CONTRIBUTORS

PREFACE

Annual Reports in Medicinal Chemistry has reached Volume 42. This is a testament to the longevity and value of the series to the Medicinal Chemistry community. This year continues in the tradition of providing seminal reviews on key topics in our field. The format for Volume 42 follows previous issues with a small change. We have included in our Immunology and Metabolic Sections case histories for recently approved drugs: Zelnorm® and Januvia® We hope to include more case histories in future volumes as we believe that these provide an intimate account of what it takes to bring a drug to market: strong science, persistence, and a little luck.

Putting together an endeavor like *Annual Reports in Medicinal Chemistry* requires the assistance and dedication of many individuals, including the Section Editors and proofreaders. Firstly, I would like to thank the Section Editors for their hard work and dedication in creating Volume 42. Joel Barrish and John Lowe joined a team of veteran Section Editors of Al Robichaud, Andy Stamford, Manoj Desai, and David Myles. I want to thank them for what was a seamless operation. Secondly, I encouraged the Section Editors to each enlist a group of proofreaders to help them ensure a consistent quality to the volume. I would like to acknowledge these proofreaders by listing their names below as a demonstration of our appreciation for their time and effort.

Bristol-Myers Squibb – Douglas Batt, Joanne Bronson, Andrew Degnan, Murali Dhar, Alaric Dyckman, Gene Dubowchik, George Karageorge, John Kadow, Lawrence Hamann, Soo Ko, Nicholas Meanwell, Richard Olson, Lawrence Snyder, John Starrett, Lorin Thompson, Christopher Zusi, Vivekananda Vrudhula, Michael Walker, and Stephen Wrobleski
Pfizer – Wade Blair, Blaise Lippa, and R. Keith Webber
Schering-Plough – Joel Harris, Timothy Kowalski, and Anandan Palani

I would also like to acknowledge the consistent quality effort of Shridhar Hedge and Michelle Schmidt for putting together our ''To-Market-to-Market'' review for the past few years.

Finally, I would like to thank Dr. Anthony Wood and Hannah Young from Pfizer, Sandwich. Tony was Editor-in-Chief in Volume 41 and Hannah Young was his key Administrative Assistant. Their gracious help in the transition to Volume 42 was much appreciated. In Volume 42, I would specifically like to thank Ms. Catherine Hathaway, who was the key Administrative Assistant for the volume.

In summary, I hope that you see Volume 42 of *Annual Reports in Medicinal Chemistry* as an integral reference for the medicinal chemist. As an Editor-in-Chief, I continue to look for ways to optimize and evolve the series. Please contact me with suggestions for improving the series (john.macor@bms.com).

John E. Macor, Ph. D.
Bristol-Myers Squibb
Wallingford, CT, USA

CHAPTER 1

Potential CNS Applications for Phosphodiesterase Enzyme Inhibitors

Nicholas J. Brandon* and **David P. Rotella****

Contents

1. INTRODUCTION

Mammalian type II phosphodiesterases (PDEs) are intracellular enzymes that hydrolyze the phosphodiester bond of the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [1]. PDE inhibition, which leads to an increase in cyclic nucleotide concentration, represents a means by which cellular signaling pathways can be influenced for therapeutic benefit. There are 11 distinct phosphodiesterases, designated as PDE1 through PDE11. In many of these gene families, a variable number of isozymes exist with additional complexity generated by alternative splicing. Over 50 distinct enzyme species have been identified to date. PDEs are localized to specific subcellular sites which allows for fine spatial and temporal control of the levels of the cyclic nucleotides [2,3]. This feature is thought to be an important contributing factor that allows the enzyme to influence selective intracellular signaling

* Wyeth Research, Discovery Neuroscience, CN8000, Princeton, NJ 08543, USA
** Wyeth Research, Chemical and Screening Sciences, CN8000, Princeton, NJ 08543, USA

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pathways in response to different stimuli, in spite of the ubiquitous intracellular distribution of cyclic nucleotides.

PDEs are classified by their substrate specificity. Some selectively hydrolyze cAMP or cGMP, while others will accept both cyclic nucleotides as substrates. The most widely studied members, cAMP-selective PDE4 and cGMP-selective PDE5, have been investigated for their potential as agents for the treatment of anti-inflammatory and erectile dysfunction disorders, respectively, with varying degrees of success [4,5]. Three PDE5 inhibitors, sildenafil, vardenafil, and tadalafil, have been approved for the treatment of erectile dysfunction, and sildenafil has been recently approved for the treatment of pulmonary hypertension. Among the other well-studied phosphodiesterases, a recent review in this series provided an update on the discovery and potential utility of PDE7 inhibitors [6].

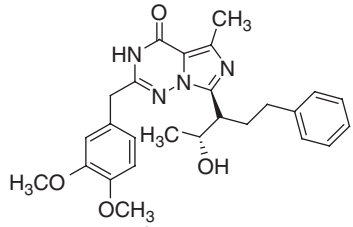
In this rapidly evolving field, the detection of PDE enzymes in the central nervous system (CNS) has stimulated interest in exploring potential applications of PDE inhibitors for treating CNS disorders such as Alzheimer's disease and other cognitive malfunctions, depression, anxiety, and schizophrenia. This review will focus on these therapeutic opportunities as well as new developments in the medicinal chemistry and biology associated with selected members of the PDE family, in particular PDEs 2, 4, 9, and 10. There have been a number of other reviews in this field in the past year that have covered selected individual PDE enzymes and potential pharmacologic applications of PDE inhibitors in CNS disorders [3,7,8].

2. PDE2 INHIBITORS

PDE2 is a mixed specificity PDE that is expressed in the CNS in the hippocampus, cortex, and striatum. This expression pattern stimulated the study of PDE2 inhibitors for treatment of cognitive disorders [9,10]. One of the most recent and thoroughly explored compounds is Bay 60-7550 (**1**). This imidazotriazinone inhibits PDE2A with an IC_{50} of 4.7 nM, and shows ~800-fold selectivity against PDEs 3B, 7B, 8A, 9A and 11A and 50-fold selectivity versus PDE1 [10].

Bayer has published the biochemical and behavioral profile of Bay 60-7550, providing very encouraging evidence for a role for PDE2 inhibition in cognitive disorders. The compound increases cGMP levels in neuronal cultures and slices, and enhances long-term potentiation (LTP) in hippocampal slices [10]. LTP as measured in the hippocampus is a long-term increase in excitatory post-synaptic potential after high-frequency stimulation of afferent pathways and is thought to be the cellular basis of learning and memory [11]. From a biological standpoint these effects are interesting, as they require the presence of a guanylate cyclase activator (Bay 41-8543). Since PDE2 is activated by cGMP binding to its GAF domains, this raises the possibility that PDE2 inhibition may enhance synaptic function only in a use-dependent manner. At the behavioral level, Bay 60-7550 improved performance in social and object recognition memory tasks and

reversed MK-801 induced deficits in T-maze spatial alternation [10]. These studies suggest that PDE2 inhibitors may be pro-cognitive and beneficial for a range of disorders.



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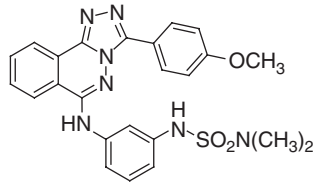
A series of triazolophthalazines were recently reported to be PDE2 inhibitors with IC_{50} values as low as 0.1 nM. In this series, compound **2** demonstrated activity in chemotaxis assays. No data were reported for effects on cyclic nucleotide levels, however, efficacy in cellular assays such as the chemotaxis assay suggests the effect is related to PDE2 inhibition in the cell types studied [12]. Pyridopyrimidines such as **3** were claimed to have IC_{50} values less than 50 nM [13].



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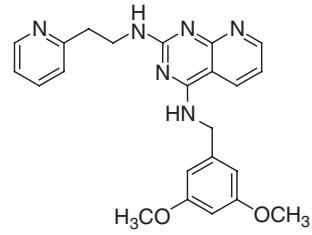

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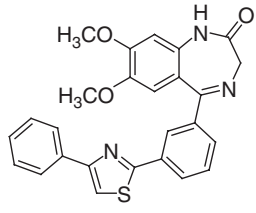
Benzodiazepinones such as **4** [14] have been claimed as PDE2 inhibitors, as have oxindoles related to **5** [15]. Compound **5** was reported to have an IC_{50} of 40 nM against PDE2. Preliminary ADME screening showed **5** to have the physicochemical and pharmacokinetic characteristics that should allow use as a tool to study PDE2 function in more detail [16].



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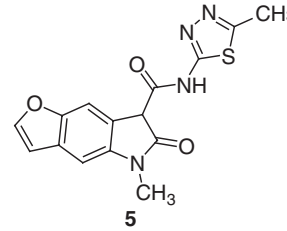

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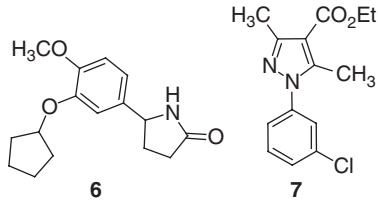
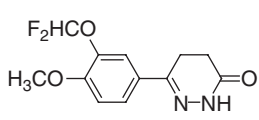
3. PDE4 INHIBITORS

PDE4 is a cAMP-specific phosphodiesterase that is widely distributed in humans. There are four PDE4 genes that encode distinct PDE4 enzymes. Of these, PDE4A, 4B, and 4D are most relevant to CNS applications. Alternative splicing of PDE4 variants leads to a range of isoforms that have been shown to have distinct subcellular localizations and functions [2]. The potential of PDE4 inhibitors in cognitive disorders was described in early reports. More recently, the effects of PDE4 inhibitors in animal models predictive of anxioytic activity, anti-depressant activity, anti-psychotic activity, stroke, and spinal cord injury have been described [7,17,18]. Rolipram (**6**), a prototypical PDE4 inhibitor, is a widely used tool compound in these experiments because of its high selectivity for PDE4 and demonstrated activity in animal models predictive of anti-depressant efficacy. Clinical development of rolipram for anti-inflammatory as well as CNS indications was limited by adverse events, such as emesis and vasculitis [7], as well as inconclusive efficacy trials.

In an effort to discover novel PDE4 inhibitors, Card and co-workers employed high throughput X-ray crystallography along with a scaffold-based approach [19]. Beginning with low molecular weight fragments with the ability to bind PDE4 as shown by X-ray crystallography, a parallel synthesis approach for optimization was employed to provide molecules with high affinity for PDE4. In one example, a pyrazole building block was derivatized with aromatic and heterocyclic substituents. The phenyl derivative **7** was the most potent analog in this series with IC_{50} values of 19 and 56 nM for PDE4B and PDE4D, respectively. In general, pyrazoles with phenyl substituents were the most active compounds in the library. A conceptually different approach to discovering novel PDE4 inhibitors comes from Rognan and co-workers beginning with the known PDE3/4 inhibitor zardaverine **8** (PDE4 $IC_{50} = 800$ nM) as a starting point [20]. The core pyridazinone template was optimized via parallel synthesis using different linkers and functional groups on the pyridazinone ring. Using three different protocols, a virtual library was created and scored after docking the candidates into the active site of human PDE4D. These virtual libraries covered three different points of attachment of the linker to zardaverine, five simple alkyl linkers and 15 different functional groups. Top scoring compounds were synthesized and evaluated, leading to the identification of compound (**9**) with a PDE4 IC_{50} value of 0.9 nM, a 4-log increase in potency from the starting point.



6 **7**



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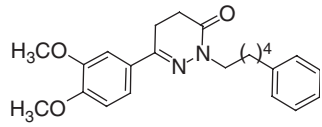

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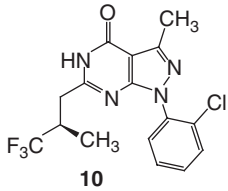
MEM1414 is a PDE4 inhibitor from Memory Pharmaceuticals that is in clinical development for potential use in Alzheimer's disease. The structure of this compound has not been disclosed. Preclinical evidence of cognitive activity was observed in rats and mice at doses as low as 1 mg/kg i.p. Available human clinical data indicate that doses up to 1000 mg/day have been tested and that the maximally tolerated dose was not reached [21]. Single and ascending multiple doses up to 400 mg revealed no significant adverse events [22]. Phase II studies using MEM1414 as monotherapy in Alzheimer's patients were initiated in mid-2006. Earlier compounds MEM1018 and MEM1091 (structures not yet disclosed) have been reported to enhance working and reference memory impaired by MK-801 in the radial-arm maze (0.1–2.5 mg/kg, IP), while blunting the amnesic effect of MK-801 on passive avoidance behavior [23].

A role for PDE4 in schizophrenia has been highlighted by recent genetic insights into this disease. PDE4B was identified to interact with DISC1, a key schizophrenia genetic risk factor [24]. DISC1 may regulate the localization and function of PDE4B. In the same report, a schizophrenia patient was identified with a balanced translocation in the PDE4B gene [24]. The genetic link between PDE4 and schizophrenia has been strengthened by a further report where single-nucleotide polymorphisms (SNPs) in PDE4B have been shown to provide protection against schizophrenia in females only [25]. Behavioral pharmacology support for the linkage between PDE4 and schizophrenia has come primarily from the Pfizer group, who have reported that rolipram is efficacious in a range of anti-psychotic models with minimal side-effects [26]. Rolipram antagonized PCP- and amphetamine-induced hyperactivity and inhibited conditioned avoidance responding. Furthermore, use of PDE4D knockout mice showed that the anti-psychotic effects of rolipram are mediated to a large extent through PDE4B [26]. Of interest to schizophrenia is a recent publication showing that chronic nicotine administration downregulates expression of PDE4 isoforms in rats [27]. The high rate of smoking in schizophrenic patients may be a form of self-medication in which nicotine intake decreases PDE4 enzyme activity by regulating its expression.

4. PDE9 INHIBITORS

PDE9 is a cGMP-specific PDE that is widely distributed in CNS tissues. PDE9 is found on chromosome 21q22.3, a region of the genome that has been linked to bipolar disorder. In addition to standard screening efforts, the extensive PDE5 inhibitor literature has been used as a starting point for PDE9 inhibitor discovery

because both enzymes are cGMP-specific phosphodiesterases. An aryl-substituted pyrazolopyrimidinone scaffold **10** was reported by Hendrix and co-workers. While the compound has moderate activity ($IC_{50} = 50$ nM) against PDE9, it shows excellent selectivity (>500-fold) against all other PDEs except PDE11, where selectivity is more modest (~50-fold) [28]. Compound **10** has been reported to elevate cGMP levels in a CHO cell line that co-expressed soluble guanylate cyclase, a cyclic nucleotide-gated ion channel, and an intracellular cGMP reporter photoprotein [28]. Thienopyrimidinones such as **11** were claimed to be potent PDE9 inhibitors ($IC_{50} \sim 20$ nM) with excellent selectivity versus PDE5 (>800-fold) [29]. The presence of a carboxylic acid may reduce the ability of this class of compounds to passively diffuse into the central nervous system.



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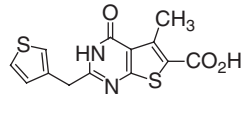

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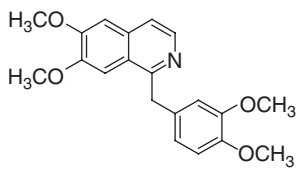
5. PDE10 INHIBITORS

PDE10 is a dual specificity phosphodiesterase that is expressed in the central nervous system [7,17]. There is increasing interest in the discovery of PDE10 inhibitors, due in part to recent reports describing the activity of papaverine (**12**) in animal models predictive of anti-schizophrenic activity. In addition, PDE10 is expressed in regions of the brain associated with neurotransmitters such as dopamine and glutamate that are thought to be involved in schizophrenia [30,31]. Dihydroisoquinoline **13**, one example from a series of derivatives claimed as PDE10 inhibitors, was reported to have a PDE10 IC_{50} value of approximately 1 nM [32]. Quinoline **14** is representative of a family of PDE10 inhibitors derived ultimately from papaverine [33]. Thienopyrimidines such as **15** were claimed to have IC_{50} values less than or equal to 500 nM [34].



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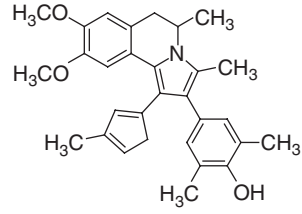

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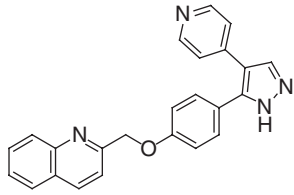

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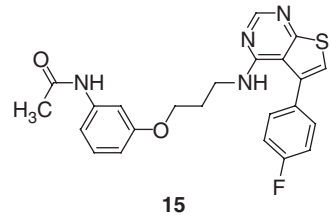

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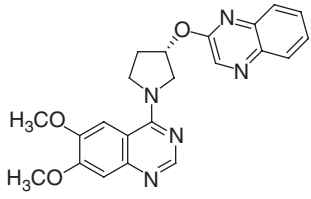
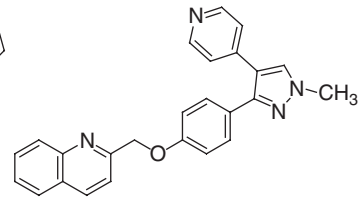
Pfizer has led the field in understanding the biology of PDE10A inhibition and developing specific PDE10A inhibitors [17]. They have published characterization of a transgenic PDE10A knockout mouse and used papaverine in a range of schizophrenia-related animal models. Observations included elevations in cAMP and cGMP, along with a reduction in activity and a reduced sensitivity to stimulants. Furthermore, PDE10A inhibition in rats and mice resulted in the disruption of the conditioned avoidance response (CAR), a model that is predictive for antipsychotic efficacy [30,31]. Papaverine has shown activity in two additional anti-psychotic models: inhibition of PCP-induced and amphetamine-induced hyperactivity [30]. In these same publications papaverine was reported not to induce catalepsy, suggesting that a PDE10A inhibitor behaves like an atypical antipsychotic with minimal liability for extra-pyramidal side effects. PQ-10 (**16**, IC_{50} 4 nM)) and MP-10 (**17**, PDE10 $IC_{50} = 0.18$ nM, 1000-fold PDE selectivity) have shown efficacy in the CAR model (ED_{50}s = 4.1, 0.7 mg/kg, respectively) and PCP locomotor reversal models (EC_{50}s = 3.8, 1.0 mg/kg, respectively), providing additional pharmacologic support for PDE10A as a potential schizophrenia target [18].

A PDE10A inhibitor may also have the potential to treat the cognitive symptoms of schizophrenia. The principal evidence for this claim is papaverine reversal of a PCP-induced deficit in the 'EDID-set shifting' assay in rats [35]. This assay translates into human behavior in the form of the 'Wisconsin Card Sorting Test (WCST)'. EDID-set shifting is a test of executive function, a measure in which schizophrenics have a robust deficit. It has also been shown recently that papaverine is efficacious in the 'Novel Object Recognition' cognition assay [36].

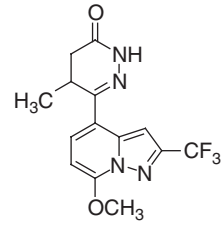
Pyrazolopyridines such as **18** were claimed to have activity as PDE10A inhibitors [37]. PDE selectivity information (comparable inhibition of PDE3A and PDE4B) provided in the patent application suggests this series of compounds may be less selective than pyrrolidinyl quinazoline **16** (PDE10A $IC_{50} = 4$ nM), which was reported to have 50- to 80-fold selectivity against PDE3A and PDE3B, respectively [38].



16 (PQ-10)



17 (MP-10)



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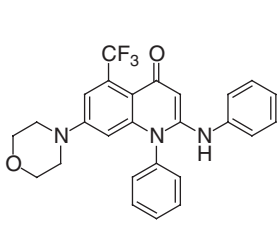
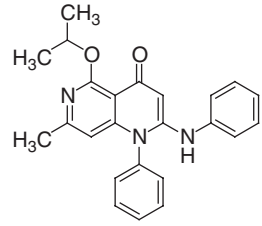
6. PDE11 INHIBITORS

A recent study examined the role of all PDEs and their potential genetic contribution to major depression disorder (MDD). Interestingly, variants in PDE9A and PDE11A were found to be associated with the diagnosis of MDD, while variants in PDE1A and PDE11A were associated with remission on antidepressants. This highlights a potential role for PDE11A in the pathophysiology of MDD. Two recent PDE11 inhibitors, **19** (PDE11A IC_{50} 0.7 nM; PDE5 selectivity >1000-fold) and **20** (PDE11A IC_{50} 3.5 nM; >1000-fold selectivity versus PDE 2, 3, 4, and 5) illustrate compounds structurally unrelated to tadalafil (**21**) that are active as PDE11 inhibitors [39,40]. Tadalafil has been shown to inhibit PDE11 with an IC_{50} of approximately 40 nM [41]. These compounds were studied as potential anti-diabetic agents. However, because of their selectivity profiles, they may be useful probes to explore PDE11A biology in the CNS.



19 (BAY 65-6207)



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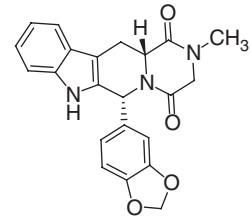

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7. SUMMARY

The interest in potential therapeutic utility of PDE inhibitors for CNS disorders is growing as evidenced by recent publications and presentations. There is an improved understanding of the roles played by second messengers cAMP and cGMP in signal transduction pathways thought to be linked with receptors and biochemical mechanisms associated with disease processes. Coupled with increasing evidence of genetic linkages between PDEs and CNS disorders, this research has provided the basic groundwork for investigation of PDEs as CNS drug targets.

There are a variety of structural classes of compounds that are active against each phosphodiesterase, and evidence suggests that selective inhibitors of PDEs can be identified. The structural diversity of PDE inhibitors provides a multitude of opportunities for development of compounds with drug-like properties. Furthermore, phosphodiesterase inhibition, which avoids direct interaction of a compound with a cell surface or nuclear receptor, may circumvent some of the target selectivity issues that can complicate receptor-based therapeutic approaches. As noted above, the specific subcellular distribution of phosphodiesterase enzymes is a key feature of their ability to modulate intracellular signaling pathways. This localization of the enzyme may minimize non-specific target

interactions. However, one potentially challenging feature in PDE inhibitor development is the prerequisite for a chemical series that demonstrates efficacy in a cellular model. The availability of cell-based assays reflective of inhibition of selective PDE enzymes is currently limited, and improved methods will be of significant value to the field. The availability of X-ray crystal structures of PDEs with inhibitors bound will no doubt aid the rational design of more potent derivatives.

Preclinical validation of PDE2, 4, 9, and 10 as targets for important disorders including schizophrenia, depression, anxiety, and cognition has been achieved. The animal models employed in these screens are predictive of the potential for efficacy in humans. At this writing, the only PDE inhibitor known to be in human clinical studies for a CNS indication is the PDE4 inhibitor MEM1414 for Alzheimer's disease. If the surge of interest in the field is a preview of the potential of the area, investigators can look forward to the discovery of PDE inhibitors that will attempt to provide human clinical validation for a range of diseases for which new treatment mechanisms are a well-recognized medical need.

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CHAPTER 2

Recent Developments in Monoamine Reuptake Inhibitors

Shuang Liu and **Bruce F. Molino**

Contents

1. INTRODUCTION

Monoamine reuptake inhibitors elevate extracellular levels of serotonin (5-HT), norepinephrine (NE) and/or dopamine (DA) in the brain by binding to one or more of the transporters responsible for reuptake, namely the serotonin transporter (SERT), the norepinephrine transporter (NET) and the dopamine transporter (DAT), thereby blocking the reuptake of the neurotransmitter(s) from the synaptic cleft [1]. Monoamine reuptake inhibitors are an established drug class that has proven utility for the treatment of a number of CNS disorders, especially major depressive disorder (MDD).

