiamnesic and neuroprotective activities of the two compounds were studied in mice intracerebroventricularly injected with $A\beta_{25-35}$ fragments. This injection of $A\beta_{25-35}$ fragments in oligomeric form leads to neuroinflammation, reactive gliosis, activation of proapoptotic caspases, oxidative stress, loss of cholinergic and hippocampal neurons, and memory impairments.

ANAVEX1-41 and ANAVEX2-73 were able to improve impairments and to prevent learning problems, inflammation, and oxidative stress caused by $A\beta_{25-35}$ fragments. Regarding the expression of caspases induced by these same fragments and associated with the cellular apoptosis pathway, ANAVEX1-41 did not prevent the expression of caspase-9 but blocked the expression of caspase-3 [61]. In addition to amyloid toxicity, the injection of $A\beta_{25-35}$ fragments seems to lead to accumulation of endogenous amyloid species and hyperphosphorylation of tau protein. A week after the injection, the APP and $A\beta_{1-42}$ levels increased in the hippocampus and the cortex, and an increase of tau phosphorylation (hyper- and abnormal) was reported in several studies following the activation evolution of several kinases (GSK-3 β , Akt). Muscarinic ligands, especially M1 and M3 agonists, increase sAPP α formation, prevent pathological amyloid fragment formation, and block tau phosphorylation by targeting kinase GSK-3 β , an activity also demonstrated by compound ANAVEX2-73 [62]. More recently, ANAVEX2-73 showed that it could preserve the integrity of the mitochondrial membrane in mice injected with $A\beta_{25-35}$ fragments by preventing an increase in the level of lipid peroxidation and of the Bax/Bcl-2 ratio [63].

Using the mAChR antagonist scopolamine with a σ 1 receptor antagonist blocked the beneficial effects of the two compounds on learning deficits induced by $A\beta_{25-35}$ fragments. Other tests helped to compare their efficiency with that of a selective σ 1 receptor agonist and a mAChR agonist. Results confirm the synergistic effect of ANAVEX1-41 and ANAVEX2-73 on the two targets and highlight their therapeutic potential in AD. Nevertheless, ANAVEX2-73's

activity lasts longer, and it is currently being assessed in a phase II clinical trial with patients suffering from mild to moderate AD.

In March 2015, Anavex Life Sciences Corp. released the first promising preclinical results of compound **ANAVEX3-71 (AF710B)**, a $\sigma 1$ receptor activator and a M1 muscarinic receptor allosteric modulator. The compound proves to be very potent at low dose. It lowers concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ and amyloid plaque concentrations in 3xTg transgenic mice, as well as expression of BACE1. In the same animal model of AD, it reduces hyperphosphorylation of tau induced by the kinases (GSK-3 β and Cdk5 inhibition) and alleviates cognitive troubles. ANAVEX3-71 also has beneficial effects on inflammation (reduction of different markers such as astrocytes and activated microglia) and on mitochondrial dysfunctions.

16.4 Parkinson's Disease

In the same way as AD is a cholinergic deficiency syndrome, PD appears as a dopaminergic one. Indeed, the neurodegeneration that accompanies PD is responsible for a neuronal loss that principally takes place in the *substantia nigra* and leads to a dopaminergic depletion in the striatum. The latter is implied in the motor disorders that constitute the characteristic symptoms of PD: tremor, rigidity, bradykinesia, and gait impairment. However, since other neuronal systems (cholinergic, serotonergic, noradrenergic) are concerned by neurodegeneration, other nonmotor and cognitive symptoms are observed in PD patients.

Even if the role of Lewy bodies in the pathogenesis of PD is not totally elucidated, they constitute a hallmark of the disease. They result from the aggregation of hyperphosphorylated α -synuclein oligomers. Other molecular causes are closely interwoven with oxidative stress, metallic ion dyshomeostasis, neuroinflammation, glutamatergic excitotoxicity, or exposure to toxins, some of them further playing a pathological chaperon role in the α -synuclein aggregation [64].