Since the introduction of tricylic antidepressants (TCAs) almost 50 years ago, monoamine reuptake inhibitors with greatly improved safety profiles have significantly enhanced the treatment of depression [2,3]. Although TCAs are very effective antidepressants, cardiovascular, anticholinergic and sedative side effects

Albany Molecular Research Inc., 30 Corporate Circle, Albany, NY, 12203, USA

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are common due to the interaction of TCAs with muscarinic, histaminic and adrenergic receptors [2]. The revolutionary introduction of selective serotonin reuptake inhibitors (SSRIs) in the 1980s allowed a much larger patient population to be treated because of the highly improved safety profile. Over the past decades, inhibitors that selectively block the reuptake of NE or DA, or two of the three neurotransmitters simultaneously, have become available for the treatment of CNS disorders including depression, anxiety, obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD), pain and urinary incontinence. Representative recent reviews on these classes of monoamine reuptake inhibitors will be cited in the following sections.

Considerable effort in the field of monoamine reuptake inhibitors is focused on improving antidepressant efficacy since 30–40% of patients do not respond to treatment with currently available agents [6,7]. An additional major objective is to enhance the onset of action. Current antidepressants typically require 2–6 weeks of treatment before clinical efficacy is seen [6]. Clinical trials exploring augmentation strategies, in which a DA reuptake inhibitor or a dual NE/DA reuptake inhibitor is combined with an SSRI, have resulted in improved efficacy in depressed patients refractory to SSRI treatment alone [4,5]. The improved results from clinical trials such as these serve to justify the considerable focus on the development of inhibitors that simultaneously block the reuptake of 5-HT, NE and DA.

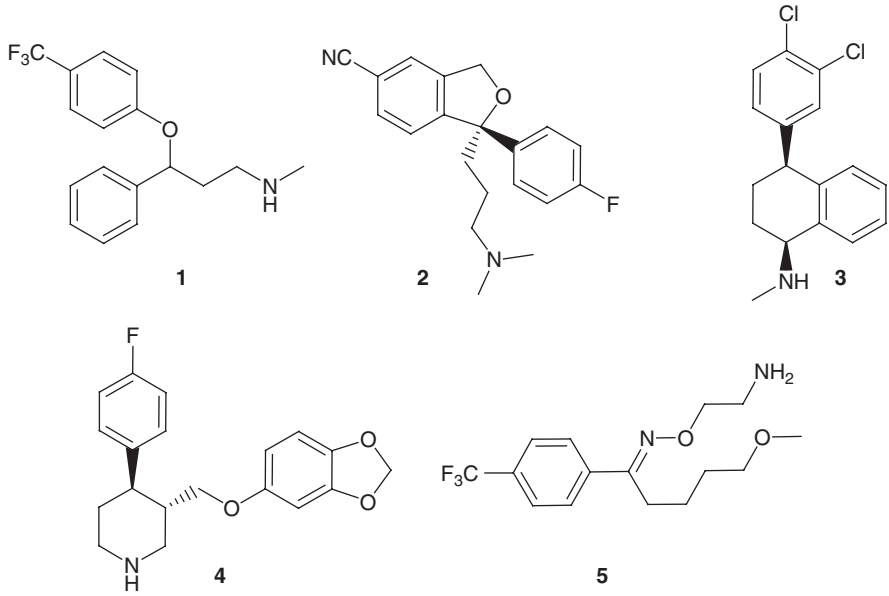
Because of the continued need for better drugs to treat depression and the opportunities for new clinical indications, efforts to discover novel monoamine reuptake inhibitors continue unabated. This review will highlight developments in the discovery of novel agents that work via monoamine reuptake inhibition primarily based on publications that have appeared between 2005 and early 2007. New clinical indications for monoamine reuptake inhibitors will also be highlighted. A comprehensive review of publications on monoamine reuptake inhibitors between 2000 and July 2005 is available [3]. Approaches for the treatment of depression involving the augmentation of monoamine reuptake inhibitors with other CNS receptor modulators, and non-monoamine-based strategies have also been reviewed recently [6–8].

2. SINGLE ACTION REUPTAKE INHIBITORS

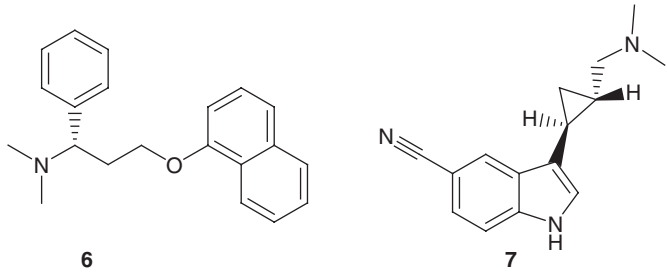
2.1 SSRIs

Since the introduction of the first approved SSRI, fluoxetine (**1**) in 1987 [9], a number of SSRIs have been developed for the treatment of depression [2]. Currently, the five most commonly prescribed SSRIs are fluoxetine, escitalopram (**2**, *S*-enantiomer of citalopram), sertraline (**3**), paroxetine (**4**) and fluvoxamine (**5**). Recent effort in the clinical development of new SSRIs has focused on the treatment of premature ejaculation (PE) by taking advantage of the ejaculation-delaying side effects of SSRIs [10]. Although SSRIs have been prescribed off-label to treat this condition, an SSRI with rapid onset of action and rapid clearance could be preferred for on-demand treatment of PE [11,12]. Dapoxetine (LY210448, **6**), an

SSRI structurally related to fluoxetine with a shorter half-life, was reported to be an effective and generally well-tolerated treatment for men with moderate-to-severe PE in clinical trial [13,14].



BMS-505130 (**7**) is a potent and selective serotonin transporter inhibitor (SERT $K_i = 0.18$ nM, NET $K_i = 4.6$ μM, DAT $K_i = 2.1$ μM). In brain microdialysis studies, **7** demonstrated a dose-dependent increase in cortical serotonin levels. Compound **7** was also active in the mouse tail suspension model [15]. Following oral administration, peak plasma concentration of **7** was reached at 1.6 h and then declined to a concentration less than 10% of C_{max} within 6 h. The short half-life of **7** might be advantageous for the treatment of PE where an acute effect to delay ejaculation followed by a relatively rapid fall in SSRI plasma concentration might be desirable.



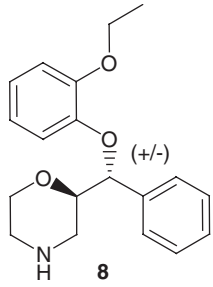
Another potential indication for SSRIs is irritable bowel syndrome (IBS). Citalopram has recently been reported to significantly improve abdominal pain, bloating, impact of symptoms on daily life, and overall well being compared

with placebo in a controlled crossover study involving 23 non-depressed IBS patients [16].

2.2 Norepinephrine reuptake inhibitors (NRIs)

The neurotransmitter norepinephrine is believed to play an important role in the etiology of depression. Approved antidepressants that belong to monoamine oxidase inhibitor (MAOI), TCA, SNRI, NRI and NDRI drug classes influence central norepinephrine function [17]. The development of selective NRI compounds has resulted in considerably fewer FDA approved drugs compared with the SSRI drug class [18]. No NRIs have been approved for treatment of depression in the U.S. and only reboxetine (**8**) has been approved for use in Europe [19]. In a recently reported double-blind study of 357 patients with major depressive disorder comparing the efficacy and tolerability of reboxetine and citalopram, both treatments produced similar efficacy [20]. However, there was a higher prevalence of sexual dysfunction in the citalopram group in this study. In another recently reported clinical trial, the combination of reboxetine and an SSRI appeared to be effective in cases of SSRI-resistant depression [21].

Atomoxetine (**9**), a selective NRI, is the first non-stimulant drug approved for the treatment of ADHD [22]. Interestingly, in a recent 12-week, randomized, double blind, placebo-controlled trial in 30 obese women, atomoxetine demonstrated modest short-term weight loss efficacy relative to placebo [23].



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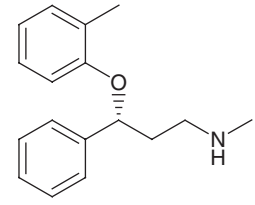

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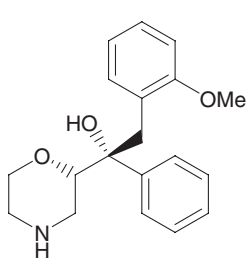
Compound **10**, representing a series of NRIs structurally similar to reboxetine, has been reported to be a potent and selective inhibitor of NET ($K_i = 3.2$ nM) [24]. In an α-methyl-*m*-tyrosine (α-MMT)-induced cortical NE depletion model in rats, **10** showed dose-dependent activity after oral administration with a measured ED_{50} of 18 mg/kg. WAY-256805 (**11**), an NRI, was recently reported to be efficacious in the mouse tail suspension model [25].

The availability of effective SERT and DAT radioligands for positron emission tomography (PET) has led to studies in which transporter occupancy of drugs can be determined in a non-invasive manner [26]. Similar studies to determine NET occupancy using PET have been hindered due to the lack of availability of a suitable NET ligand. Progress towards the discovery of NET

ligands for PET was recently reviewed [26]. A reboxetine-derived radioligand, (*S*,*S*)-[^{11}C]MRB (**12**) has been reported to have potential as a NET ligand for PET studies [27].



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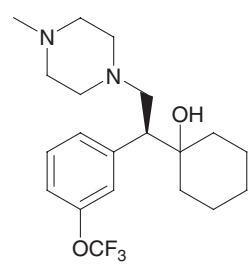

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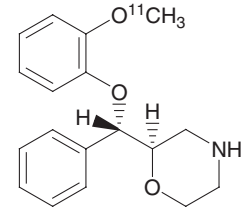

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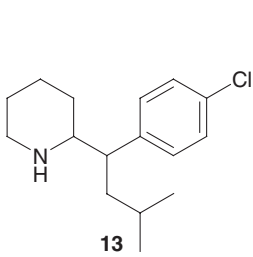
2.3 Dopamine reuptake inhibitors

The dopamine transporter has been a target for developing pharmacotherapies for a number of CNS disorders including ADHD, stimulant abuse, depression and Parkinson's disease. Several excellent reviews in this area have been recently published [28–30]. The dopamine reuptake inhibitor methylphenidate has been successfully used for decades in the management of ADHD in children and adolescents. It remains a first-line treatment along with amphetamine for this disorder [31,32].

Slow-onset, long duration dopamine reuptake inhibitors with reduced potential for substance abuse have been suggested as therapies for psychostimulant addiction [33–35]. A series of slow-onset, long duration *N*-alkyl analogues of methylphenidate were recently reported to have enhanced selectivity for the dopamine transporter [34]. A representative compound is **13**, an RR/SS diastereomer (DAT $K_i = 16$ nM, SERT $K_i = 5900$ nM, NET $K_i = 840$ nM). In a locomotor activity assay in mice, **13** has a slow onset of activity (20–30 min) with peak activity occurring between 90 and 120 min. In contrast, both methylphenidate and cocaine are active within 10 min and reach peak activity within 30 min.

In a prodrug approach, compound 30,640 (**14**) demonstrated potential for use as maintenance therapy in psychostimulant addiction treatment [35]. The N-demethylation of **14** provides indatraline, a potent triple monoamine reuptake inhibitor. Compound **14** showed cocaine-like effects, but with a slow-onset, long-lasting profile. Treatment with **14** (2 mg/kg i.p.) produced a slow-onset, long-lasting increase (300–400%) in extracellular dopamine levels in the nucleus accumbens in rats. Compound **14** (3 or 5 mg/kg i.p.) also produced a significant (~30%) slow-onset, long-lasting enhancement of electrical brain-stimulation reward, which was additive with that of cocaine (5 mg/kg i.p.). When given to cocaine-administering rats, **14** significantly inhibited cocaine self-administration,

with a long-lasting profile.



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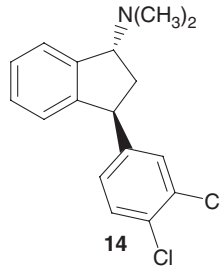

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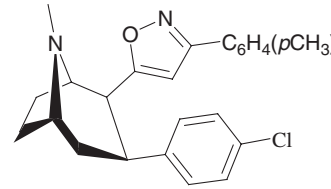

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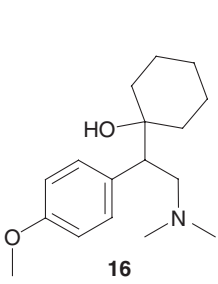
A series of DAT selective 3-phenyltropanes have been reported to have potential for treatment of cocaine abuse [33,36,37]. RTI-336, **15** (reuptake $IC_{50} = 4.1$ nM) was the most potent among these tropane derivatives in locomotor activity and drug discrimination; it was less stimulatory than cocaine, and had the slowest onset and longest duration of action. It also reduced self-administration of cocaine in rats and rhesus monkeys. Interestingly, in rhesus monkeys trained to self-administer cocaine, when coadministrated with either citalopram or sertraline, **15** produced significantly more robust reductions in cocaine self-administration compared with **15** alone [38].

3. DUAL REUPTAKE INHIBITORS

3.1 SNRIs

There are three approved drugs, venlafaxine (**16**), duloxetine (**17**) and milnacipran (**18**), in the serotonin-norepinephrine reuptake inhibitor (SNRI) class. Whereas milnacipran blocks 5-HT and NE reuptake with almost equal potency, venlafaxine and duloxetine block 5-HT reuptake preferentially [39–41]. Clinical evidence shows that SNRIs have comparable efficacy in the treatment of MDD compared with antidepressants in the SSRI class. An advantage with SNRIs appears to be the ability of alleviating chronic pain associated with, and independent of depression [42–44].



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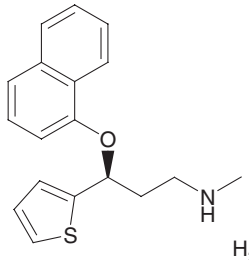

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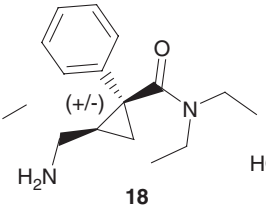

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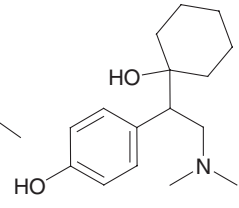

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As the first SNRI drug approved, venlafaxine has become one of the first-line choices for depression and anxiety disorder [45,46]. An active metabolite, desvenlafaxine (**19**), is also under clinical development for the treatment of major depressive disorders [47]. Preclinical studies also indicate that **19** may be effective in relieving vasomotor symptoms associated with menopause (e.g., hot flushes and night sweats) [47,48]. Desvenlafaxine is reported to be in clinical development for the treatment of fibromyalgia and neuropathic pain, as well as vasomotor symptoms associated with menopause [68].

In recently reported clinical trials, venlafaxine extended release was found effective and well tolerated in short-term and continuation treatment of patients with posttraumatic stress disorder [49,50].

In addition to treating MDD [51–53], duloxetine was approved as the first agent for the treatment of painful diabetic neuropathy in the U.S. [54–56]. It also has been used for stress urinary incontinence in women in Europe [57,58]. In 2007, duloxetine was approved for the treatment of generalized anxiety disorder in the U.S.

Duloxetine in combination with ibuprofen was reported to exert a significant synergistic interaction in rodents both for reducing acetic acid-induced writhing and carrageenan-induced thermal hyperalgesia, but was additive for reversing mechanical allodynia [59]. These results suggest that duloxetine and ibuprofen in combination may provide a useful approach to the clinical treatment of persistent pain, particularly inflammation-related pain.

Using the carrageenan pain model in rats, a synergism between serotonergic and noradrenergic reuptake inhibition was reported [60]. The selective NRI thionisoxetine (0.03–10 mg/kg, i.p.) produced complete reversals of carrageenan-induced thermal hyperalgesia and a greater than 80% reversal of carrageenan-induced mechanical allodynia. In contrast, the SSRI drugs paroxetine, sertraline and fluoxetine had little or no effect in the carrageenan model. In the presence of fluoxetine, the potency of thionisoxetine in reversing carrageenan-induced hyperalgesia and allodynia was increased by ~100-fold, while brain concentrations of thionisoxetine were increased by only 1- to 5-fold. These results indicate that, in the carrageenan model, dual serotonergic-noradrenergic reuptake inhibition produces synergistic analgesic efficacy.

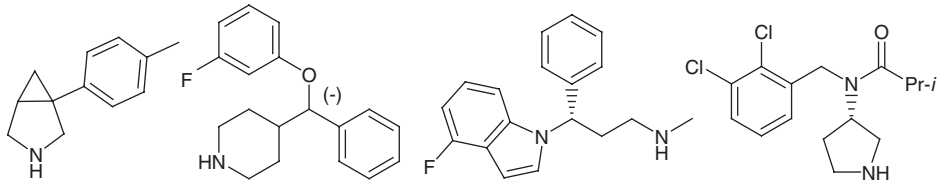
A dose-finding study of duloxetine based on duloxetine-associated SERT occupancy was recently reported using PET [61]. SERT occupancies increased dose responsively and correlated well with the plasma concentration of duloxetine. It was found that 40 mg or more duloxetine was needed to attain 80% occupancy, and 60 mg of duloxetine could maintain a high level of SERT occupancy with once-a-day dosing.

Milnacipran is currently available for use as an antidepressant in several countries outside the U.S. It is also under clinical development to assess its potential role in the treatment of fibromyalgia syndrome [62,63]. In a rat model of neuropathic pain, milnacipran, administered intrathecally, produced dose-dependent anti-allodynic effects at doses between 3 and 100 μg for up to 7 h [64]. The anti-allodynic effect of 30 μg of milnacipran was attenuated by intrathecal coadministration of a serotonin receptor antagonist or a norepinephrine receptor

antagonist. The anti-allodynic effects were not produced by intrathecal administration of paroxetine or maprotiline (a TCA and strong NET inhibitor). These findings suggest that simultaneous inhibition of serotonin and noradrenaline reuptake in the spinal cord is essential to mediate anti-allodynic effects.

Another SNRI, bicifadine (**20**), formerly under clinical development for chronic low back pain, is now being developed for neuropathic pain [65,66]. Flufenoxine, also known as F-98214-TA (**21**), was reported to display greater potency than several reference antidepressants in animal models predictive of antidepressant and anxiolytic activities [67]. SEP-227162 (structure undisclosed) is another SNRI reportedly undergoing clinical development [68].

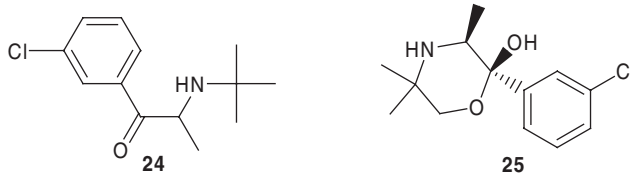
A series of 3-(1*H*-indol-1-yl)-3-arylpropan-1-amines was recently reported as a new class of SNRIs [69]. Compound **22** exhibited potent inhibition of SERT and NET ($IC_{50} = 9$ and 12 nM, respectively). A number of *N*-(pyrrolidin-3-yl) carboxamide derivatives were reported as SNRIs in a recent patent application [70]. Compound **23** showed potent inhibition of 5-HT and NE reuptake ($IC_{50} = 12$ and 23 nM, respectively).



20 **21** **22** **23**

3.2 Norepinephrine dopamine reuptake inhibitors (NDRIs)

After more than a decade of use, bupropion (**24**) is considered a safe and effective antidepressant, suitable for use as first-line treatment. In addition, it is approved for smoking cessation and seasonal affective disorder. It is also prescribed off-label to treat the sexual dysfunction induced by SSRIs. Bupropion is often referred to as an atypical antidepressant and has much lower affinity for the monoamine transporters compared with other monoamine reuptake inhibitors. The mechanism of action of bupropion is still uncertain but may be related to inhibition of dopamine and norepinephrine reuptake transporters as a result of active metabolites [71,72]. In a recently reported clinical trial, bupropion extended release (XL) had a sexual tolerability profile significantly better than that of escitalopram with similar remission rates and Hospital Anxiety and Depression (HAD) total scores in patients with MDD [73].



24 **25**

In a placebo-controlled, 8-week prospective trial involving 162 adult patients diagnosed with ADHD, subjects were treated with up to 450 mg/day of bupropion XL [74]. Bupropion XL responders (53%) exceeded placebo responders (31%) at week 8 with a significantly greater proportion of bupropion XL responders as early as week 2, suggesting that bupropion XL is an effective non-stimulant treatment for adult ADHD. Several randomized clinical trials have also shown efficacy for this drug in promoting weight loss in obese patients [75].

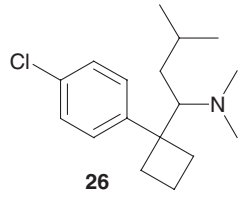
One of the metabolites of bupropion, radafaxine (GW 353162, **25**), is being studied as a treatment for obesity in clinic trials [76]. It is also reported to be in clinical development for restless leg syndrome, neuropathic pain, bipolar disorder and fibromyalgia [68].

4. TRIPLE REUPTAKE INHIBITORS (TRIs)

Triple reuptake inhibitors (TRIs), which inhibit reuptake at all three transporters, have attracted considerable interest in recent years [77]. The involvement of dopamine reuptake in the etiology of depression and other CNS disorders has been recognized [29,30]. As a result, TRIs have been proposed to offer a faster onset of action and improved efficacy for depression over currently prescribed single or dual action monoamine reuptake inhibitors. Historically, the mesocorticolimbic dopamine pathway is thought to mediate the anhedonia and lack of motivation observed in depressed patients [78,79]. In addition, methylphenidate, both immediate release and extended release formula, has been found to be effective as an augmenting agent in treatment-resistant depression [4]. Furthermore, clinical studies using the combination of bupropion and an SSRI or SNRI have showed improved efficacy for the treatment of MDD in patients refractory to the treatment with SSRIs, SNRIs, or bupropion alone [5,80,81].

A recent study further supported the involvement of dopamine in the mechanism of antidepressants [82]. In this study, the antidepressant-like effect of citalopram, paroxetine, desipramine and imipramine in the mouse forced swim test (FST) was compared with and without dopamine depletion. It was found that lesioning with 6-OHDA did not affect the response of mice to desipramine and imipramine, whereas dopamine depletion abolished the antidepressant-like effect of citalopram and paroxetine. These results suggest that the antidepressant-like effect of SSRIs in the FST requires the activation of dopaminergic pathways.

Although it is often classified as a SNRI, sibutramine (**26**) is metabolized *in vivo* to produce metabolites that have varying degrees of inhibition of NE, 5-HT and DA reuptake [83,84]. It has been approved for the control of obesity in the U.S. and many other countries.



26

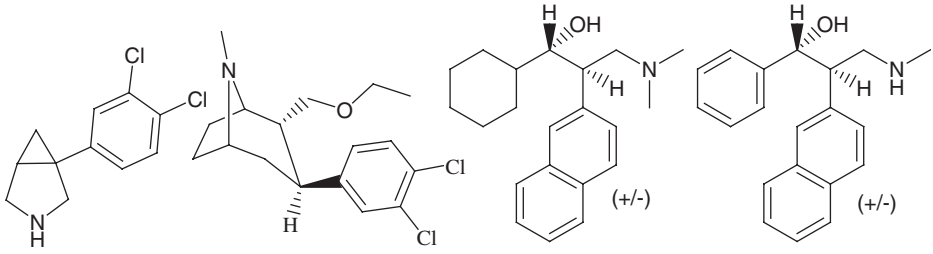
Both enantiomers and the racemate of 1-(3,4-dichlorophenyl)-3-azabicyclo [3.1.0]hexane, **27a–c**, have been reported to be in development. The racemate, DOV 216,303, inhibits the reuptake of NE, 5-HT and DA with IC_{50} values of 20, 14 and 78 nM, respectively [85]. DOV 216,303 is active in tests predictive of antidepressant activity, including the mouse FST (minimum effective dose = 10 mg/kg), reversal of tetrabenazine-induced ptosis and locomotor depression. DOV 216,303 was also reported to be well tolerated in phase I clinical trials [85,86]. In a phase II study designed to explore safety and tolerability in depressed individuals, patients received either DOV 216,303 (50 mg, b.i.d.) or citalopram (20 mg, b.i.d.) for two weeks [85]. It was found that the side effect profile was not remarkably different between the two treatment groups. In addition, time-dependent reductions in Hamilton Depression Scores (HAM-D) were similar for both groups.

The (+)-enantiomer, DOV 21,947, is approximately twice as potent at NET and SERT as DOV 216,303. The minimum effective dose in both mouse tail suspension and rat FST models is 5 mg/kg [87,88]. The (−)-enantiomer, DOV 102,677, is less potent than DOV 216,303 across all three transporters [89]. It is active in the FST in rats with a minimum effective dose of 20 mg/kg. DOV 102,677 is as effective as methylphenidate in reducing the amplitude of the startle response in juvenile mice, without notably altering motor activity. It is reportedly under development for the treatment of alcohol abuse and alcoholism [68].

SEP-225289 (structure not disclosed) is reported to be a triple reuptake inhibitor in early clinical development [68].

Tropane derivative tesofensine, also known as NS2330 (**28**), is reported to be a triple reuptake inhibitor. Its efficacy as a monotherapy in early Parkinson's disease was evaluated in a clinical trial; however, it did not provide significantly greater benefits than placebo [90]. NS2330 is also reported to be in clinical trials for obesity [68]. NS2359 (GSK 372475, structure not disclosed), also a triple reuptake inhibitor, is reportedly in clinical development for depression and ADHD, as well as addictive disorders [68].

Structurally related to venlafaxine, PRC025 (**29**) and PRC050 (**30**) were reported to be triple reuptake inhibitors. Both exhibited antidepressant efficacy at 5 mg/kg in the rat FST [91].



(+/-)-27a
(+)-27b
(-)-27c

28 **29** **30**

5. CONCLUSION

The search for new drugs based on monoamine reuptake inhibition continues in an effort to find more effective treatments for MDD as well as a variety of other diverse clinical indications. The movement toward novel monoamine reuptake inhibitors with broader transporter reuptake inhibitor profiles (SERT, DAT and NET) is driven by the substantial patient populations that do not respond to the drugs in the SSRI class as well as the need for drugs with faster onset of action. Results from clinical trials where SSRIs are augmented with drugs like methylphenidate or bupropion support the benefit of adding DA reuptake inhibition in the design of new monoamine reuptake inhibitors. Other studies of this kind are likely to further elucidate the factors important for making better antidepressants.

The recent approval of the SNRI duloxetine for the treatment of diabetic neuropathy reinforces the utility of this drug class in the treatment of neuropathic pain. Other largely untapped areas which remain to be exploited with this drug class include sexual dysfunction, such as premature ejaculation, irritable bowel syndrome, obesity, neurodegenerative diseases such as Parkinson's disease, restless leg syndrome, and substance abuse and addiction. It is apparent that considerable opportunities for drug discovery will exist in this area for some time to come.

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CHAPTER 3

Secretase Inhibitors and Modulators for the Treatment of Alzheimer's Disease

Richard E. Olson and **Lawrence R. Marcin**

Contents

1. INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the developed world, affecting more than 15 million people worldwide and >50% of

Bristol-Myers Squibb, Richard L. Gelb Center for Pharmaceutical Research and Development, Neuroscience Chemistry, Wallingford, CT 06492-7660, USA

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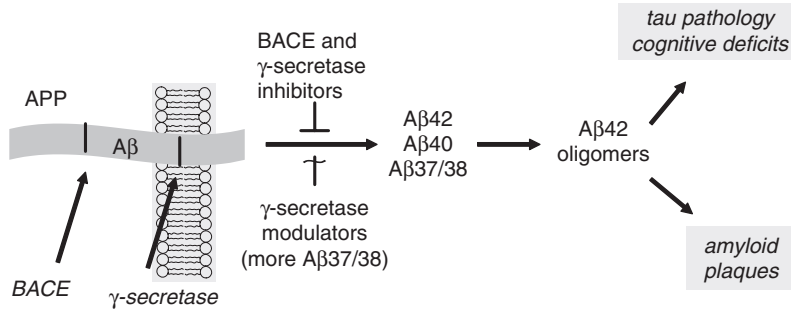


Figure 1 Amyloid cascade hypothesis and intervention points.

individuals over the age of 85 in the USA [1,2]. A defining characteristic of AD pathology is the presence of extraneuronal plaques composed of β-amyloid peptides (Aβ). Aβ is a 37–42 amino acid fragment excised from the transmembrane (TM) region of amyloid precursor protein (APP) by the sequential action of β-site APP cleaving enzyme (BACE) and γ-secretase (Figure 1). The ''amyloid cascade hypothesis'' posits an initiating role for Aβ42 in AD, in which accumulation of Aβ42 leads to neurotoxicity, tau hyperphosphorylation and aggregation, and clinical decline [3]. The amyloid cascade hypothesis is supported by the genetics of familial forms of AD. In particular, mutations in the Aβ region of APP as well as numerous mutations in the γ-secretase-associated proteins presenilin-1 and -2 result in increased Aβ42, or increased Aβ42/Aβ40 ratios, and lead to early-onset AD [4].

While plaques are a hallmark of AD pathology, the number of plaques is not clearly associated with disease severity. Recent studies suggest that soluble, oligomeric forms of Aβ have a role in neurotoxicity and memory loss [4,5]. Oligomeric forms of Aβ have been shown to cause synaptic dysfunction and disrupt long-term potentiation (LTP) in cell culture, and affect behavior in transgenic mice [6,7]. Strategies for reducing Aβ42, and Aβ42-derived oligomers, include reducing Aβ synthesis through inhibition of BACE and γ-secretase, and shifting the Aβ species formed by γ-secretase to shorter forms of Aβ, such as Aβ38, using γ-secretase ''modulators.'' This review will summarize recent findings relevant to these targets, with a focus on advances in inhibitor design strategies reported since these topics were last reviewed in this journal [8,9].

2. INHIBITORS OF BACE

2.1 BACE function

Two BACE homologs have been identified. BACE1 and BACE2 are members of the pepsin-like family of aspartyl proteases [10]. BACE1 (β-secretase, BACE, memapsin-2, Asp-2) was identified nearly a decade ago as the aspartyl protease responsible for the cleavage of APP at the β-secretase site. Consequently, BACE1 has become a highly pursued therapeutic target for the treatment of AD [11].

The design of BACE inhibitors that cross the blood–brain-barrier (BBB) and maintain effective drug concentrations in the brain has proven to be exceedingly difficult [12]. While the first identified inhibitors were highly peptidic, considerable progress has been made toward reducing molecular weight, polar-surface area, rotatable bonds, and number of hydrogen bond donors/acceptors. Despite these advances, P-glycoprotein (Pgp)-mediated efflux of BACE inhibitors at the BBB has proven to be a major hurdle in achieving brain efficacy in animal models [13–19]. Currently, there are no published disclosures of BACE inhibitors in human clinical trials.

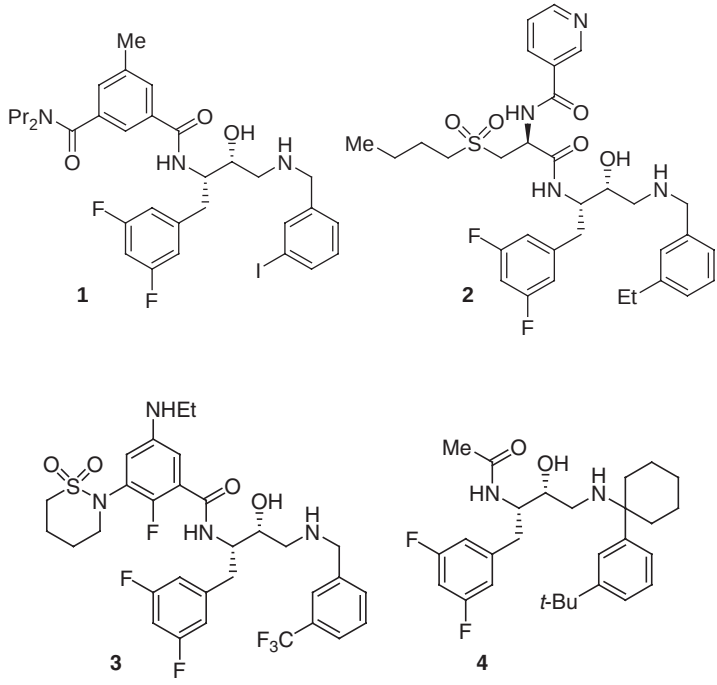
Very little is known about other physiological roles of BACE1. A recent publication suggests that BACE1 and APP processing are critical for cognitive, emotional, and synaptic functions as indicated by behavioral studies of BACE1 knockout mice [20]. Two cautionary reports have also appeared which provide evidence that BACE1 processing of neuregulin-1 is required for nerve myelination in both the central and peripheral nervous system [21,22]. While nerve myelination is critical in early life, it remains to be determined whether BACE1 cleavage of neuregulin-1 is required for maintenance of the myelin sheath in mature animals.

BACE2 is not implicated in the pathogenesis of AD, but has been associated with the onset of dementia in Down's syndrome [23], is overexpressed in certain forms of cancer [24], and may be involved in human muscle biology [25]. A recent publication has described the crystal structure of a BACE2/inhibitor complex [26]. While the degree of off-target selectivity that is necessary for the development of a well-tolerated BACE1 inhibitor remains unclear, the authors have identified structure-based opportunities by which further improvements in selectivity against BACE2 might be realized.

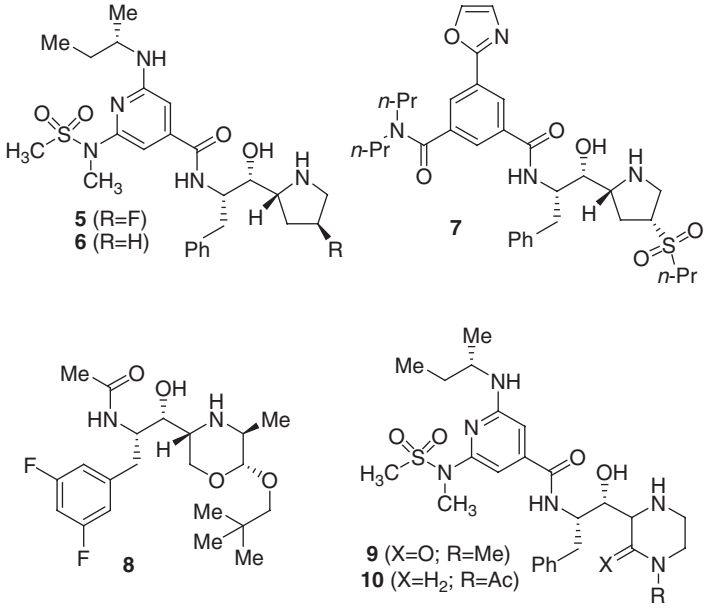
2.2 Hydroxyethylamine (HEA) inhibitors

BACE inhibitors utilizing the HEA scaffold comprise a majority of recently reported structures. These include the first accounts of inhibitors with *in vivo* efficacy in animal models. The activity of isophthalate-derived inhibitors (e.g. **1**) with excellent enzyme and cellular potency (BACE1 $IC_{50} = 5\,nM$, Aβ $IC_{50} = 3\,nM$) has been detailed in a full article [27]. An X-ray crystal structure of **1** complexed with BACE1 reveals seven key hydrogen bonds with the protein. Interatomic distances are consistent with a hydrogen bond between the hydroxyl group of **1** and a single catalytic aspartate (Asp228). Two additional hydrogen bonds are evident for the protonated secondary amine with the second catalytic aspartate (Asp32) and the carbonyl oxygen of Gly34. An optimized series of sulfone-bearing HEA inhibitors that incorporate a variety of P2 side chains, including pyridyl amide **2** (BACE1 $IC_{50} = 2\,nM$, Aβ $IC_{50} = 1\,nM$), has been disclosed [28]. A literature report has described HEA inhibitor **3** (GSK188909), which demonstrated potent BACE1 activity ($IC_{50} = 5\,nM$) and good selectivity with respect to other aspartic proteases, including BACE2 ($IC_{50} = 170\,nM$), cathepsin D ($IC_{50} = 2600\,nM$), and renin ($IC_{50} = 1490\,nM$) [13]. Good cellular potency (Aβ $IC_{50} = 5\,nM$) is reported for reduction of both Aβ40 and Aβ42.

Acute administration of **3** (250 mg/kg, orally (PO)) with a Pgp inhibitor to transgenic mice resulted in significant decreases in soluble Aβ42 (55%) in brain extracts. Dosing for 5 days with **3** (250 mg/kg, PO, bid) without a Pgp inhibitor resulted in smaller, but still significant, decreases in Aβ42 (23%). Considerable effort has been focused on the design of lower-molecular-weight HEA inhibitors, which are typically P3 truncated and modestly expanded into the S1′ or S2′ sites [29–32]. Three patent applications have disclosed C(3) acetamides with *in vivo* efficacy [29–31]. For example, compound **4** (BACE1 $IC_{50} = 47$ nM, Aβ $IC_{50} = 12$ nM) is reported to have 20% oral bioavailability, and inhibit the production of Aβ40 in the cortex and plasma by 62% and 50%, respectively, when dosed at 100 mg/kg, in transgenic mice [30].

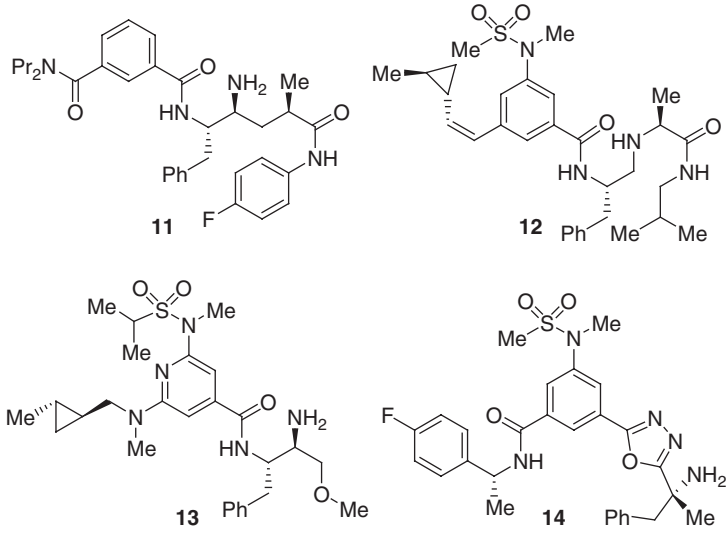


Several variants of ring-constrained HEA analogs, including 4-substituted pyrrolidines, have been disclosed. For example, excellent BACE1 ($IC_{50} = 1.8$, 3.9 nM) and cell activity (Aβ $IC_{50} = 13$, 15 nM) was reported for the representative pyrrolidines **5** and **6**, respectively [33]. A 4-*n*-propylsulfonylpyrrolidine **7** has a reported BACE1 $IC_{50} < 100$ nM [34]. Alkoxymorpholine **8** exhibited modest enzyme and cellular potency (BACE1 $IC_{50} = 78$ nM, Aβ $IC_{50} = 95$ nM), but was reported to lower Aβ levels in transgenic mice by 39, 40, and 25% in the plasma, cerebrospinal fluid (CSF), and cortex, respectively [11]. Piperazine-2-ones and piperazines have also emerged as highly potent BACE1 inhibitors [35,36]. Compounds **9** and **10** demonstrated excellent BACE1 inhibition ($IC_{50} = 1.0$, 3.0 nM) and good cellular activity (Aβ $IC_{50} = 41$, 45 nM), respectively [35,36].



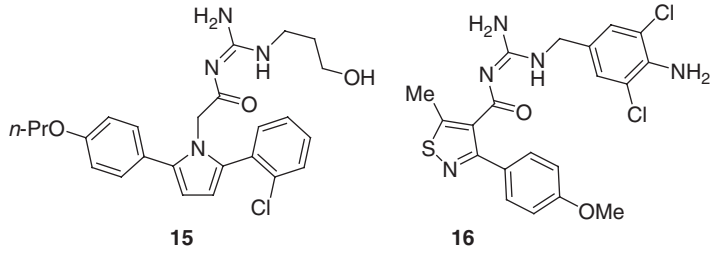
2.3 Amine inhibitors

Recent literature reports have shown that potent inhibition of BACE is possible with certain primary amines. The concept of replacing the alcohol isostere with a primary amine has been demonstrated within the context of the hydroxyethylene (HE) class of BACE inhibitors [37]. For example, aminoethylene (AE) **11** exhibited good enzyme and cellular potency (BACE $IC_{50} = 26\,nM$, Aβ $IC_{50} = 180\,nM$) [37]. A crystal structure of **11** complexed with BACE1 revealed that the primary amine of the inhibitor makes hydrogen bond contacts to both catalytic aspartates, Asp32 and Asp228, in a similar fashion to that of the hydroxyl group in HE complexes. Additional efforts in this series have focused on reducing the number of amide bonds. Toward this end, the secondary amine **12**, bearing a *cis*-vinylcyclopropane as a benzamide P3 replacement, was reported to be highly potent (Aβ $IC_{50} = 23\,nM$), but still susceptible to Pgp efflux [14]. Removal of the prime-side amide and conversion of the isostere to a primary amine resulted in isonicotinamides, such as **13**, that displayed modest cellular activity (Aβ $IC_{50} = 845\,nM$) and were reported to be moderate efflux substrates in a cell line expressing human Pgp [18]. Replacement of the internal P1 amide with an 1,3,4-oxadiazole has been described [17,38]. The resulting tertiary carbinamine inhibitors, such as **14**, displayed very good enzyme and cell-based activity (BACE1 $IC_{50} = 12\,nM$, Aβ $IC_{50} = 65\,nM$), but significant *in vitro* Pgp efflux was reported to limit the *in vivo* utility of such compounds. An X-ray crystal structure of **14** complexed with BACE1 revealed minor distortions of the linker region, due to incorporation of the five-membered heterocycle.



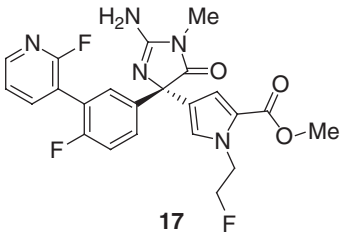
2.4 Acylguanidine and related heterocyclic inhibitors

Acylguanidines are a relatively new class of aspartic acid protease inhibitors. Details surrounding the evolution of an acylguanidine-derived BACE1 inhibitor **15** (BACE1 $IC_{50} = 110$ nM) from a high-throughput screening lead have recently appeared [39]. The unique hydrogen-bonding interactions of the guanidine moiety with the catalytic aspartates have been revealed through X-ray crystallography. This particular series has exhibited consistently poor Aβ cellular potency. A recent patent application has disclosed a series of isothiazole-derived acylguanidines as BACE inhibitors [40]. The dichloroaniline **16** has a reported BACE1 $IC_{50} < 100$ nM.



Several patent applications have appeared which claim BACE1 activity for a wide variety of aminoheterocycles. Imidazolones, such as **17**, are reported to display BACE1 potency (IC_{50}) in the range of 10–100 nM [41]. Spiropiperidine imidazolones, such as **18**, have reported BACE1 IC_{50} values in the range of 1–1000 nM [16]. 2-Aminoquinazolines, including example **19** (BACE1 $IC_{50} = 21$ nM), have displayed very good *in vitro* potency. A variety of

pyrimidinone derivatives have been claimed as BACE inhibitors, as exemplified by compound **20** (BACE1 $IC_{50} = 36\,nM$) [42]. Application of fragment screening by high-throughput X-ray crystallography has produced the aminopyridine lead **21** (BACE1 $IC_{50} = 690\,nM$) [43,44]. Optimization of napthyl and coumarinyl biarylpiperazines has resulted in derivatives with good enzyme potency but poor cell-based activity (e.g. **22**, BACE1 $IC_{50} = 630\,nM$, Aβ $IC_{50} = 2.5\,mM$) [45].



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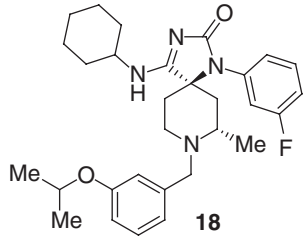

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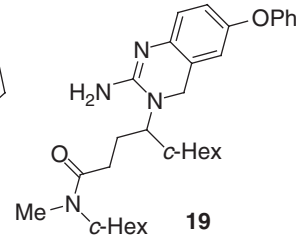

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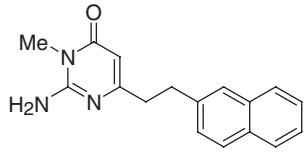

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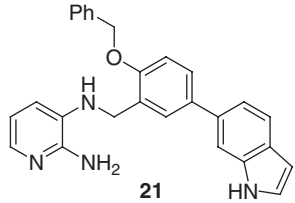

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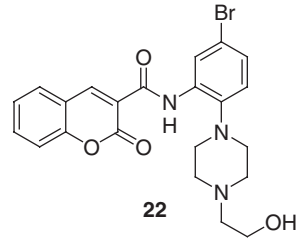

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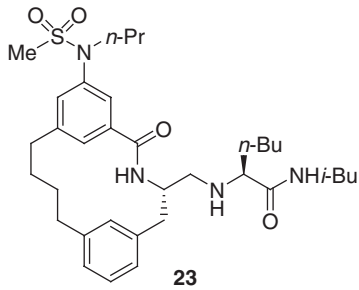
2.5 Macrocyclic inhibitors

Macrocyclic BACE inhibitors have emerged from efforts to optimize the potency and biopharmaceutical properties of known inhibitor scaffolds by increasing rigidity and reducing peptidic character. Macrocycles in which the P1 and P3 subunits are joined have been disclosed for HEA [46,47], primary amine [19,48], and other peptidomimetic inhibitors [49,50]. A publication has described the design and synthesis of P1–P3 macrocyclic aminoethylene BACE inhibitors, such as **23** (BACE1 $IC_{50} = 4\,nM$) which exhibited good cellular potency (Aβ $IC_{50} = 76\,nM$), improved permeability, and reduced Pgp susceptibility [48]. Most significantly, **23** provided a reduction of 25% in Aβ40 levels in brain extracts, when dosed at 100 mg/kg (IV bolus) in a transgenic mouse model. Good cellular potency (Aβ $IC_{50} = 40\,nM$) has been reported in a patent application [47] for a single example of a 15-membered aminopyridine macrocycle **24**. A recent patent disclosure has illustrated an alternative strategy that connects the P1′ and P2 subunits of γ-lactam-derived HEA inhibitors [51]. Several of the examples, including compound **25**, have reported BACE1 IC_{50} values of less than 10 nM.