The dopaminergic deficiency, as a result of the degeneration in PD, led to propose a symptomatic approach of the latter, based on the improvement of the neurotransmission through the administration of levodopa, the physiological precursor of dopamine. Levodopa remains today the standard drug against PD, but its long-term efficiency is compromised by disabling motor complications such as dyskinesia. To avoid the latter, dopaminergic receptor (DR) agonists can be associated with levodopa. They counteract the dopamine depletion by activating D₁R, D₂R, or D₃R. Alternatively, MAO-B inhibitors, such as selegiline or rasagiline, are used to reduce the physiological degradation of dopamine that these enzymes are in charge of in the CNS.

Some of the commercially available drugs against PD activate, in a synergic manner, both D₁R and D₂R (apomorphine) or D₂R and D₃R (ropinirole, pramipexole, rotigotine). These dopaminergic agonists certainly act therefore as MTDL but only in a non-disease-modifying manner. Novel D₂/D₃R agonists are currently developed. Some of them claim a disease-modifying effect through

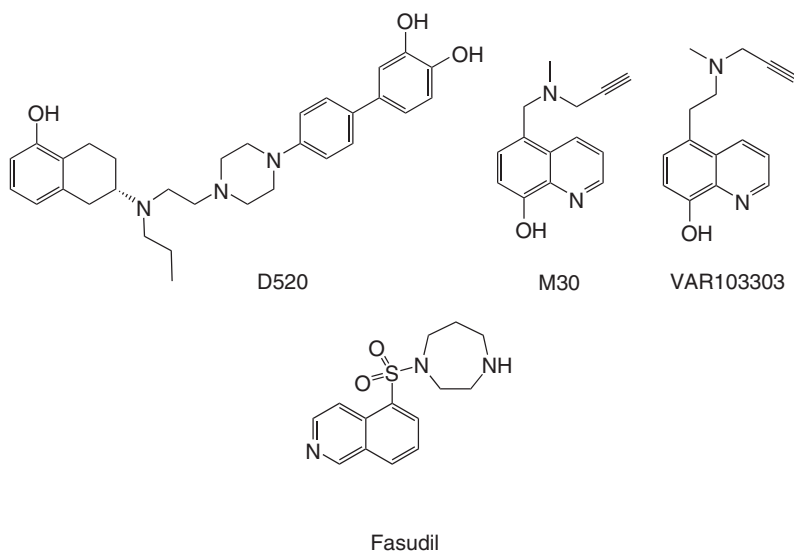


Figure 16.21 Structures of D520, M30, VAR103303, and Fasudil.

additional neuroprotective properties such as antioxidant, metal chelating, or α -synuclein aggregation inhibition (e.g., D520) (Figure 16.21) [65–69].

Some other MTDL with a potential therapeutic interest against PD are currently under investigation such as MAO-A/B inhibitors with additional AChE inhibitory activities (e.g., ladostigil already mentioned as potential pleiotropic AD agent) or metal chelating properties (e.g., M30 or VAR103303) (Figure 16.21) [70, 71].

Finally, fasudil deserves to be cited as an interesting MTDL for PD treatment with Rho-associated kinase (ROCK) and GSK3 β inhibitory activities associated with effects on the suppression of inflammatory responses and the promotion of antioxidant (Nrf2) or neurotrophic factors (GDNF) (Figure 16.21) [72].

Despite a few examples of MTDL specifically designed against PD, this pleiotropic approach remains much less developed against AD. However, according to some authors, one can also explain the recent failures occurring during the clinical trials devoted to this pathology by the too much selectivity of the tested drug for a single target without taking into account the multifactorial aspect of PD. The same authors think that a pleiotropic approach could bring response to this pitfall [73].

16.5 Conclusion

As detailed in this chapter, the multifactorial origin of the neurodegenerative diseases is in our opinion one of the reasons for which no disease-modifying agents emerged until now. In this chapter we have described a number of promising agents developed according to the MTDL strategy that reach clinical trials. Some of them appear with capacity to bring the proof of concept according to which MTDL is not only a nice concept but also an effective therapeutic approach.

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