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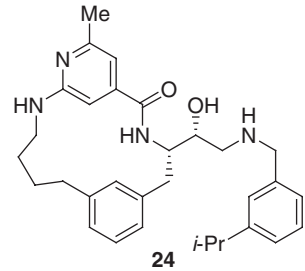

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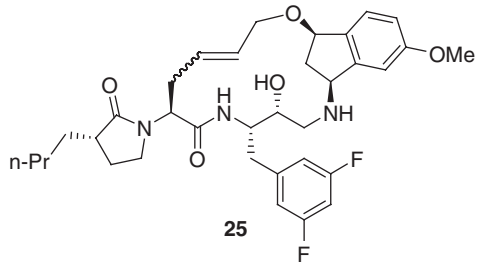

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3. INHIBITORS OF γ-SECRETASE

3.1 γ-Secretase structure and function

Inhibition of γ-secretase has been extensively explored as a means of reducing Aβ synthesis [52–59]. The γ-secretase complex is comprised of the C- and N-terminal fragments of presenilin-1 or -2 together with the TM proteins nicastrin, Aph-1 and Pen-2, and is the result of a highly regulated assembly process [60]. Heterogeneity in the γ-secretase complex arising from homologs of Aph-1 (Aph-1a/b) has been characterized [61]. Presenilin contains asparate residues in TM-6 and TM-7, which are essential to proteolytic function [62]. The putative catalytic aspartates reside in GxGD motifs, which characterize a new class of intramembrane-cleaving peptidases [62,63]. Results from cysteine-scanning studies are consistent with these residues being in close proximity to one another and having access to solvent in a cavity within the complex, consistent with proteolytic events taking place in a compartment separated from the hydrophobic membrane environment [64]. Electron microscopy-derived images also reveal a central cavity as well as pore-like structures, which may allow cleavage products to exit the complex [65,66]. Recent studies on the C-terminal processing of C99, produced by BACE cleavage of APP, suggest that γ-secretase mediates endoproteolytic ε-cleavages (Aβ48 or -49) of C99 which are followed by carboxypeptidase-like cleavages which produce shorter forms of Aβ (Figure 2) [67,68]. Increases in Aβ42 associated with disease-causing mutations in presenilin may result from incomplete γ-secretase processing [69–71].

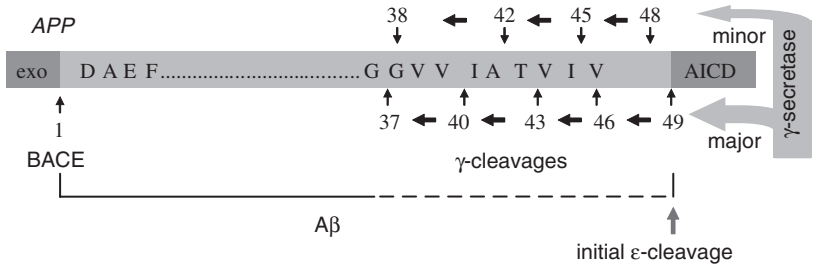
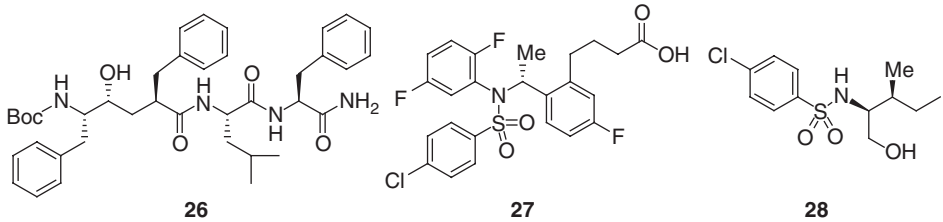


Figure 2 γ-Secretase processing: ε- and γ-cleavages.

3.2 Efficacy and toxicity

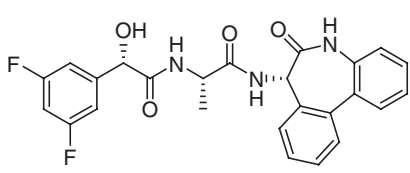
A challenge for the use of γ-secretase inhibitors to block Aβ42 synthesis involves the fact that γ-secretase processes other substrates, including Notch, a highly-conserved receptor involved in cell-fate decisions [57]. Considerable evidence exists that γ-secretase-mediated cleavage of Notch and release of the Notch intracellular domain (NICD) are essential to Notch function, and that toxicities associated with γ-secretase inhibition in preclinical models are due to interference with Notch signaling [52,57]. Whether γ-secretase inhibitors can differentially inhibit APP vs. Notch processing is unclear. The allosteric relationship between binding sites for substrate, catalytic-site inhibitors such as **26** (L-685458), and small molecule inhibitors such as azepinones and sulfonamides, may offer the potential for substrate-based selectivity [72–76]. A study of cellular processing of Notch and APP using catalytic-site directed inhibitors, including **26**, found little evidence of selectivity. On the other hand, a 15-fold separation between APP and Notch processing potency for sulfonamide **27** (BMS-299897) has been reported, and a Notch-sparing inhibitor of γ-secretase, carbinol sulfonamide **28**, with a cellular EC_{50} for Aβ42 inhibition of 4,089 nM vs. an EC_{50} for Notch cleavage of 20,000 nM, was recently described [77–79].



26 **27** **28**

Recent studies have examined the separation of APP and Notch processing effects *in vivo*. Dibenzazepinone **29** (LY-411,575) demonstrates significant reductions in plasma, CSF, and brain Aβ in transgenic mice [80–83]. Several toxicities attributed to inhibition of Notch processing, including effects on the intestine, thymus, and spleen, have also been characterized. Doses associated with partial inhibition of cortical Aβ40 levels by **29** in mice did not cause intestinal changes

and had reduced impact on other organs [83]. Sulfonamide **27** and sulfone **30** (MK-560) have been tested in multiday studies in mice and rat, respectively, at doses that lowered brain Aβ without evidence of Notch-related toxicities [78,84]. A cognitive benefit of γ-secretase inhibition in a mouse model of AD has been reported. An ammelioration of the deficit in contextual fear conditioning in Tg2576 mice was demonstrated with the γ-secretase inhibitor **31** (DAPT) [85].



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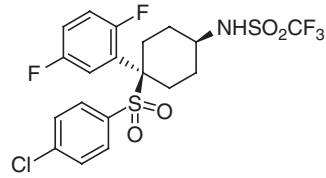

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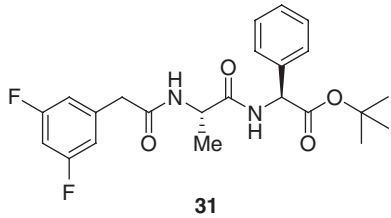

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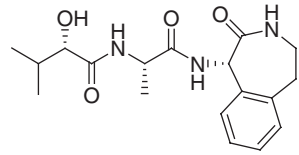

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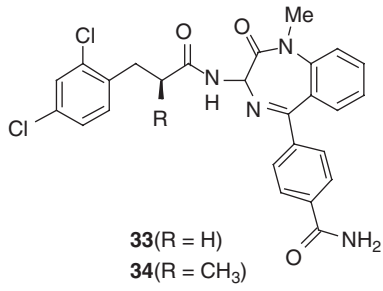
3.3 Clinical evaluation of γ-secretase inhibitors

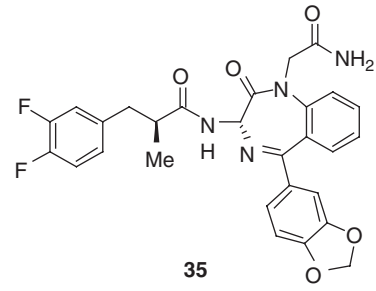
Clinical evaluation of azepinone **32** (LY-450,139) in Alzheimer's patients has been reported. Rises in plasma Aβ, but no significant effects on CSF Aβ, were observed. Similar effects on plasma Aβ were observed in guinea pigs treated with **32** [86–88]. Clinical testing for γ-secretase inhibitors GSI-953 and MK-0752 (structures undisclosed) has been initiated [89–91]. Significant reductions in CSF Aβ40 (35% at 12 h) after treatment with MK-0752 have been observed. Based on the role of Notch signaling in tumorigenesis, γ-secretase inhibitors are being evaluated as potential cancer therapeutics [92,93]. The development of γ-secretase inhibitors for this indication may provide important clinical information on the relationship between γ-secretase-mediated Aβ reduction and side effects due to Notch inhibition.

3.4 Azepinones

Further elaborations on the dipeptide-azepinone theme present in **29** and **32** have been described. Benzodiazepine **33** was transformed through SAR studies to the more potent α-substituted analog **34** and the potent carboxamide **35** ($IC_{50} = 1.2$ nM), which demonstrated 22% bioavailability in rats, but poor brain levels (plasma and brain AUC = 2.9 vs. 0.17 μM · h, respectively) [94,95]. Potent homoaldol **36** (Aβ $IC_{50} = 0.06$ nM) and related benzodiazepine derivatives have been reported [96]. Caprolactam **37** (Aβ $IC_{50} = 17$ nM) resulted from modification

of a related hydroxamic acid [97]. Optimization of a screening hit resulted in the potent noncyclic phenacyl amide **38** (Aβ $IC_{50} = 1$ nM), which did not reduce brain Aβ in Tg2576 mice [98]. Benzodiazepine analog **39** had improved pharmacokinetic properties and reduced brain Aβ 43% in the same model after a dose of 200 μmol/kg [99].





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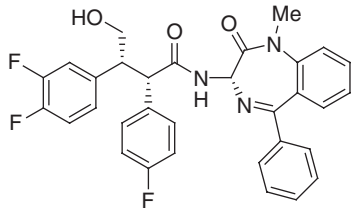

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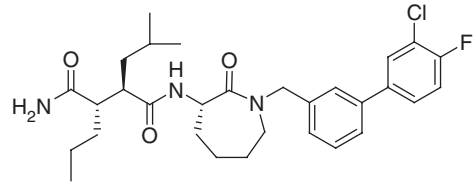

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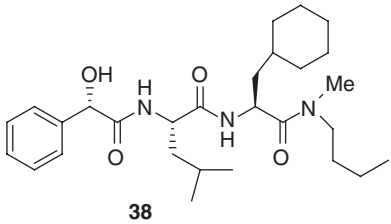

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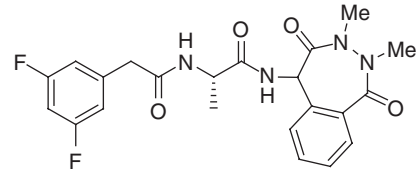

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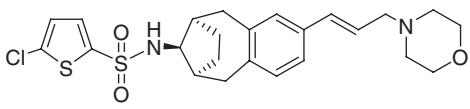
3.5 Sulfonamides and sulfones

Numerous γ-secretase inhibitors featuring sulfonamide- and sulfone-based scaffolds have been disclosed. Bicyclononane thiophene sulfonamide **40** reduced brain Aβ in transgenic mice by 50% after a dose of 100 mg/kg [100]. High potency (Aβ $IC_{50} = 0.5$ nM) and improved oral activity ($ID_{50} = 17$ mg/kg) was found in a series of related sulfamides represented by **41** [101]. Tetrahydroquinoline (**42**) and piperidine (**43**–**44**) sulfonamides have been developed [102–104]. Elaboration of the piperidine series with the cyclopropyl substituent present in **44** improved *in vitro* potency (Aβ $IC_{50} = 2.1$ nM in membrane assay) and *in vivo* activity in transgenic mice (plasma Aβ = 2% of control after oral dose of 30 mg/kg). Reductions of Aβ in the cortex were reported to be comparable to those observed in plasma.



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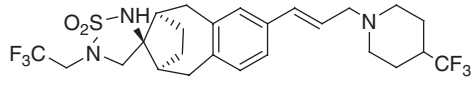

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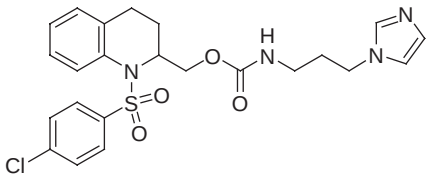

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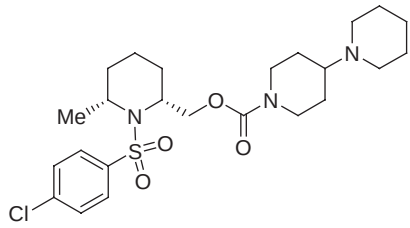

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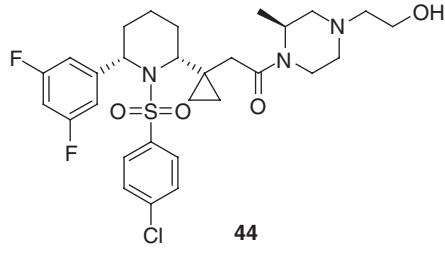

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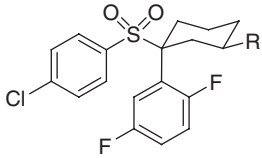
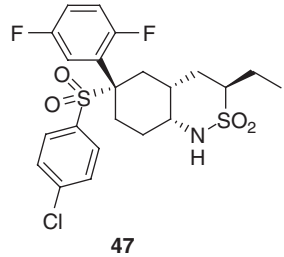
Sulfone-based γ-secretase inhibitors including cyclohexane **45** (Aβ $IC_{50} = 3\,nM$) have been reported [105]. Variations on this series include 3- and 4-substituted analogs such as **46** and **30**, and highly potent bicyclic systems such as **47** (Aβ $IC_{50} = 0.06\,nM$), which was found to lower brain Aβ in mice with an ED_{50} of 3.9 mg/kg [106–108]. Piperidine sulfone **48** resulted from modification of hits from a pharmacophore-based computational search [109].



45 (R = H)
46 (R = $CH_2CH_2CH_2CO_2Me$)



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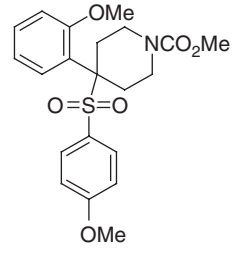

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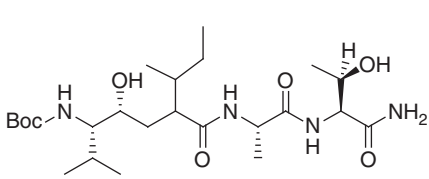
3.6 Peptidic isosteres and other γ-secretase inhibitors

To explore the SAR of catalytic site ligands such as **26**, by more closely aligning inhibitor structure with substrate, a hydroxyethylene analog of the Aβ40 V–I

cleavage site (**49**) was prepared, but found to be inactive (Aβ $IC_{50} > 10{,}000$ nM), indicating that further understanding of the interaction of peptide isosteres with γ-secretase is needed [110]. Synthetically accessible hydroxyethylureas have been used to explore the steric and stereochemical requirements of the active site [111–113]. In one study, the P2-Val derivative **50** demonstrated an IC_{50} of 70 nM in cells. Additional small molecule inhibitors of γ-secretase with diverse structures have been described, including triazine **51** and enones **52** and **53** [114,115].



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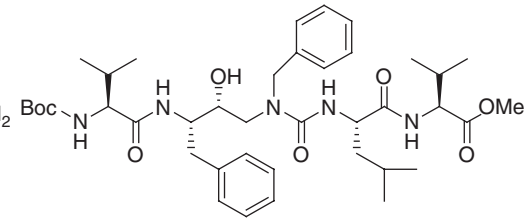

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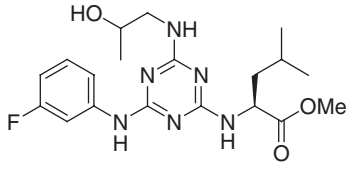

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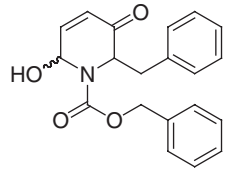

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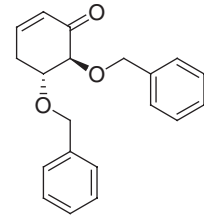

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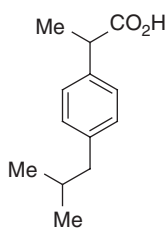
4. MODULATORS OF γ-SECRETASE

In 2001, a subset of nonsteroidal anti-inflammatory drugs (NSAIDs), including ibuprofen (**54**), indomethacin (**55**), and sulindac sulfide (**56**), were found to reduce cellular secretion of Aβ42 [116]. Importantly, the reduction in Aβ42 levels was determined to be independent of cyclooxygenase (COX) activity. Mass spectrometry experiments revealed that the decrease in Aβ42 was accompanied by a dose-dependent increase in the production of Aβ38 [116]. Shorter Aβ species are reportedly less toxic to cells than Aβ40 and Aβ42, and inhibit Aβ42 aggregation [117]. Subsequent radiolabeling experiments [73] and fluorescence resonance energy studies [118] have suggested that these particular NSAIDs bind to an allosteric site on the presenilin subunit of the γ-secretase complex, and induce a change in the enzyme conformation which favors the production of smaller, nonamyloidogenic Aβ peptide fragments. Interestingly, it has been demonstrated that Aβ42 lowering NSAIDs also modulate the cleavage pattern of the γ-secretase substrate Notch [119], and the cleavage of substrate by signal peptide peptidase, another GxGD intramembrane-cleaving peptidase [120]. However, unlike inhibitors of γ-secretase, NSAID γ-secretase modulators do not inhibit ε-cleavage of APP to afford amyloid intracellular domain (AICD) nor

do they inhibit S3-cleavage of Notch and release of NICD [121]. Since γ-secretase modulators have the potential to reduce the production of Aβ42, without the side effects associated with Notch inhibition, the design of γ-secretase modulators for the treatment of AD has become a rapidly expanding field [56,122,123].



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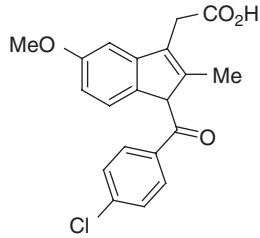

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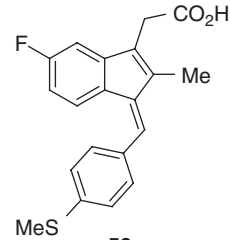

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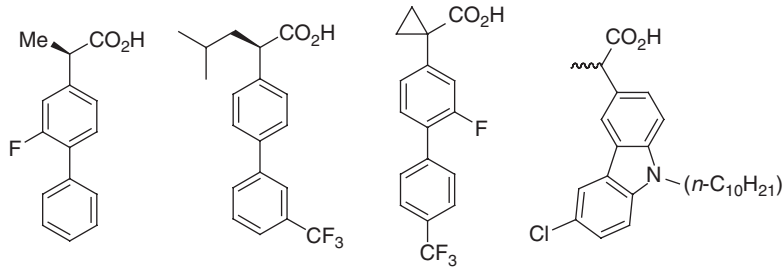
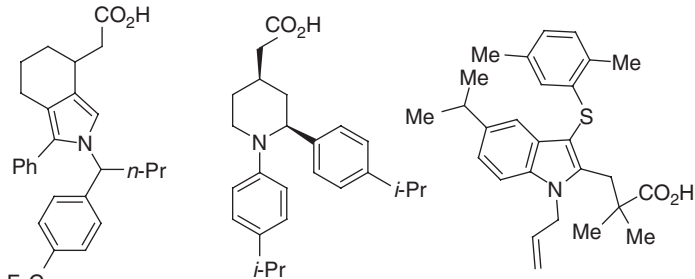
4.1 NSAIDs and NSAID-like compounds as modulators

Human epidemiological studies support a decreased risk of developing AD with long-term NSAID usage [124,125]. However, it is unclear whether the decreased risk can be attributed to the known anti-inflammatory properties of these agents or their recently discovered γ-secretase modulator activity. In cells, IC_{50} values for the inhibition of Aβ42 secretion by **54**, **55**, and **56** range from 25 to 250 μM [116]. In transgenic mice, doses of 50 mg/kg/day of these same three NSAID modulators have been reported to effect 30–39% reductions in brain Aβ42 [116,126]. It should be noted that not all research groups have been able to replicate these results and one group has reported complications with dose limiting toxicity [127]. Certain NSAIDS have also been reported to elevate brain Aβ42 levels by 20–80% in mouse models [126–129]. Recent studies with NSAID-like modulators have focused on developing compounds with reduced COX activity. Clinical evaluation of the (*R*)-enantiomer of the NSAID flurbiprofen is in progress [126,130]. (*R*)-Flurbiprofen (Flurizan, **57**) (Aβ42 $IC_{50} = 100$ μM) has been shown to be nearly equipotent to the racemate in its ability to lower Aβ42 levels, but it lacks significant activity against COX. In a transgenic mouse model, **57** demonstrated a 34% reduction in brain Aβ42 levels after 3 days of oral dosing at 50 mg/kg. Interestingly, drug concentrations in the brain only reached 1.5–2.5 μM, values well below the *in vitro* Aβ42 IC_{50}. In phase II clinical trials of Flurizan, patients with mild-to-moderate AD receiving 800 mg bid had results marginally superior to placebo. However, a post hoc analysis of those individuals who achieved a plasma drug exposure >75 μg/ml did achieve statistically significant outcomes with two of three clinical endpoints. This has been interpreted as an overall slowing of cognitive and behavioral decline of 36–62% compared with placebo. Phase III investigations with **57** are reportedly underway [130]. Novel biaryl acetic acids for the treatment of AD have been disclosed. The biphenyl pentanoic acid **58** is reported to lower production of Aβ42 in the brains of single transgenic mice by 36% [131]. CHF5022 (**59**) is twofold more potent *in vitro* than Flurizan in inhibiting Aβ42 secretion and displayed higher systemic and brain exposure

[129,132]. *N*-Alkyl carprofen analogs (e.g. **60**, Aβ42 $IC_{50} = 2.9\,\mu M$) were found to be nearly devoid of COX-1 and COX-2 activity [133,134]. Multiple patent applications have disclosed a variety of alkanoic acids that are based on tetrahydroindole (**61**) [135], piperidine (**62**) [136], and indole (**63**) [137] scaffolds, which selectively lower Aβ42.



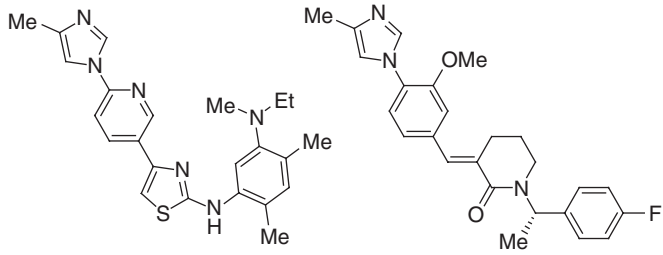
57 **58** **59** **60**



61 **62** **63**

4.2 Other modulators of γ-secretase

Compounds structurally distinct from NSAIDs can act as modulators of γ-secretase. A patent application has disclosed a series of diarylaminothiazoles (e.g. **64**) that inhibit the cellular production of Aβ42 with IC_{50} values $<200\,nM$ [138]. Cinnamide compounds capable of reducing Aβ42 production, while simultaneously enhancing the production of Aβ37, have been claimed in a recent application [139]. The preferred piperidone **65**, for which a process patent has been published [140], has a reported cellular Aβ42 $IC_{50} = 80\,nM$.



64 **65**

5. SUMMARY

Inhibition of BACE and γ-secretase, and modulation of γ-secretase, represent promising strategies to reduce Aβ peptides in brain. Reports of BACE inhibitors with *in vivo* activity in animal models have appeared from several laboratories, demonstrating that the challenge of brain penetration for this class of inhibitors is not insurmountable. Considerable progress has been made in understanding Notch-related toxicities of γ-secretase inhibitors, and Aβ reductions in the absence of these side effects have been reported. The field of γ-secretase modulators is relatively immature, but late stage clinical studies are in progress with an NSAID-derived modulator. There is anticipation that data addressing the validity of the amyloid hypothesis will be reported in the near future as BACE and γ-secretase targeted therapies move into clinical development.

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CHAPTER 4

Recent Advances in Drug Discovery of Histamine H_3 Antagonists

Robert L. Hudkins and **Rita Raddatz**

Contents

1. INTRODUCTION

Drug discovery efforts in the histamine GPCR receptor family, now some 75 years old, are currently focused on the H_3 receptor (H_3R), identified in 1983 [1] and finally cloned in 1999 [2]. The H_3R is an attractive drug target for the potential treatment of central nervous system (CNS) diseases due to its role in modulating a variety of CNS functions. H_3Rs are expressed predominantly on the presynaptic terminals of CNS neurons, where they function as inhibitory auto- and hetero-receptors. H_3 antagonists can therefore function to increase the release of various neurotransmitters, including histamine, acetylcholine, norepinephrine, serotonin and dopamine [3–6], and thus have potential utility in addressing a variety

Discovery Research, Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA

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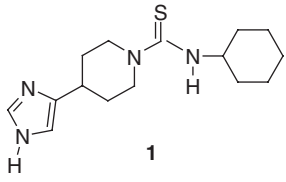
of CNS disorders, including deficits in wakefulness and attention, attention-deficit hyperactivity disorder (ADHD), various dementias, schizophrenia and obesity.

Initial work in the field focused on imidazole analogs of the natural ligand, histamine [7]. This led to the identification of several useful research tools that helped advance the biology, e.g., thioperamide (**1**) [8] and ciproxifan (**2**) [9]. To date however, imidazole-based H_3 antagonists have not successfully advanced through the drug development process. These compounds have numerous liabilities and poor drug-like properties, including metabolic degradation by histamine *N*-methytransferase (HNMT), poor off-target selectivity, cytochrome P450 inhibition and poor blood–brain barrier penetration. A further confounding factor has been the species differences in the *in vitro* activities of this compound class, with high affinity for rodent receptors and lower affinity for human H_3 receptors [10]. An early clinical candidate, GT-2331 (**3**), had even more complex pharmacology due to the imidazole group, showing mixed agonist/antagonist-like effects in different systems [11]. The imidazole H_3 class has been extensively reviewed and will not be discussed further here [7,12,13].



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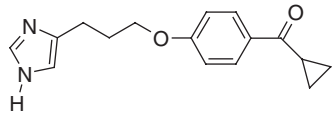

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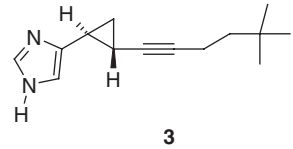


3

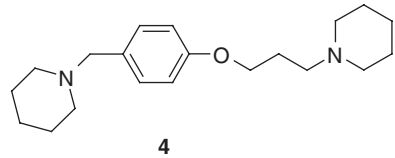
The search for H_3 antagonists with drug-like properties has been almost exclusively focused on amine-based compounds. These tertiary amine scaffolds exhibit an extremely broad diversity of structural classes and pharmacophores, and demonstrate an inherent tolerance of the H_3R to accommodate considerable functional substitutions with large lipophilic aryl groups, polar hydrogen bond donor and acceptor groups or additional basic amines, accounting for the variety and often simplicity of H_3 scaffolds. Moreover, drug-like tertiary amines with reduced side effect liabilities have been identified and advanced into clinical evaluation. Many excellent reviews have been recently published on H_3 chemistry and biology [13–20]. An important aspect in understanding the H_3R and its ligands is the high degree of constitutive activity *in vitro* and *in vivo*. As a result, compounds previously classified as antagonists may in effect decrease the constitutive activity of the H_3 receptor and appropriately be classified as inverse agonists. The concept of constitutive activity at H_3R, the nature of this pharmacological action, the consequences of inverse agonism and its relationship to receptor function and efficacy have been discussed in these reviews [13,17–20]. The current chapter will focus on recent advances in H_3 drug discovery, clinical candidates and their therapeutic applications, and the design of drug-like molecules.

2. CLINICAL APPLICATIONS OF H_3 RECEPTOR ANTAGONISTS

2.1 Sleep/wake

Treatments for excessive daytime sleepiness and narcolepsy are now recognized as important areas for therapeutic intervention. Histaminergic projections from the tuberomammillary nucleus of the posterior hypothalamus project to several brain regions, including those that control sleep/wake and arousal states [21]. The pronounced effect of histaminergic tone on sleep/wake states is evidenced by the sedative effects of centrally active H_1 antagonists. Conversely, increased activation of central H_1 receptors through various mechanisms, including H_3 antagonism, is wake-promoting in a number of species (reviewed in ref. 22). However, unlike the psychostimulant amphetamine, H_3 antagonists do not induce behavioral sensitization or sleep rebound in animals at wake-promoting doses [23]. Among the H_3 antagonists examined preclinically for wake-promoting effects [22] was JNJ-5207852 (**4**) which had potent H_3 antagonism and wake-promoting activity in rats and mice and was ineffective on sleep in $H_3^{-/-}$ mutant mice [24].

Narcolepsy is a disabling sleep disorder characterized by excessive daytime sleepiness and sudden loss of muscle tone (cataplexy). Antagonism of H_3Rs is anticipated to not only reduce excessive somnolence, but also prevent inappropriate transitions into paradoxical sleep states thought to underlie cataplectic episodes. Interestingly, the H_3 antagonists thioperamide (**1**) and JNJ-5207852 decrease episodes of cataplexy in a genetically narcoleptic Doberman Pinscher model [25], further supporting a potential role for this class of compound in treating narcolepsy.



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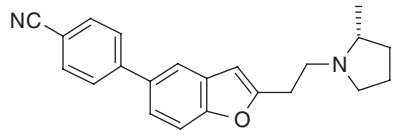
2.2 Cognitive disorders

H_3 receptor blockade results in the release of neurotransmitters in brain regions associated with memory and learning, such as the cerebral cortex, amygdala and hippocampus. Early imidazole-based H_3 ligands were active in rodent models of learning. More recently, selective H_3 antagonists have been tested in a variety of rodent models of enhanced normal memory and in models of chemical and age-related cognitive impairment. The variety of animal models and species used makes direct comparison of the activity of H_3R antagonists difficult, but the efficacy shown for highly potent and selective compounds in a number of rodent models supports the potential utility of H_3 antagonists in enhancing cognitive function.

ABT-239 (**5**), a potent H_3R antagonist, was effective at low doses (0.1 mg/kg s.c.) in a repeat trial inhibitory avoidance task in SHR pups [26], a model involving aspects of attention, impulsivity and learning that is thought to be relevant to characteristics of ADHD. ABT-239 was active in a social recognition

model of short-term memory in aged and adult rats and in a water maze model, demonstrating effects on different aspects of cognitive impairment [27]. ABT-239 was also active in the prepulse inhibition (PPI) startle model [26], a model of sensory gating proposed to be related to schizophrenia. JNJ-5207852, another potent H_3 compound, ameliorated learning and memory deficits in PTZ-kindled mice in a variety of cognitive models [28]. However, the compound was inactive when tested at 5 mg/kg s.c. in a water maze model in these mice [25].



5

2.3 Obesity

The importance of CNS histaminergic systems in energy homeostasis is well established [17]. However, the potential role for H_3R modulation in the treatment of obesity remains controversial in the absence of clinical validation. Conflicting data regarding the activity of H_3 agonists and antagonists in rodent feeding models, along with the mildly obese phenotype of H_3R knockout mice, have raised concerns regarding the utility of H_3 antagonists in the treatment of obesity [29]. However, structurally distinct compounds including NNC 38-1049 (**19**), NNC 38-1202 (**21**) and A-417022 (**7**) have strengthened the case that H_3 antagonists may have utility in the treatment of obesity. Observations of decreased triglyceride levels with H_3 antagonist treatment and the presence of H_3 receptors in peripheral tissues involved in energy expenditure suggest further potential applications for H_3 antagonists in metabolic disorders [17,30].

The H_3R is expressed on histamine-releasing neurons in the hypothalamus, a brain region involved in the regulation of food intake and energy expenditure. One proposed mechanism for the potential antiobesity effects of H_3 antagonists involves enhanced histamine release, resulting in increased stimulation of H_1 receptors [30]. This concept is supported by a suggested link between blockade of the H_1 histamine receptor by atypical antipsychotics and the overt weight gain associated with the clinical use of these agents [31]. While several H_3 antagonists decrease weight gain in rodent models of obesity, not all potent, CNS-permeable H_3 compounds are active in these models [17]. Several hypotheses have been proposed to explain the differential effects of H_3 antagonists in feeding models, but such differences have yet to be defined at the molecular level to allow differential *in vitro* screening to identify potential antiobesity compounds.

2.4 Analgesia

Conflicting evidence exists with both agonists and antagonists in pain models that confound the potential role of H_3 antagonists in analgesia [12,32]. The H_3

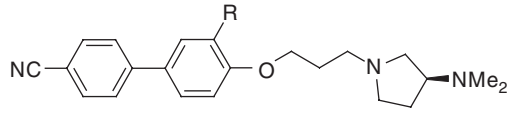
agonist immepip administered systemically or intrathecally had analgesic effects in models for mechanical but not thermal pain, the latter a profile absent in H_3R knockout mice [33]. In contrast, the non-selective antagonist, thioperamide had antinociceptive effects in hotplate and writhing tests [34]. A large number of H_3 antagonists have been claimed for the use and treatment of neuropathic pain [35] and include ABT-239 and JNJ-5207852.

3. PHARMACOPHORES AND MODELING

To date the application of homology modeling to develop pharmacophore models has received limited attention. A common pharmacophore for H_3 antagonists can be generated to account for the variety and simplicity of H_3 scaffolds [15]. The simple model is defined by a basic amine (pK_a 8.8–10.2) spaced 2–4 atoms from a central hydrophobic core with a large binding region off the core that can accommodate high chemical diversity and space. A similar model was described by JNJ to explain the SAR for a diamine scaffold [36]. The basic amine was hypothesized to interact with the highly conserved aspartic acid 114 in helix 3 [37] of the 7-transmembrane domain. Rhodopsin-based homology models have attempted to predict features of the large binding pockets between helices 5 and 6 and also between helices 3, 4 and 5 [38] as well as to explain species-related H_3 receptor heterogeneity [39].

4. DESIGN STRATEGIES AND STRUCTURE–ACTIVITY ADVANCES

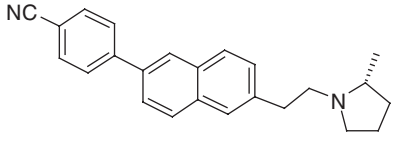
An early biphenyl candidate, A-331440 (**6**) has been reported to be a competitive, potent inverse agonist with balanced activity at human and rat H_3Rs with good oral bioavailability [40]. While A-331440 was active in obesity models, its development was precluded by genotoxicity issues [41]. The latter was eliminated by a tactical *ortho*-substitution on the phenoxy central core by fluorine to provide A-417022 (**7**). A-417022 and the 3,5-difluoro analog (A-423579) also produced prolonged weight loss over a 28-day period in a rat diet-induced obesity model [17,41].



6 R = H
7 R = F

A design strategy applied to enhance the overall drug-like properties and selectivity of the biphenyl scaffold was to rigidify the skeleton and reduce the number of rotatable bonds in the amine sidechain. Constraining the phenoxypropyl sidechain into a ring produced the novel benzofuran scaffold [42]. Extensive SAR elaboration around the benzofuran identified ABT-239 (**5**) [43,44]. Although ABT-239 had an impressive *in vivo* profile for cognition enhancement [26,45], it had limitations, including high plasma protein binding and high brain to plasma

partitioning. The development of ABT-239 was halted due to cardiovascular liabilities [19]. ABT-239 inhibits [^{3}H]dofetilide binding to the hERG potassium channel with a K_i value of 195 nM. ABT-239 has a high clogP of 5.2, which was believed to contribute to the high brain partitioning (B/P > 34), high bound fraction in human brain homogenates *in vitro* and phospholipidosis [19]. Naphthalene analogs have even higher clogP and brain to plasma ratios than the corresponding benzofuran analogs [45]. For example, compound **8** has a clogP of 5.9. ABT-834 is a backup compound in clinical trials for ADHD, although the structure has not been disclosed [15,16]. Protective patents related to compound **9** have published, including disclosing the crystal structure of various salt forms [46]. To improve the drug-like properties by lowering the logP, compounds **10** (A-688057) and **11** (A-687136) were identified [47]. Compared to ABT-239, A-688057 showed improved hERG activity (K_i of 9 μM for dofetilide binding), a lower $\log D_{7.4}$ (2.05), improved brain to plasma ratio of 3.4, with a low potential for phospholipidosis and genotoxicity. A-688057 demonstrated efficacy at low doses (0.1 mg/kg s.c.) in a repeat trial inhibitory avoidance task in SHR pups. A-688057 displayed only moderate oral bioavailability in rat (26%), dog (30%) and monkey (8%) [47], and exhibited potent CYP2D6 activity, which could halt its advancement to man.



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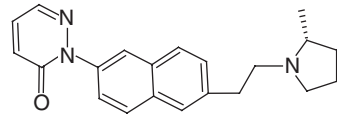

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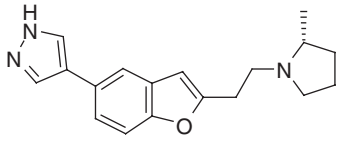

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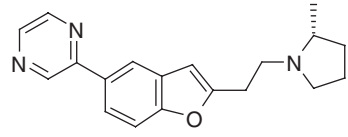
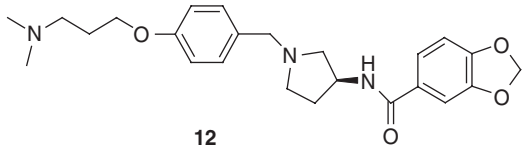


11

The benzazepine clinical candidate GSK189254 (**14**) was identified via a focused screen on the phenoxypropylamine pharmacophore **12**. Conformational constraint led to **13**. The observation that **13** could be binding in the reverse mode guided replacing the propylpiperidine with various aryl- or heteroaryl-ethers to eventually identify GSK189254 **14** [48]. GSK189254 is in Phase II (http://www.clinicaltrials.gov/ct/show/NCT00366080) for narcolepsy. In a second Phase I study (http://www.clinicaltrials.gov/ct/show/NCT00387413) directed to neuropathic pain, the safety and efficacy of GSK189254 will be investigated in the electrical hyperalgesia (EH) model in healthy volunteers. GSK189254 demonstrated high potency for recombinant human H_3Rs *in vitro* ($pA_2 = 9.06$) and for rat H_3R blockade *in vivo* ($ID_{50} = 0.05$ mg/kg p.o.), with greater than 10,000-fold selectivity for H_3 versus other receptors [49]. GSK189254 was efficacious across a panel of models designed to test different cognitive domains in rodent at

0.3–3 mg/kg p.o., reversing scopolamine-induced deficits in passive avoidance tasks, improving performance of aged rats in a water maze model and improving memory in an object recognition task. Sustained efficacy with repeat dosing (7 days) was also demonstrated in the object recognition task as well as in an attention set shifting task [49]. GSK239512 (structure not disclosed) is reportedly in Phase I for the treatment of dementia in Alzheimer's disease [50].



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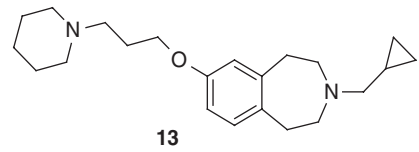

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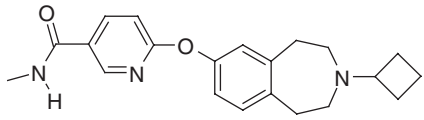

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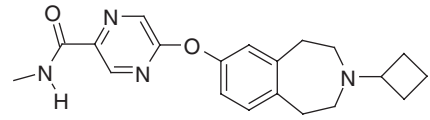
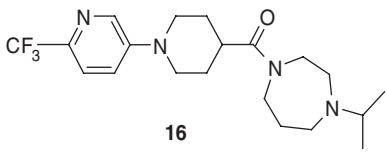


15

The pyrazine benzazepine analog **15** (GSK207040) had subnanomolar affinity for human ($K_i = 0.21$ nM) and rat receptors ($K_i = 0.83$ nM) and was a full inverse agonist ($EC_{50} = 0.63$ nM) [51]. The rat oral bioavailability of GSK207040 was 88% with an i.v. $t_{1/2}$ of 2.6 h. GSK207040 p.o. inhibited *ex vivo* [^{3}H]-*R*-α-methylhistamine binding ($ED_{50} = 0.03$ mg/kg) and was also active in the rat dipsogenia model. In pathophysiologically relevant pharmacodynamic models, GSK207040 reversed scopolamine-induced amnesia in a passive avoidance paradigm and reversed capsaicin-induced reductions in paw-withdrawal threshold [51]. GSK has also disclosed pyrazolo[3,4-d]azepines [52] and thiazolo[4,5-d]azepines [53] heterocyclic azepine cores. Incorporation of the basic nitrogen into a diazepine ring is represented by **16** (GSK334429) [51]. GSK334429 displayed subnanomolar affinity for human and rat receptors and had good oral bioavailability and intrinsic pharmacokinetic properties in rat. GSK207040 and GSK334429 produced antinociceptive activity in the capsaicin model of secondary allodynia/hyperalgesia, a model of neuropathic pain [51]. The piperazine amide, **17** was reported by GSK as a peripherally selective H_3 antagonist for treatment of inflammatory and allergic diseases, particularly allergic rhinitis [54]. It is reported to have low CNS penetration with good H_3/H_1 selectivity.



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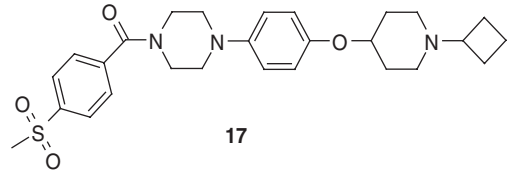
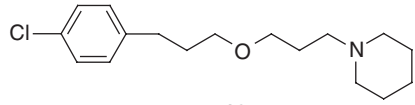


17

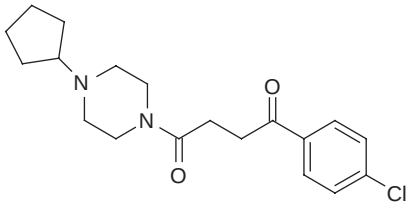
BF-2649 (**18**) is a potent and selective H_3 antagonist reportedly in clinical trials for a number of potential indications, including cognitive enhancement, appetite

control, schizophrenia and antiepileptic activity. *In vitro*, BF-2649 potently inhibited the recombinant human H_3R ($pA_2 = 9.5$) [55]. Based on binding K_i values, BF-2649 was 230-fold selective over the human H_1 receptor and greater than 200-fold selective over other targets. BF-2649 was active at 15 mg/kg i.p. in a novel object recognition task and in several mouse models of schizophrenia [55]. At 5 mg/kg i.p., it reduced both methamphetamine and MK-801-induced motor hyperactivity, while at 3 mg/kg i.p. the compound reversed apomorphine-induced disruption of PPI. Known antipsychotic agents as well as H_3 antagonists are active in these models, suggesting a role for H_3 receptor antagonists in schizophrenia [56,57]. Additional efficacy for BF-2649 was noted in a patent application [58], including an antiepilepsy trial in 12 patients, a satiety effect in six volunteers when given olanzapine, and in a 36 patient wake/vigilance assessment. BF-2649 (10 mg/kg p.o.) increased wake time in both cats and mice [55,59].



18

Using an acyl piperazine as the amine scaffold, a series of 4-aryl butyric acids was reported as high affinity H_3R ligands [60] for treating obesity. NNC 38-1049 (**19**) had an hH_3 K_i value of 1.2 nM and reduced cumulative food intake and body weight in a diet-induced obesity model (DIO) [61]. To further improve the brain penetration and lower the polar surface area, the central acyl-amide was replaced by a quinoline ring resulting in **20** (hH_3 K_i = 1.8 nM) [62]. Further modification of the piperazine led to the *S*-2-((pyrrolidin-1-yl)methyl)pyrrolidine scaffold [63]. NNC 38-1202 (**21**) was effective in a diet-induced rodent model of obesity [64] maintaining weight reduction at 2.5 mg/kg p.o. q.d. in a 50-day rat study [30]. NNC 38-1202 reduced food intake at 0.1 mg/kg s.c. in rhesus monkeys [65]. Constraining the cinnamic acid into a benzofuran moiety produced a new series **22**, (H_3 $K_i = 4$ nM) [66].



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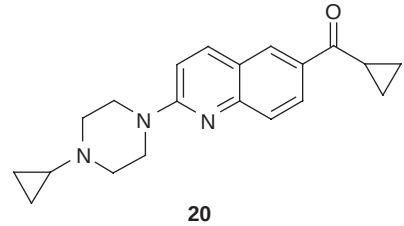

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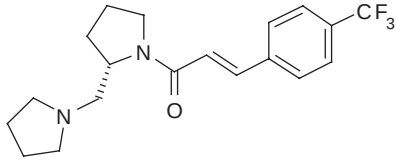

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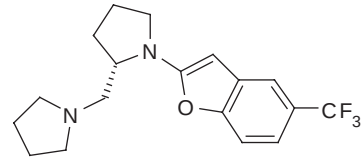
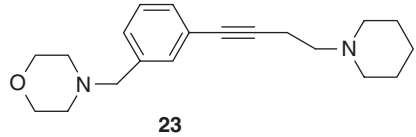


22

Following an initial HTS hit, a series of diamine-based H_3 antagonists with enhanced affinity exemplified by JNJ-5207852 (**4**), a reported neutral antagonist, were identified [56,67]. Intriguingly, while JNJ-5207852 increased wakefulness without affecting motor activity, it had no effect on food intake in leptin-deficient *ob/ob* mice. JNJ-5207852 had an exceptionally long bio-half-life and brain residency time greater than 48 h after a single i.v. injection [25]. Dibasic molecules also have a high propensity to induce phospholipidosis-toxicity that occurs due to high partitioning of a compound into lipid bilayers that impairs normal phospholipid turnover. JNJ-5207852 was a potent inducer of phospholipidosis. Replacing the piperidine of **4** with a morpholine to lower the pK_a yielded JNJ-10181457 (**23**), a neutral antagonist with approximately 10-fold lower hH_3 affinity. JNJ-10181457 had a shorter brain residence time [25] and was wake-promoting in mice and rats [68,69] and decreased episodes of cataplexy in the narcoleptic Doberman Pinscher model [25]. JNJ-10181457 also improved acquisition in a repeat trial passive avoidance task in spontaneously hypertensive rat (SHR) pups at 10 mg/kg s.c. [25]. JNJ-10181457 was ^{11}C labeled as a potential PET ligand [70]. The imidazopyridine **24** (JNJ-6379490) had good oral bioavailability in rat and dog and increased wake time at 0.6 mg/kg s.c. in rats. However, even at doses as high as 10 mg/kg, wake-promoting activity only occurred in the first 2 h after administration [25]. Constraining the chain into a 4-hydroxypiperidine ring and incorporating the less basic morpholine produced **25**, a compound that is also wake-promoting in rats [71]. In May 2005, the novel, orally active, selective H_3 antagonist JNJ-17216498 (structure undisclosed) reportedly entered clinical trials for narcolepsy and was well tolerated in humans [72]. There has been no further information on this compound.



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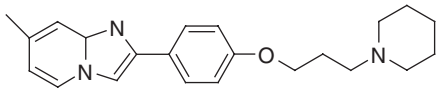

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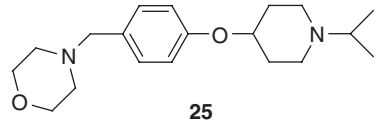
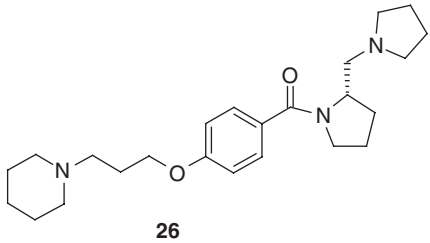


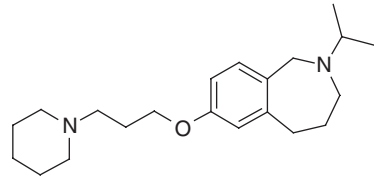
25

A series of diamine amides based on the phenoxypropyl amine scaffold was reported. Amide **26** displayed an hH_3 K_i of 1 nM and was selective versus other histamine receptors [73]. The chirality was removed via cyclic diamines to produce tetrahydroisoquinolines, tetrahydroquinolines, benzazepines and indolines [74]. The benzazepine **27** displayed picomolar hH_3 binding affinity. Pharmacokinetic issues were also identified with this diamine series, with i.v. half-lives of 10–12 h in the rat. A strategy to remove the diamine skeleton and prepare new H_3

cores was demonstrated with the pyrazin-2-ylsulfanyl **28** and benzenesulfonyl **29** [75,76].



26



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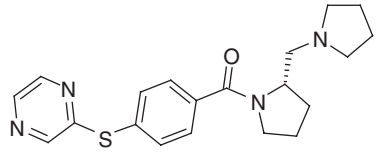

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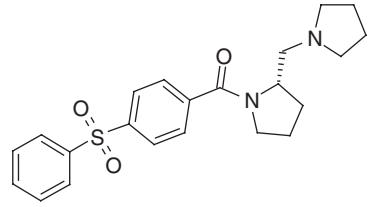
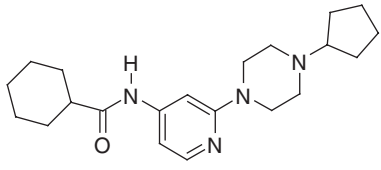


29

A series of piperazine-based antagonists was disclosed as potential obesity agents [77]. Compound **30** shows an hH_3R K_i of 11 nM. Quinazolone **31** was highlighted, and may be of further interest as the specific synthesis and a crystal structure was disclosed [78].



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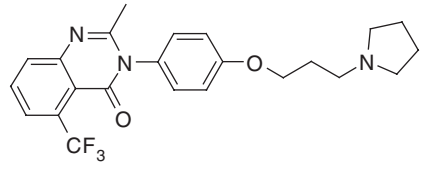
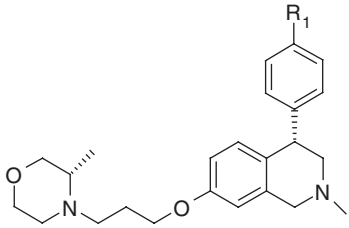


31

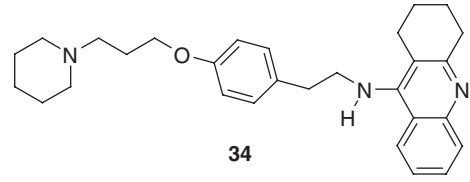
5. H_3 ANTAGONISTS WITH A DUAL MECHANISM

Patients suffering from depression are frequently diagnosed as having cognitive impairment and fatigue. Marketed SSRIs address the mood aspects of depression but fail to improve the cognitive and fatigue issues, and in many cases actually contribute to these phenotypes. Wake-promoting drugs, e.g. modafinil, have been proposed for use in treating the excessive sleepiness characteristic of depression [79]. JNJ reported dual H_3 and SERT activity in a series of tetrahydroisoquinolines for treating depression [80,81]. A 4-methoxy or thiomethyl aryl substituent was preferred. Compounds **32** and **33** displayed high affinity for both hH_3 ($K_i = 5.6$ and 4.0 nM) and hSERT ($K_i = 2.7$ and 13 nM) and also had moderate activity for norepinephrine (NET) and dopamine transporters (DAT). Dual H_3 and AChE

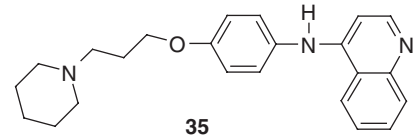
activity was reported for a series of tacrine analogs to treat the cognitive deficits in Alzheimer's disease [82]. Compound **34** (FUB833) (hH_3 $K_i = 0.33$ nM) had an IC_{50} of 2.6 nM for AChE. The series also showed activity for BuChE and HNMT, the main histamine metabolizing enzyme in brain. A series of compounds with dual H_3 antagonist/HNMT activity was reported [83]. Compound **35** had sub-nanomolar affinity for H_3 and an IC_{50} of 51 nM for HNMT. Schering–Plough reported dual H_1/H_3 antagonists prepared by linking chloropheniramine with imidazole alkyl amines for allergic rhinitis [84].



32 R_1 = OMe
33 R_1 = SMe



34



35

6. FUTURE PERSPECTIVES

There is an increasing appreciation for the complexity of the H_3 receptor (species sequence differences, splice isoforms, localization, constitutive activity) that adds to the challenges in drug discovery. However, despite these complexities, significant advances have been made in medicinal chemistry to identify novel amine-based cores for drug design, and in synthesizing a large number of highly potent and selective H_3 antagonists with efficacy in a variety of preclinical models of cognition, sleep and obesity. To date no peer-reviewed clinical efficacy data for an H_3 antagonist are available, reflecting the numerous hurdles and challenges in developing safe drug-like H_3 antagonists. Since the H_3R has the potential to treat a large number of CNS diseases, additional efforts with newer compounds will no doubt lead to a better understanding of the target and the properties required for therapeutic efficacy.

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CHAPTER 5

Recent Advances in the Treatment of Insomnia

Albert Palomer, Marta Príncep and **Antonio Guglietta**

Contents

1. INTRODUCTION

Insomnia is defined as a condition of unsatisfactory quantity and/or quality of sleep, which persists for a considerable period of time, including difficulty in falling asleep, difficulty in staying asleep, or early final wakening [1]. Insomnia is the most

Ferrer Internacional S.A., Juan de Sada 32, 08028 Barcelona, Spain

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common sleep complaint within the sleep disorders and is classified by the duration of the problem as well as the underlying pathophysiology. According to duration, it is considered transient (less than 4 weeks), short term (from 4 weeks to 3–6 months) and chronic (more than 6 months [2] but new trends consider chronic insomnia more than 30 days [3]). Insomnia is also named primary when is a condition *per se* and secondary when is arising as a symptom of a comorbid disorder.

Disease prevalence ranges from 5 to 35% of the adult population, with variations reported depending on the methods used in epidemiological studies [4]. Insomnia in the pediatric population is less understood, with prevalence estimates of 10–30% when bedtime refusal and night waking are included. The need for pharmacological management of pediatric insomnia and improvement in knowledge of the safety and efficacy of drugs was identified in the Sleep in American Poll 2006 [5]. A complete guide to publicly available sleep-related data was published in 2006 [6].

Insomnia is associated with severe daytime dysfunction and low performance, related to traffic accidents and work absenteeism [7], with increasing medical burden, attributable to both direct medical and indirect costs [8].

Although insomnia affects millions of people worldwide, few seek medical advice and only 14% report using sleep aids [9]. Nevertheless, the worldwide insomnia market was estimated at over $2.2 billion in 2004 and is forecast to grow at an annual rate of 10.2%, with the market predicted to approach $3.6 billion in 2009 [10].

Indeed, a pharmacological approach is the first line treatment in transient insomnia. Meanwhile, a behavioral or non-pharmacological approach is the recommended therapy for chronic insomnia, together with intermittent aid of pharmacological treatment [3].

Current drug therapy for insomnia includes γ-aminobutyric acid A ($GABA_A$) receptor agonists, melatonin receptor agonists, over-the-counter (OTC) products, antidepressants and antihistamines. Among these, only $GABA_A$ receptor agonists (benzodiazepines and non-benzodiazepine 'Z-drugs') and melatonin receptor agonists are approved for insomnia therapy. Moreover, despite the fact that insomnia is often a chronic condition, only two medications, eszopiclone (non-benzodiazepine structure) and ramelteon (melatonin receptor agonist), have been approved with no time limitation by the U.S. Food and Drug Administration (FDA). All other medications are limited to less than 35 days but are commonly used off-label for chronic conditions.

Marketed compounds display well-known efficacy in inducing sleep onset, but many fail in the maintenance of sleep throughout the night due to short half-lives. On the other hand, longer acting compounds, such as the benzodiazepines, elicited significant next-day adverse effects. Therefore, the balance between sustained efficacy and adequate pharmacokinetic profile remains to be solved.

Moreover, in March 2007 the FDA requested label changes for all sleep disorder drug products from the manufacturers, to strengthen and expand the language concerning potential risks [11].

Due to these unmet needs for efficacy and safety, interest is rising in many companies [12] in searching for new compounds acting on the clinically validated mechanisms of action ($GABA_A$ and melatonin) or for emerging new therapies

(orexin/hypocretin or serotonin/histamine). The goal is to improve hypnotic efficacy, to modulate the pharmacokinetic profile and to reduce major side-effects. How these new approaches attempt to accomplish this goal is summarized hereinafter.

2. $GABA_A$ RECEPTOR MODULATORS

$GABA_A$ receptor agonists used for insomnia fall into two classes: 'Z-drugs' and benzodiazepines. Zolpidem, a 'Z-drug', is the market leading compound in this therapeutic group and will enter the generic market in 2007. This fact has triggered the search for alternatives with improved properties, e.g. increased duration of action or elimination of day-after and rebound effects. The search strategies fall into three main categories: innovative formulations and devices for older compounds, enantiomerically pure molecules and alternative structures. We have attempted here to review the strategies to improve 'Z-drugs' together with the recent medicinal chemistry advances in this area and the pharmacological and clinical outcome, taking into consideration that the pharmacology of GABA receptors and their therapeutic relevance has been recently reviewed by M. Chebib *et al*. in this series [13].

2.1 Non-benzodiazepine hypnotics 'Z-drugs'

'Z-drugs' are the most frequent treatment for insomnia with the compounds zolpidem, zaleplon and zopiclone marketed for transient insomnia and eszopiclone (S-isomer of zopiclone) for both transient and chronic insomnia. These non-benzodiazepine structures display efficacy equivalent to benzodiazepines for insomnia. The half-lives, ranging from 1.5 to 5 h, are claimed to be enough for sleep maintenance and to avoid daytime sedation. Moreover, 'Z-drugs' have a reduced propensity for inducing tolerance, withdrawal and abuse compared to benzodiazepines [14]. However, like benzodiazepines, they are classified as schedule IV drugs by the Drug Enforcement Administration (DEA).

Innovation on formulations to improve pharmacokinetics (see Table 1) is a common strategy to address the generic introduction of zolpidem (2007 in the US).

Table 1 Summary of new formulations in development for 'Z-drugs'

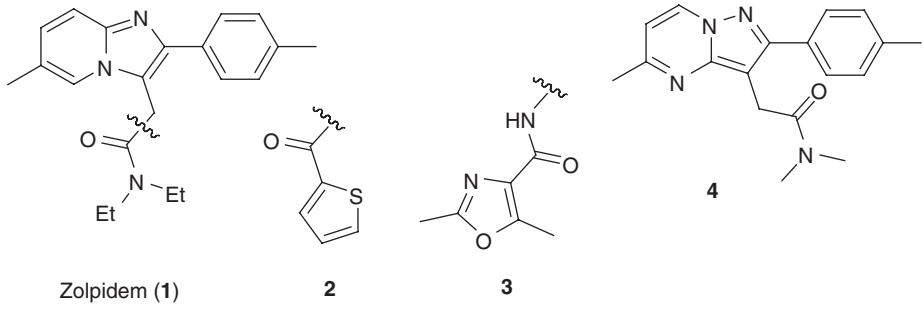
Compound	Originator	Status
Zolpidem MR	Sanofi-Aventis	Launched-Phase IV studies
Zolpidem Flash Dose (fast dissolving tablet)	Biovail	FDA approved
Zolpidem I2R (lingual spray)	NovaDel	Phase II (pivotal studies in 2007)
TransOral zolpidem	TransOral Pharmaceuticals	Phase II
Zolpidem BEMA film	BioDelivery Sciences	IND filling in 2007
Zolpidem intranasal	Fabre-Kramer	No data available
Zaleplon-ER	King Pharmaceuticals	Discontinued 2005

Eszopiclone, the latest approved 'Z-drug', in a 6-month study in patients with chronic insomnia and following studies to 12 months, demonstrated significant decrease of sleep latency and awakenings and improvement in total sleep time. Importantly, daytime function and alertness were not affected, nor was there evidence of tolerance [15].

2.2 Recent advances in 'Z-drugs' research

Novel 'Z-drugs' should be specially designed to address the duration of action issue, assuring sleep maintenance, or to improve sleep onset. Indiplon, currently in pre-registration, is being developed in two formulations. The immediate-release capsule has the advantage of quick clearance, resulting in rapid sleep onset and reduced risk of next-day impairment. The modified-release formulation delivers two doses, one at bedtime and one in the middle of the night, achieving rapid sleep initiation and maintenance throughout the night. In mid-2006, the FDA issued an approvable letter for the immediate-release capsule, but additional documentation was requested from the originator, Neurocrine Biosciences. In contrast, the modified-release formulation received a not approvable letter and Neurocrine Biosciences expects to re-submit the NDA in brief [16].

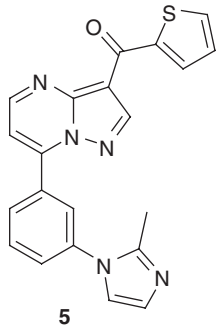
Imidazo[1,2-a]pyridine, the central scaffold in Zolpidem (**1**), has been extensively explored in the search for insomnia drugs. Fang *et al.* [17] have described compounds bearing such heterocycles but lacking the side chain amide group. **2** inhibits [^{3}H]-flunitrazepam binding to central receptors with an IC_{50} value of 36 nM and to peripheral receptors with an IC_{50} of 180 nM. *In vivo*, this compound was active at 10 mg/kg in the maze test for anxiety [17]. In addition, Falco *et al.* have described compounds bearing an inverse amide group side chain (**3**) that display sedative-hypnotic action (94% inhibition of motor activity) following i.p. administration in mice [18]. Azaisosteres of zolpidem, pyrazolo[1,5-a]pyrimidines, have been identified as selective ligands for Bz/$GABA_A$ receptor subtypes. Compound **4** has been described to have affinity only for α1β2γ2 subtype ($K_i = 31$ nM) and revealed sedative and anxiolytic-like properties without any amnesic and myorelaxant effects in rodents [19].



Zolpidem (**1**) **2** **3** **4**

Pyrazolo[1,5-a]pyrimidine, the central scaffold in zaleplon, is present in **5**, **6** and **7**. Compound **5** inhibits the binding of tritiated benzodiazepine in synaptosomal fractions from rat cortex [20] and **6** and **7** inhibit the α1 $GABA_A$ subunit with $K_i = 53$ nM and 17 nM, respectively, and showed sedative-hypnotic action following i.p. administration to mice (<90% inhibition of motor activity)

[21]. In addition, the positional isomer, imidazo[1,5-a]pyrimidine, is present in **8**, a compound that inhibits the α1 $GABA_A$ subunit by 81.1% at 0.1 μM [22].



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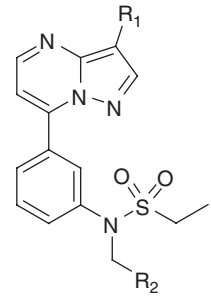
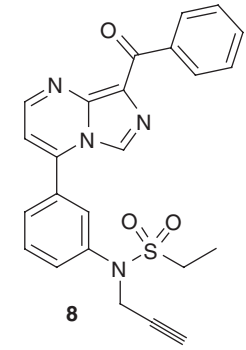


6, R_1 = CN, R_2 = Et
7, R_1 = NO_2, R_2 = Et



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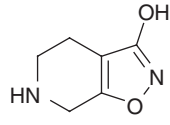
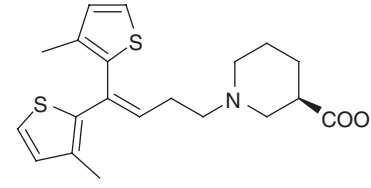
2.3 Other $GABA_A$ receptor modulators

Gaboxadol (**9**) is a selective extrasynaptic GABA receptor agonist in late-stage investigation for the treatment of insomnia. The action of this compound was extensively reviewed in Chebib *et al*. [13] and updated in Wafford *et al*. [23]. Nevertheless, the sponsor companies Merck & Co., Inc., and H. Lundbeck A/S announced in March 2007 that the results from recently completed clinical studies do not support further development and announced the discontinuation of their joint development program for gaboxadol.



Gaboxadol (**9**)



Tiagabine (**10**)

Tiagabine (**10**), a GABA reuptake inhibitor launched for epilepsy, is undergoing clinical trials for insomnia. Tiagabine 4, 6 and 8 mg significantly increased slow-wave sleep, with a significant decrease in Stage 1 sleep. Tiagabine was generally well tolerated, with doses of less than 6 mg having tolerability profiles similar to that of placebo. The 8-mg dose, however, was associated with troublesome adverse events [24].

A phase II study of EVT-201, a partial positive modulator of $GABA_A$ receptor, has recently been initiated in the US in elderly patients with chronic insomnia with the maintenance as primary endpoint (no structure disclosed).

NG2-73 is a $GABA_A$ receptor partial agonist that, according to the information given by the company, modulates preferentially the α3 subunit – a subunit that is hypothesized to be associated with sleep induction – and is undergoing phase II trials for chronic insomnia with primary endpoints measuring sleep onset as well as maintenance (no structure disclosed) [25].

3. MELATONIN RECEPTOR AGONISTS

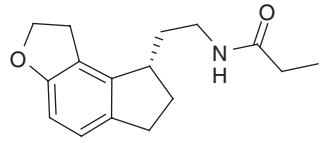
Melatonin, secreted by the pineal gland, is produced at night to aid the body in regulating sleep-wake cycles. The amount of melatonin the body produces decreases with age, which may explain why the elderly suffer from insomnia more frequently than the general population. Melatonin may be helpful in treating patients with insomnia, however, its short half-life (minutes) limits its therapeutic use. A melatonin prolonged-release formulation (Circadin®) is currently undergoing a phase III study in the treatment of insomnia patients with low endogenous melatonin [26].

To mimic melatonin action and increase the half-life is the goal of melatonin receptor agonists, which are the more recent addition to the insomnia therapeutic armamentarium. These compounds, in addition to use for insomnia, may have potential application in the synchronization of disturbed circadian rhythms, sleep disturbances in the elderly, seasonal depression and jet lag, to name a few. Furthermore, studies have shown that melatonin receptor agonists do not induce any of the hypothermic, hypotensive or bradycardic effects caused by melatonin in humans [27,28].

Melatonin receptor agonists and their relevance for the treatment of sleep disorders and major depression have been previously reviewed in *Ann. Rep. Med. Chem.*, volume 39 [29]. Since then, ramelteon has been approved, representing an important milestone for the proof of concept of this target, and has opened new possibilities for research.

3.1 Ramelteon

Takeda's melatonin (MT1/MT2) receptor agonist ramelteon (**11**) was approved and launched in 2005 in the U.S., indicated for the treatment of primary insomnia characterized by difficulty with sleep onset. It is the first prescription medication for insomnia with a novel mechanism of action to reach the US market in 35 years. It is also the first and only prescription sleep medication that has not exhibited potential for abuse and dependence, and as such is not designated as a scheduled substance by the DEA. Moreover, ramelteon was also filed in late March 2007 in E.U. for primary insomnia.



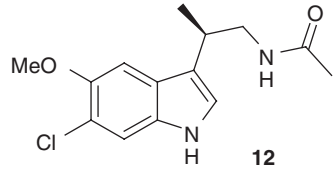
Ramelteon (**11**)

In controlled clinical trials in patients with primary insomnia, ramelteon 4–32 mg demonstrated significant reduction in latency to persistent sleep (LPS) compared with placebo. In elderly patients, objective and subjective LPS were also reduced at doses of 4 and 8 mg. Data on total sleep time are more variable,

depending on the clinical trial evaluated, but significant improvements are reported at 4 and 8 mg [30]. Most common adverse effects noted to date appear minor, i.e., headache (7%), dizziness (5%) and somnolence (5%). The last published clinical trials evaluated the potential effects of ramelteon 16 mg on apenic and hypopneic events in individuals with obstructive sleep apnea, due to the lack of depressant effects on nervous system, demonstrating no worsening of sleep apnea [31].

3.2 MLT agonists in clinical development for insomnia

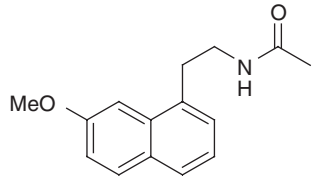
Compound **12** (LY-156735/PD-6735) is a melatonin MT1 and MT2 agonist currently undergoing phase II trials for the improvement of sleep onset latency in patients with primary insomnia. In 2001, the compound was assigned orphan drug status for the treatment of circadian rhythm sleep disorders in blind patients with no light perception. The compound has been shown to be safe and well tolerated. In addition, it dose-dependently reduces objective polysomnographic sleep parameters without producing any morning-after psychomotor impairment, as well as reducing the subjective time to fall asleep in patients with moderate to severe primary insomnia [32–37]. **12** demonstrated chronobiotic efficacy in healthy volunteers undergoing simulated shift lag [38] and showed adequate pharmacokinetics, pharmacodynamics and safety at doses of 20–40 mg in healthy volunteers [39].



12

VEC-162 is a dual MT1/MT2 melatonin agonist in phase II trials (no structure disclosed). A study in 39 subjects revealed that the compound (10–100 mg p.o.) dose-dependently, and on initial administration, advanced phases of melatonin circadian rhythm by up to 5 h. Sleep analysis also confirmed that these effects are associated with improved overall sleep efficacy, reduced sleep latency and attenuated rapid-eye-movement (REM) sleep. VEC-162 may therefore be beneficial for the management of sleep-wake disorders in subjects who rapidly shift their circadian phase [40]. The announced phase III study aimed at evaluating the safety and efficacy of VEC-162 compared to placebo in healthy subjects with induced transient insomnia has recently ended the patient recruitment phase [41].

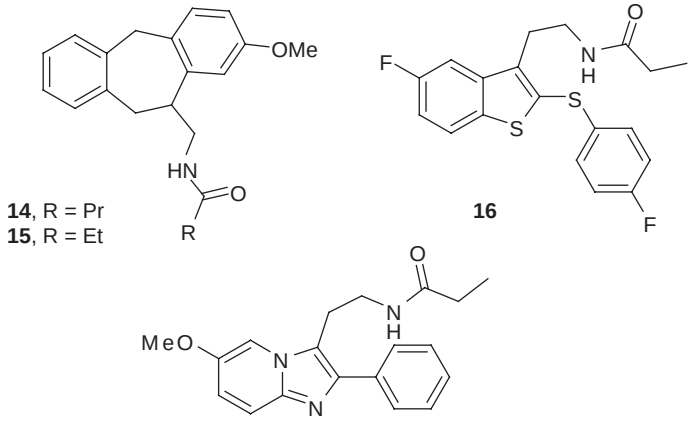
Agomelatine (**13**) represents an outsider within this group, because it is not in development for insomnia but for depression and anxiety. Although it is a MT1/MT2 receptor agonist in the picomolar range, it also displayed potent affinity for the $5HT_{2C}$ receptor ($IC_{50} = 270$ nM). This compound was extensively reviewed in 2004 [29,42], and, since then, new phase III trials for major depressive disorder were completed. However, the European Medicines Agency (EMEA) refused authorization in July 2006 due to concerns about its effectiveness [43].



Agomelatine (**13**)

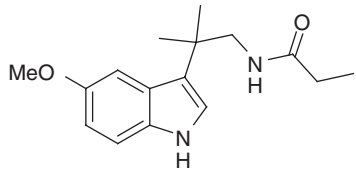
3.3 New MLT agonist structures

Tricyclic compounds, namely those bearing the scaffold dibenzo[a,d]cycloheptene, have been described as melatonin receptor ligands with MT2 receptor selectivity. Noticeably, **14** is described as an MT2 selective receptor antagonists, with affinity comparable to that of melatonin, while **15** produced a noticeable reduction of GTPγS binding at MT2 receptor, thus being among the few inverse agonists described to date [44]. Audinot *et al*. have disclosed the compound S-70254 that selectively binds to the MT2 receptor with a K_d of 7.0 pM compared to an affinity higher than 1.0 nM for MT1, but no structure is available for this compound [45]. Other melatonin receptor modulators described by the same group bear the benzothiophene sub-structure, **16**, with affinity for the human MT1/MT2 receptors (IC_{50} = 80/1 nM) [46] or the imidazopyridine moiety, **17** (IC_{50} = 29/8 μM) [47].



17

A series of melatonin analogues has been used to investigate the nature of the binding site of the melatonin receptor. The agonist/antagonist potency was measured using the pigment aggregation response of a clonal line of *Xenopus laevis* melanophores. In this assay β,β-dimethylmelatonin (**18**) showed high agonistic potency on *Xenopus* with ED_{50} of 0.0072 nM (0.063 nM for melatonin) [48]. Recently, melatonin receptor ligands with the general formula **19** were described but with no specific activity reported [49].



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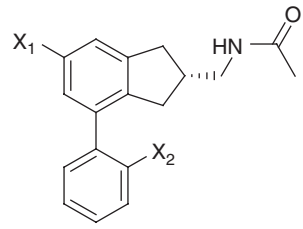


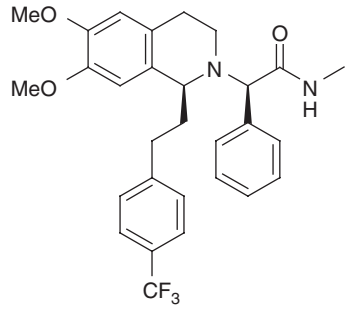
19,X_1,X_2 = Halogen

4. OREXIN RECEPTOR ANTAGONISTS

Orexins (hypocretins) are a class of neuropeptides first described in the late 1990s. These neuropeptides are produced specifically by a very small number of dedicated neurons located in the hypothalamus [50,51]. They have been shown to regulate the sleep/wake cycle by eliciting wakefulness, as well as being involved in appetite regulation and energy homeostasis. Orexins A and B bind to two central nervous system receptors, designated orexin-1 (OX1) and orexin-2 (OX2). Modulation of OX1 and OX2 receptors is being pursued as a promising and novel strategy for the treatment of sleep-wake disorders, including insomnia, narcolepsy and restless legs syndrome, and for obesity [52,53].

The sequences and functions of orexins-A and B are similar to each other, but the high sequence homology (68%) is limited to their C-terminal regions (residues 15–33). The sequence of the N-terminal region of orexin-A (residues 1–14), containing two disulfide bonds, is very different from that of orexin-B. Tomoyo *et al.* determined the structure of orexin-A using two-dimensional NMR [54] and Lang *et al.* used the shortest active analog and the L-alanine and L-proline replacement scans to screen for important peptide regions and amino acid residues. The Orexin A peptide was identified as the first analog with OX1 receptor preference while orexin B, [A27]orexin B and [P11]orexin B peptides are highly potent OX2 receptor selective (>1000-fold) compounds [55]. The potential value of Orexin A or B peptidic agonists in therapeutics will be demonstrated by the results with the existing pharmacological tools SB-668875 [56], h-Orexin B(10–28), [P11]h-Orexin B(6–28) and [A27]h-Orexin B(6–28) [55].

Subsequent to the extensive medicinal chemistry exploration of Orexin antagonism, its utility in the treatment of sleep disorders in man has been reported recently. This important milestone for the therapeutic validation of the target results from the OX1/OX2 receptor antagonist ACT-078573 (**20**) [57]. SB-649868 has also been announced to be in phase II clinical development, but neither the structural formula nor the results have been reported to date [58,59]. Moreover, insomnia treatments based on orexin modulation may be addressed by not only receptor antagonism but by inhibition of pathways related to the genesis of the bioactive peptides Orexin A or B, e.g. inhibition of Orexin-converting enzyme [60].



ACT-078572 (**20**)

ACT-078573 (**20**) is the first oral orexin receptor antagonist that penetrates the blood–brain barrier and is capable of inducing a transient and reversible blockade of the two receptors, OX1 and OX2 [61]. In animal models, the administration of **20** resulted in a dose-dependent decrease in alertness and increased non-REM and REM sleep. The compound, administered at oral doses ranging from 10 to 300 mg/kg, dose-dependently decreased alertness in rats and exhibited increased duration of REM and non-REM sleep, indicating no intrusive REM sleep that is characteristic of narcolepsy. In dogs, treatment with **20** (10–100 mg/kg p.o.) resulted in dose-dependent reductions in mobility and also induced signs of clinical somnolence.

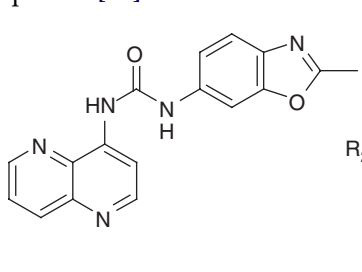
Clinical results with **20** have been recently reported from a phase I study that enrolled 70 healthy male human subjects. In this study, morning administration of the drug (200 mg and above) reduced alertness and latency to sleep stage 2 and increased time spent in sleep stage 2 with an overall improvement of sleep efficiency and total sleep time. These effects disappeared 6.5 h after drug administration [57].

Currently the safety and efficacy of **20** is being evaluated in a Phase II study. The program is focused on the evaluation of the sleep induction and maintenance in insomnia patients but also will attempt to demonstrate orexin antagonists effects on improved sleep and side effect profiles compared to current $GABA_A$ receptor modulators.

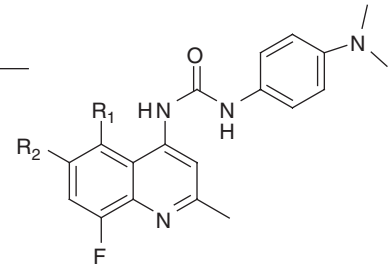
4.1 *N*-quinolinyl-*N*′-phenyl ureas and *N*-quinolinyl cynamamides

Quinolinyl compounds were first reported by Chan *et al*. as potent orexin receptor antagonists with excellent selectivity for OX1, good brain permeability and *in vivo* activity following i.p. dosing [62,63]. In fact, compounds SB-334867 (**21**), SB-408124 (**22**) and SB-410220 (**23**) displayed high affinity for the OX1 receptor in both whole cell ($K_i = 99$, 57 and 19 nM, respectively) and membrane formats ($K_i = 38$, 27 and 4.5 nM, respectively). Meanwhile, native orexin peptides A and B display affinities for the OX1 receptor with K_i values of 318 and 1516 nM, respectively. In addition, calcium mobilization studies showed that all three are functional antagonists of the OX1 receptor, with potencies in line with their affinities, and with ~50-fold

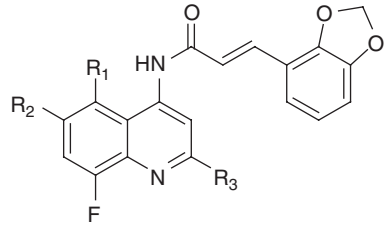
selectivity over the OX2 receptor [64]. Similar quinolinyl compounds bearing a cinnamyl group, exemplified by **24** and **25**, have also been described but affinity was not reported [65].



GSK-334867A (**21**)



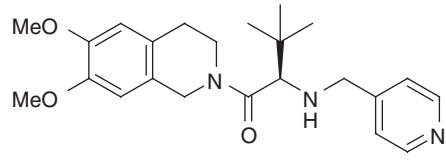
GSK-408124 (**22**) R_1=H, R_2=F
GSK-410220 (**23**) R_1=F, R_2=H



24, R_1=H, R_2=F, R_3=CH_3
25, R_1=F, R_2=H, R_3=H

4.2 1,2,3,4-tetrahydroisoquinolines, piperazines and morpholines

Since the first OX1/OX2 receptor antagonist bearing the 1,2,3,4-tetrahydroisoquinoline sub-structure was described by Aissaoui *et al*. [66], this scaffold has been widely utilized producing interesting compounds like OX2 receptor selective antagonist **26** (IC_{50} = 40 nM, OX1/OX2 ≥250-fold) [67,68]. Notwithstanding, ACT-078573 belongs to this structural class [61,69,70]. Interestingly, similar disposition of substituents onto the piperidine, piperazine and morpholine heterocycles or even the five-membered pyrrolidine has also rendered active compounds, exemplified by structures **27**, **28** and **29**, [71–82].



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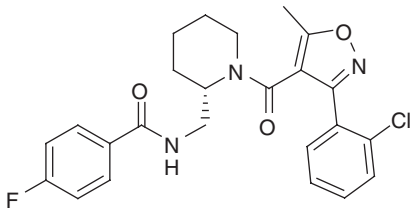

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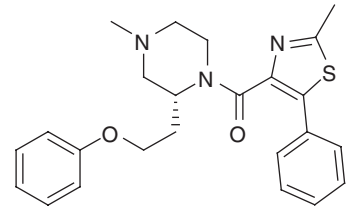

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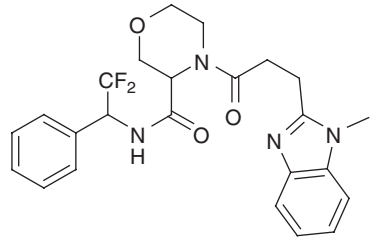

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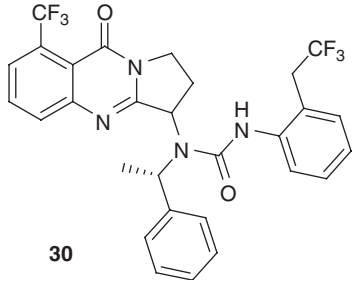
4.3 Pyrrolo- and pyrido[2,1-b]quinazolinones and 3,4-dihydroquinoxalin-3-ones

2,3-Dihydro-2*H*-pyrrolo[2,1-b]-quinazolinones and 7,8,9,11-tetrahydro-6*H*-pyrido[2,1-b]quinazolinones as well as 3,4-dihydroquinoxalin-3-ones have rendered OX1/OX2 receptor antagonists as described by Aissaoui *et al*. However, no activity has been reported to date for this family of compounds exemplified by analogs **30** [83] and **31** [84]



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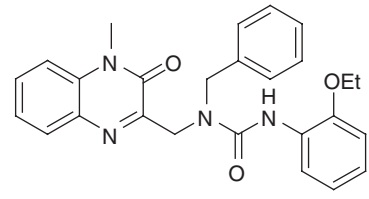

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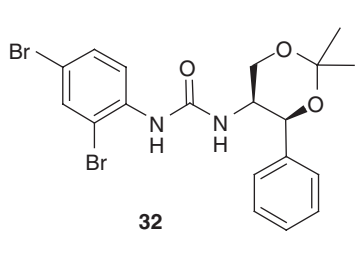
4.4 Other Orexin antagonists

4-Phenyl-[1,3]dioxane **32** has been reported to bind the OX2 receptor with a pK_i of 8.3 and a pK_b of 7.9, and is 600-fold selective for OX2 over OX1 [85,86]. JNJ-10268752 (no specific structure has been disclosed) has been described as a selective OX2 receptor antagonist (K_i OX1/OX2 = 18/2, 500 nM, respectively) [87]. Compounds exemplified by **33** have been reported as OX2 receptor antagonists (IC_{50} = 9 nM) selective versus OX1 (IC_{50} = 1870 nM) [88]. Recently, open chain amide **34,** sulfonamide compound **35** [89,82], and spirocyclic sulfonamide **36** [90]

have been described as high affinity Orexin receptor ligands.



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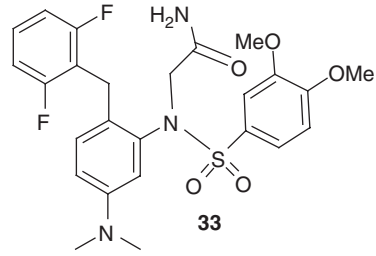

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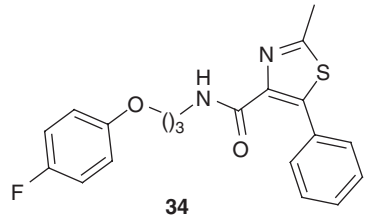

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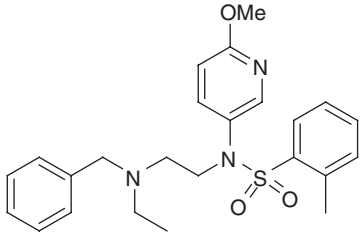

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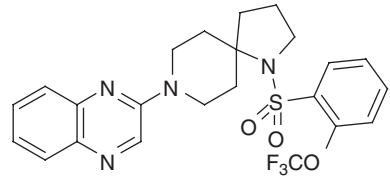

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Aside from chemical innovation, biological strategies focus on new methods to study the effect of this neurotransmitter system on insomnia as well as on the possibilities for faster screening of new chemical entities, i.e, zebrafish models [91].

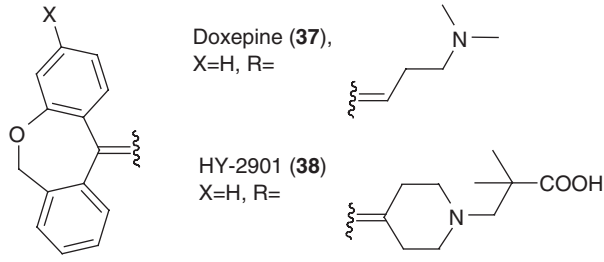
5. HISTAMINERGIC AND SEROTONINERGIC MODULATORS

Histamine neurons, located in the tuberomamillary nuclei of the hypothalamus, and serotonin (5-HT) neurons of the raphe nuclei play an important role in the sleep-wake cycle, being part of the ascending arousal system. Both neuronal systems discharged maximally during waking, diminished during SWS and ceased during REM [92]. However, 5-HT neurons, mainly focused on movement and postural-muscle tone, continue to be active during less aroused waking-states. Meanwhile histamine neuron activity is mainly related to cortical arousal. Therefore, antagonist or inverse agonist compounds acting on post-synaptic 5-HT and histamine receptors may avoid wakening. Many companies are currently developing compounds with these mechanisms of action for treating insomnia, thus addressing the lack of abuse potential associated with GABAergic compounds. In this respect, an innovative approach would be a dual mechanism of action: H_1 inverse agonist and 5-HT_{2A} receptor modulator. Compound

HY-10275 (no structure reported), having such a dual mechanism, has been recently reported to meet the primary and secondary endpoints in the initial phase II trial at doses of 1 and 3 mg in adults with transient insomnia [93].

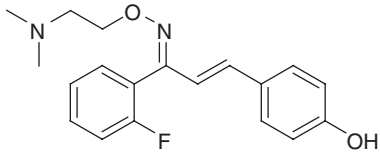
5.1 Histamine receptor modulators

Based on the well-known sedative effects of histamine H_1 receptor antagonists [94], three compounds have advanced to clinical development: Doxepin, HY-2901 and NBI-75043. Doxepin (**37**), a mixed H_1/H_2 receptor antagonist, has completed a successful phase III program for the treatment of insomnia. Doses of 3 and 6 mg in elderly adults with primary insomnia were associated with significant and dose-dependent improvements in sleep maintenance, duration and onset [95]. The doxepin analogue, HY-2901 (**38**), has been shown to have high affinity for histamine H_1 receptors ($K_i = 69.5$ nM) and to dose-dependently prolong non-REM sleep in rats (0.3–30 mg/kg). Regarding NBI-75043, this compound is the most recent H_1 receptor antagonist entering phase I clinical development for insomnia, but neither the structure nor preliminary results have yet been disclosed.

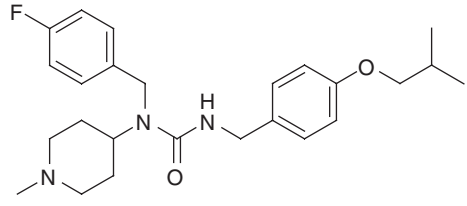


5.2 Serotonin receptor modulators

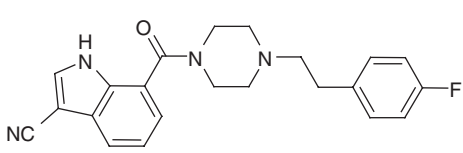
Serotonin related compounds devoted to sleep disorders mainly act via 5-HT_{2A} receptors. Compounds in clinical development reported with this mechanism of action are: Eplivanserin, Pimavanserin, Pruvanserin, Volinanserin and APD-125.



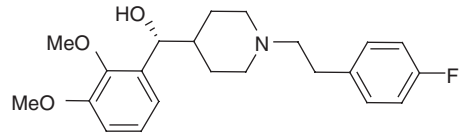
Eplivanserin (**39**)



Pimavanserin (**40**)



Pruvanserinn (**41**)



Volinanserin (**42**)

Eplivanserin (**39**) is a 5-HT_{2A} antagonist initially developed for a broader spectrum of psychiatric disorders but that has been tested recently for insomnia. Within this latter indication, phase II studies showed benefits in sleep maintenance, but not in induction [9]. Compound **39** is currently in phase III, to assess the efficacy for the treatment of sleep maintenance insomnia, evaluating both sleep and daytime functioning [96].

Pimavanserin tartrate (**40**, ACP-103) is a 5-HT_{2A} receptor inverse agonist currently in phase II clinical development as an antipsychotic agent and for insomnia, focused on sleep maintenance, evaluating slow-wave-sleep at doses of 5 and 20 mg [97,98].

Pruvanserin hydrochloride (**41**, EMR-62218/LY-2422347) is a 5-HT_{2A} antagonist in phase II for the treatment of insomnia [99]. Safety and tolerability studies of 5 and 15 mg compared with placebo are ongoing and completion was expected in November 2006.

Volinanserin (**42**, MDL-100907) is a selective 5-HT_{2A} antagonist discontinued for schizophrenia and is currently undergoing phase II clinical trials for the treatment of insomnia. However, no data has been reported recently for this indication.

APD-125 (structure not disclosed) is a 5-HT_{2A} inverse agonist that recently began phase II studies for chronic insomnia at 10 and 40 mg doses [9]. In phase I testing, APD-125 improved measurements associated with sleep maintenance, including increases in the duration of slow wave sleep not associated with changes in the percentage of time in REM sleep. APD-125 was not associated with any limiting next-day cognitive or motor impairments [100].

6. CONCLUDING REMARKS

Insomnia treatment has been traditionally dominated by benzodiazepines and $GABA_A$ modulating compounds. However, since the early 2000s the landscape has changed completely with new mechanism of actions approved for the treatment of insomnia (melatonin receptor agonists) or in advanced clinical phases (orexin antagonists and histamine/serotonin antagonists). Furthermore, new agents are necessary to address different symptoms of insomnia and the major drawbacks of current treatments. Newer targets as 5-HT_7 receptor antagonists, *N*-acetyl-transferase inhibitors, adenosine modulators, oleamide-related compounds or circadian clock proteins (CLOCK, MOP3 and MOP4), are being discussed for sleep disorders and may open a myriad of challenging opportunities for innovative medicinal chemistry.

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CHAPTER 6

Advances in Transient Receptor Potential Modulators

Anil Vasudevan and **Michael E. Kort**

Contents

1. INTRODUCTION

The sensation of temperature in humans and other vertebrates is primarily, if not solely, mediated by members of the transient receptor potential (TRP) family of cation channels. Recent work indicates that TRPs respond to a variety of stimuli including specific ligands, temperature, acid, salt concentration, and second messenger signaling [1]. As such, TRPs act as multimodal signal integrators. This gene family represents ~20% of all ion channels found in the body. Since TRP channels are only distantly related to voltage-gated channels, they present an opportunity to identify selective ''first in class'' drugs. Each TRP channel subunit consists of six putative transmembrane spanning segments (S1–6), a pore-forming loop between S5 and S6, and intracellularly located NH_2 and COOH termini [2]. Assembly of channel subunits as homotetramers or heterotetramers results in the formation of cation-selective channels. The TRP superfamily comprises >30

Neurological Diseases Research, Abbott Laboratories, Abbott Park, IL 60064, USA

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members and can be divided into seven families, based on amino acid homology [3–6]. The four major families are (1) the vanilloid TRP (TRPV) channels, activated by a variety of signals including vanilloid compounds, such as capsaicin, noxious signals, hypotonic cell swelling and heat; (2) melastatin-related TRP (TRPM) channels, which have diverse functional properties such as controlling Mg^{2+} entry, modulating the membrane potential, and sensing cold and menthol in sensory neurons; (3) the TRPC family, containing seven members, which are activated through PLC-coupled receptors; and (4) the TRPA (Ankyrin) family, comprised of only one member, TRPA1, which is modulated by extra- and intracellular calcium.

Many of the thermoreceptor channels display significant ligand promiscuity and can be activated by additional modalities, such as hypotonicity and mechanical stretch (TRPV2, TRPV4), extracellular acidification (TRPV1, TRPV4), and numerous exogenous and endogenous chemical ligands (TRPV1: vanilloids and cannabinoids; TRPV4: arachidonic acid metabolites and menthol, icilin, and bradykinin). As TRP channels are involved in a diverse number of biological processes, including thermosensation, vascular inflammation, homeostasis of ionic gradients in cells, sensation of irritant stimuli, tumor progression, neural cell signaling, and flow sensing in the kidney, they are attractive targets for therapeutic drug development. Detailed reviews on the regulation and molecular properties of TRP channels have been published within the past year [7,8]. This review focuses on the modulators of the various TRP channels and their role in understanding the function of these channels, as well as the potential therapeutic benefits of targeting TRP ion channels.

2. SMALL MOLECULE TRP MODULATORS

2.1 TRPV1 (vanilloid receptor)

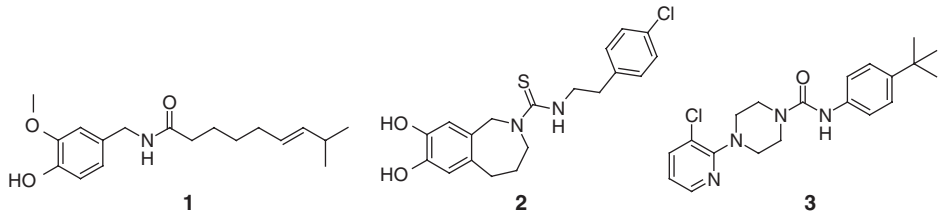
Among the various members of the TRP superfamily, the vanilloid-1 receptor (TRPV1) has emerged as a particularly attractive target for the treatment of acute and chronic pain. Regarded as a polymodal molecular integrator in nociception, TRPV1 is a nonselective cation channel localized on sensory neurons in C- and Aδ-fibers in sensory ganglia. It is gated by noxious heat, acidic pH, and capsaicin (**1**), the active component in hot chili peppers. Abundant evidence has demonstrated that TRPV1 is also modulated by numerous inflammatory mediators, including growth factors, neurotransmitters, peptides or small proteins, endogenous lipids, chemokines, and cytokines [9]. Activation of TRPV1 results in the release of molecules associated with pain transmission, such as calcitonin gene-related peptide (CGRP), substance P, and glutamate [10]. Mounting evidence for the existence of functional TRPV1 in both central and peripheral sensory neurons further implicates this receptor in pain perception and suggests that TRPV1 may be involved in numerous physiological processes [11].

Despite their initial irritant properties, capsaicin and related TRPV1 agonists ultimately reduce sensitivity to painful stimuli by desensitizing the receptor.

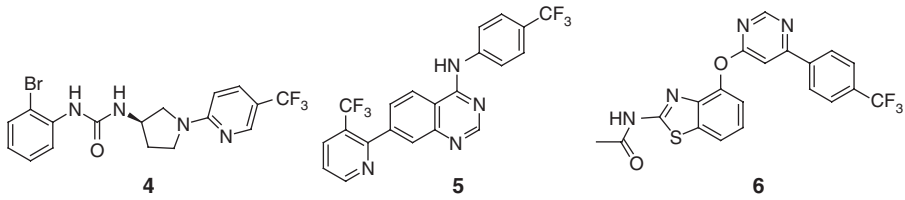
For example, capsaicin is currently marketed as a topical analgesic [12]. Clinical trials have also been initiated with an injectable formulation of a capsaicin-like agonist (ALGRX 4975) for the potential treatment of postsurgical pain, tendonitis, and posttrauma neuropathy [13]. Analogs of the diterpenoid resiniferatoxin have also been investigated for their TRPV1 agonist properties [14].

The analgesic profile in animal pain models generated by TRPV1 receptor blockade using either genetic (knockout animals) [15,16] or pharmacological (small molecule) [17] approaches has provided compelling data for use of TRPV1 antagonists as therapeutics. TRPV1 receptor antagonists dose-dependently block direct activation by capsaicin *in vivo*, and have been shown to be potent and efficacious in preclinical animal pain models associated with low pH and thermal hyperalgesia, such as acute and chronic inflammation. In this regard, the behavioral effects of TRPV1 antagonists are in close agreement with the phenotype observed in TRPV1 knockout mice when challenged with inflammatory agents such as carrageenan, mustard oil, complete Freund's adjuvant (CFA), and capsaicin. Since TRPV1 serves as a key nodal point in pain transmission pathways, pharmacological blockade by small molecule TRPV1 receptor antagonists may provide broad-spectrum applications for pain management.

In contrast to their agonist counterparts, the characterization of the analgesic profile of small molecule TRPV1 antagonists, based on early leads such as capsazepine (hTRPV1 IC_{50} 365 nM, **2**) and BCTC (hTRPV1 IC_{50} 34 nM, **3**), has been a more recent and rapidly developing area [18].



The small molecule TRPV1 antagonist SB-705498 (**4**, hTRPV1 IC_{50} 32 nM), a biaryl urea with *in vivo* efficacy in the capsaicin-induced hyperalgesia model in rat, [19] entered Phase II clinical trials for dental pain and migraine, but development was recently discontinued. NGD-8243 (structure not disclosed), which may have emanated from aminoquinazolines such as **5** (hTRPV1 $IC_{50} = 1.1$ nM) or a related chemical series, is under development for the potential treatment of pain, asthma, and cough suppression and is in Phase II studies for evaluation of dental pain (molar extraction) [20]. AMG-517 (**6**), a potent 4,6-disubstituted pyrimidine (hTRPV1 $IC_{50} = 0.8$ nM) has completed Phase I trials and will be evaluated for efficacy in inflammatory pain. Upon oral administration, this compound demonstrated preclinical efficacy in the carrageenan-induced thermal hyperalgesia and CFA-induced thermal hyperalgesia models of inflammatory pain [21].



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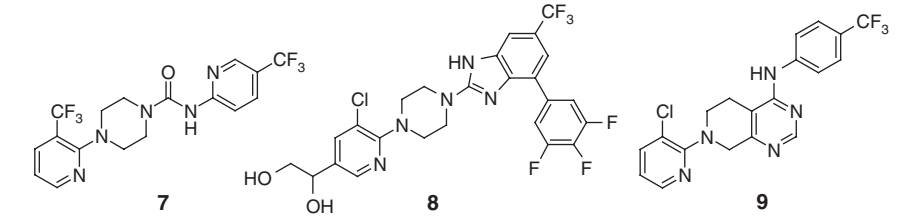
The design of TRPV1 antagonists continues to be an area of active preclinical research in the pharmaceutical industry as evidenced by the steady profusion of primary and patent literature in 2006 [22,23]. Interestingly, the structures of many newer TRPV1 antagonists remain based largely on the capsazepine (biaryl urea) and BCTC (diaryl piperazine) motifs.

Pyridinylpiperazine ureas, including JNJ-17203212 (**7**, hTRPV1 $IC_{50} = 65\,nM$) [24] and the more highly decorated imidazole isostere **8** (hTRPV1 $IC_{50} = 0.9\,nM$) [25], display excellent oral bioavailability and full efficacy in blocking capsaicin-induced flinching in rat. JNJ-17203212 also showed antitussive efficacy in an induced cough model in guinea pig. TRPV1 antagonists similar in structure to **9** are characterized by low nanomolar inhibition of acid- and capsaicin-induced calcium flux [26]. 5,6-Bicyclic derivatives including benzisoxazoles such as **10** [27] and indazolones such as **11** [28] have also been described. A series of closely related patents disclose BCTC-like derivatives generalized by **12** wherein one of the piperazine nitrogen atoms is replaced by a carbon atom to form a tetrahydropyridine or fully saturated piperidine ring [29–32]. In a related strategy, replacement of the piperidine core in **12** with a phenyl group provides potent biarylcarboxybenzamide TRPV1 antagonists analogous to **13** [20,33].



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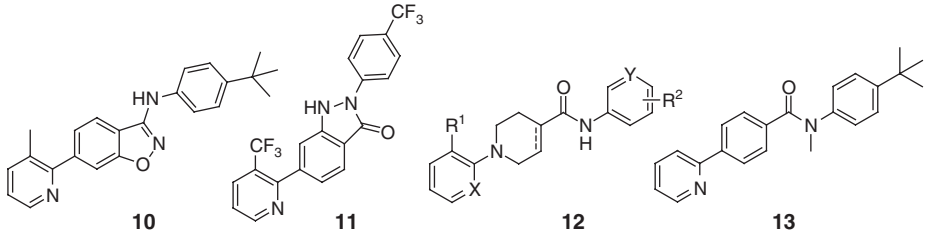
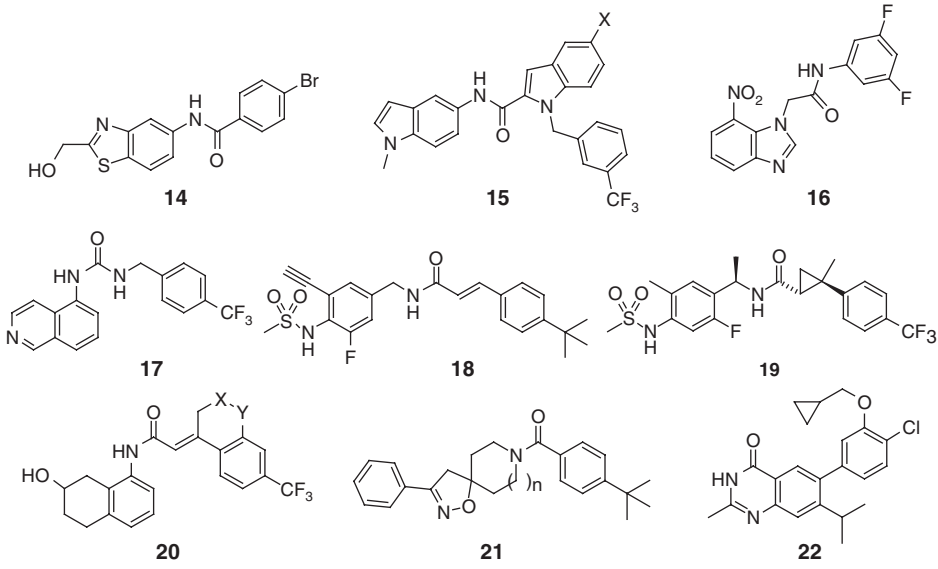


10 11 12 13

Recent reports have emerged of several TRPV1 antagonists possessing a biaryl amide (**14–16**), urea (**17**), or urea isostere (**18–20**) scaffolds. Bicyclic derivatives **14–16** block capsaicin- or pH-stimulated calcium influx in FLIPR-based assays

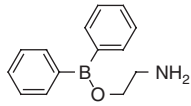
with IC_{50} values in the 10 nM range [34–36]. The 5-isoquinoline urea **17** (hTRPV1 $IC_{50} = 4$ nM) exhibited 46% oral bioavailability and *in vivo* activity in animal models of visceral and inflammatory pain [37,38]. Replacement of one of the urea nitrogens with either an olefinic (e.g. **18** or **20**) [39,40] or cyclopropyl (**19**) linkage is well tolerated [41]. TRPV1 antagonists have been identified which incorporate a spirocyclic isoxazoline–piperidine core (e.g. **21**) between the aromatic pharmacophore elements [42]. A series of recently developed 6-aryl-7-isopropylquinazolinone lead structures (e.g. **22**) represents a significant departure from traditional TRPV1 antagonist design [43].



2.2 TRPV3

TRPV3 is activated by heat (>33°C) and, unlike most thermo-TRPs, is expressed in mouse keratinocytes and not in the dorsal root ganglia [44]. TRPV3 null mice have strong deficits in response to innocuous and noxious heat but not in other sensory modalities; hence, TRPV3 has a specific role in thermosensation. The natural compound camphor, which modulates sensations of warmth in humans, has been shown to be a specific activator of TRPV3 [45]. Camphor activates cultured primary keratinocytes but not sensory neurons; this activity was abolished in TRPV3 null mice. 2-Aminoethoxydiphenyl borate (2-APB, **23**), which inhibits store-operated Ca^{2+} channels and IP3 receptors, activates recombinant TRPV3 with an EC_{50} of 28 μM [46]. 2-APB also sensitizes TRPV3 to activation by heat and represents a potentially useful tool for the physiological analysis of TRPV3 and the identification of clinically useful TRPV3 antagonists. However, since 2-APB also activates TRPV1 and TRPV2, as well as other TRPC channels, this ligand is insufficient to investigate the utility of selective modulators of TRPV3. Recently, a series of β-mercapto amide containing TRPV3 inhibitors (**24**),

with excellent selectivity over TRPV1 and TRPV6, has been reported [47].



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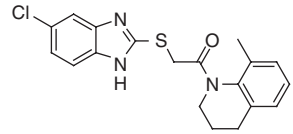

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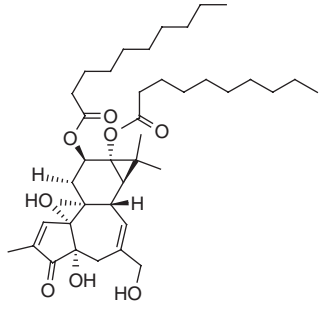
2.3 TRPV4

TRPV4 is a mechanosensitive, nonselective cation channel that is activated under hypotonic conditions and serves as an osmoreceptor. TRPV4 is expressed in brain, liver, kidney, heart, testis, and salivary glands [48]. TRPV4 knockout mice at 8 weeks of age were normal, but those at 24 weeks revealed significantly higher thresholds of auditory brainstem response [49]. These and other studies suggest that disruption of TRPV4 causes delayed-onset hearing loss and makes the cochlea vulnerable to acoustic injury. In addition, TRPV4 shows some unexpected gating promiscuity: it can be activated by cell swelling, ligand stimulation (e.g. phorbol ester **25**) [50], or heat. Since modulation of TRPV4 has been shown to play a role in attenuation of cartilage breakdown as well as a reduction in the production of matrix degradating enzymes, recent efforts have been aimed at identifying TRPV4 modulators [51]. A series of morpholine and piperidine containing agonists (**26**) has recently been reported [51].



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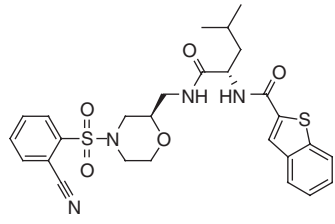

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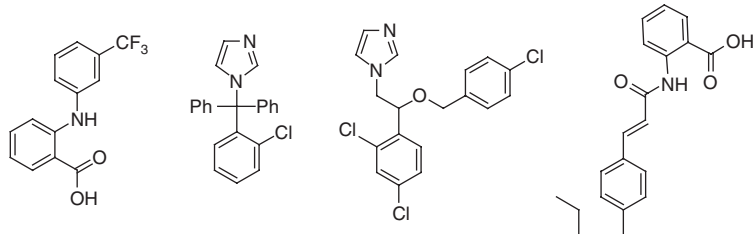
2.4 TRPM2/TRPM5/TRPM8

Like other TRP channels, TRPM2 is a Ca^{2+}-permeable, nonselective cation channel. A unique feature of TRPM2 is its activation by ADP-ribose and species that arise during oxidative stress, for example, NAD+ and H_2O_2. These properties have led to proposals that this channel may play a role in cell death produced by pathological redox states [52]. The lack of specific antagonists of this channel has made these hypotheses difficult to test. However, using patch-clamp electrophysiology, the nonsteroidal anti-inflammatory compound flufenamic acid (FFA, **27**) has been shown to inhibit recombinant human TRPM2 (hTRPM2),

as well as currents activated by intracellular ADP-ribose in the CRI-G1 rat insulinoma cell line [53]. These experiments suggest that FFA may be a useful tool for studies of TRPM2 function. Using similar experimental setups, antifungal imidazoles clotrimazole (**28**) and econazole (**29**) were shown to inhibit ADP-ribose-activated currents in HEK-293 cells expressing recombinant hTRPM2 [54]. For both compounds, concentrations in a range from 3 to 30 μM produced an essentially complete inhibition of the TRPM2-mediated current, suggesting that imidazole antifungals could be useful tool antagonists for future studies of TRPM2 function.

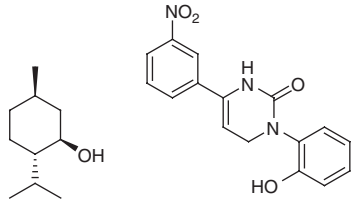


27 **28** **29** **30**

Using patch-clamp and calcium-imaging techniques, extracellular application of 20 μM *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA, **30**) completely blocked ADP-ribose induced whole-cell currents and H_2O_2-induced Ca^{2+} signals ($IC_{50} = 1.7\,\mu M$) in HEK293 cells transfected with human TRPM2. ACA (20 μM) also blocked currents through human TRPM8 ($IC_{50} = 3.9\,\mu M$) and TRPC6 ($IC_{50} = 2.3\,\mu M$) expressed in HEK293 cells [55].

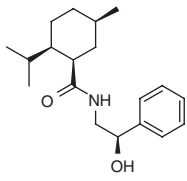
Though there are no reported small molecule modulators of TRPM5, deletion studies suggest that TRPM5 is an important factor in taste responses, and the consequences of eliminating TRPM5 expression vary depending upon the taste quality and the lingual taste field [56]. Therefore, the study of TRPM5 may provide insight into fundamental mechanisms of taste transduction.

Two of the best-known modulators of TRPM8 activity are menthol (**31**) and icilin (**32**). Menthol is effective at inducing calcium influx at ~10–100 μM, while icilin is effective in the 0.1–1 μM range. While the residues responsible for activation of TRPM8 by menthol have been identified [57], recent results demonstrate that the activation of TRPM8 by icilin and cold, but not menthol, is modulated by intracellular pH in the physiological range [58].



31 **32**

TRPM8 knockdown data indicate that it could represent an important neuronal axis that can be exploited in chronic sensitized pain states [59]. Additionally, modulators of TRPM8 have potential utility for the treatment of asthma, chronic obstructive pulmonary disease, and allergic rhinitis [60]. Based on expression patterns, TRPM8 modulators could even play an important role in prostate cancer. Recently, menthol-based TRPM8 agonists (**33**) have been reported to significantly inhibit the growth of TRPM8 positive tumors in mice by as much as 77% at well-tolerated doses [61,62].



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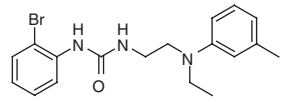

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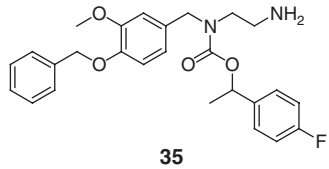

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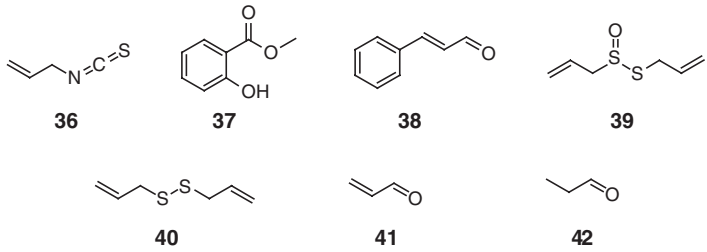
Several TRPV1 antagonists have been reported to be potent TRPM8 inhibitors, raising the possibility that there exists significant structural homology between these two ion channels and perhaps synergies in their gating mechanisms. Capsazepine (**2**) and BCTC (**3**), prototypical TRPV1 antagonists, are also TRPM8 inhibitors ($IC_{50} = 18\,\mu M$ and 143 nM, respectively), whereas the ring-opened analog SB-452533 (**34**) was also quite potent ($IC_{50} = 533\,nM$) [63,64]. In addition to these TRPV1 antagonists, benzyloxycarbamates, such as **35**, have also been reported to be potent TRPM8 inhibitors ($IC_{50} = 0.2\,\mu M$) in a cell-based Ca^{2+} influx assay in HEK293 cells [65].

2.5 TRPA1

TRPA1 which is the sole known member of the TRPA family, is a nonselective cation channel expressed in subsets of the dorsal root and trigeminal ganglia. Chemical activators of TRPA1 include isothiocyanates (allylisothiocyanate, **36**), methyl salicylate (in wintergreen oil, **37**), cinnamaldehyde (in cinnamon, **38**), allicin (**39**), diallyl disulfide (**40**, in garlic), and acrolein (**41**) [66–69]. Despite being able to recognize multiple aliphatic and aromatic compounds, TRPA1 displays a surprising level of discrimination. For example, TRPA1 is activated by acrolein (**41**) but is insensitive to propanal (**42**) [70].



Additional studies indicate that blocking TRPA1 in sensory neurons might provide a fruitful strategy for treating cold hyperalgesia caused by inflammation and nerve damage [71]. Two recent studies highlight the inherent chemical reactivity and cross-linking of TRPA1 by reactive ligands such as **36** and **38** [72,73].

3. CONCLUSION

There has been a steady increase in the fundamental understanding of the roles of TRP channels in human conditions. However, more selective modulators of many of these channels are required to fully understand their role in physiological and pathophysiological conditions.

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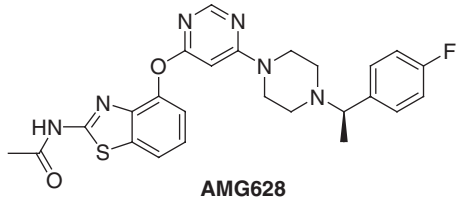

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CHAPTER 7

Case History: JANUVIA™ (Sitagliptin), a Selective Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type 2 Diabetes

Ann E. Weber* and **Nancy Thornberry****

Contents

1. INTRODUCTION

Diabetes is a global epidemic affecting more than 240 million people worldwide. The incidence of this disease is growing at an alarming rate, with 380 million cases predicted by 2025. Each year over 3.8 million people die from complications of diabetes, including heart disease, stroke and kidney failure. The vast majority

* Department of Medicinal Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA
** Department of Metabolic Disorders, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA

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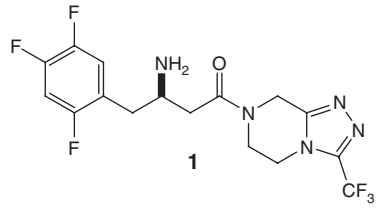


Figure 1 JANUVIA™ (sitagliptin).

(90–95%) of cases are type 2 diabetes, largely resulting from the increasing prevalence of obesity and sedentary lifestyles [1].

Despite the availability of a range of agents to treat type 2 diabetes, glucose control remains suboptimal, with less than 50% of patients achieving stated glycemic goals. In addition, current therapies have limited durability and/or are associated with significant side effects such as GI intolerance, hypoglycemia, weight gain, lactic acidosis and edema [2]. Thus, significant unmet medical needs remain. In particular, safer, better tolerated medications which provide increased efficacy and long-term durability are desired. JANUVIA™ (sitagliptin, **1**, Figure 1), a dipeptidyl peptidase IV (DPP-4) inhibitor, represents a promising new approach to the treatment of this disease.

2. PATHOGENESIS OF TYPE 2 DIABETES

The pathogenesis of type 2 diabetes involves a set of three primary defects: insulin resistance, insulin secretory dysfunction, and hepatic glucose overproduction. Insulin resistance is a common predisposing defect, and is believed to occur as a consequence of obesity in most individuals. As long as an individual maintains insulin secretion adequate to compensate for insulin resistance, plasma glucose levels remain normal; however, if β-cell function declines, and the pancreas is no longer able to produce adequate amounts of insulin to compensate for the insulin resistance, hyperglycemia – and subsequently, diabetes mellitus – results. Not only does this β-cell defect lead to hyperglycemia and the onset of diabetes, the progressive decline in β-cell function during the course of diabetes leads to the need for more and more complex treatment regimens to manage glucose control in diabetic patients, and ultimately, to the need for insulin. As expected from the pathogenesis of type 2 diabetes, therapies that increase the circulating concentrations of insulin have proven therapeutically beneficial in the treatment of type 2 diabetes [2]. Indeed, sulfonylureas and related insulin secretagogues currently represent 42% of the total worldwide oral market, with sales in excess of $1.7 billion, notwithstanding mechanism-based side effects of hypoglycemia and weight gain. In addition, current insulin secretagogues commonly fail to maintain adequate glycemic control, and may contribute to the progressive decline in β-cell function. Thus, current unmet medical needs in the treatment of type 2 diabetes include insulin secretagogues which are

glucose-dependent, decreasing the risk for hypoglycemia, and which do not lead to weight gain.

3. RATIONALE FOR THE USE OF DPP-4 INHIBITORS TO TREAT TYPE 2 DIABETES

Inhibitors of DPP-4, a proline selective serine protease, are a new therapeutic approach to the treatment of type 2 diabetes [3,4]. DPP-4 inhibitors function, at least in part, as indirect stimulators of glucose-dependent insulin secretion, and these agents may address a number of the unmet medical needs noted above. The DPP-4 inhibitor induced increase in insulin secretion is believed to be mediated primarily via stabilization of the incretin hormone glucagon-like peptide-1 (GLP-1), which has a clearly established role in glucose-dependent insulin biosynthesis and secretion. Continuous infusion of GLP-1 or subcutaneous administration of GLP-1 analogs to diabetic humans has resulted in normalization of both postprandial and fasting glucose [5]. For example, sub-chronic (6 week) continuous infusion of GLP-1 resulted in profound and significant decreases in fasting plasma glucose, and substantial improvement in HbA_{1c}, a marker of overall glycemic control [6]. A GLP-1 agonist, BYETTA® (exenatide), originally identified as a salivary protein in a lizard species, has been approved for use in patients with type 2 diabetes as a subcutaneously administered peptide, and multiple other GLP-1 analogs are in development. An alternate oral strategy involves the use of DPP-4 inhibitors to increase the concentrations of endogenously released GLP-1.

DPP-4 inhibitors have at least three potential advantages over currently available oral insulin secretagogues: first, because the incretin peptide GLP-1 increases insulin in a strictly glucose-dependent manner (i.e., when glucose levels are below normal, no stimulation of insulin secretion occurs), a low risk of hypoglycemia would be expected. Second, since the use of GLP-1 analogues has led to decreased appetite and weight reduction, a DPP-4 inhibitor, that acts through augmentation of GLP-1, would be expected to provide either weight loss or at least no gain in weight. Finally, DPP-4 inhibitors may have long-term beneficial effects on β-cell function in that GLP-1 stimulates both insulin biosynthesis and secretion, and is suggested to have a role in regulation of β-cell mass [7].

Early in the Merck Research Laboratories (MRL) program, evidence that DPP-4 inhibitors may be useful for treatment of type 2 diabetes mellitus was published, including studies showing that *Dpp4*$^{-/-}$ mice (i.e., genetically deficient in DPP-4, or "knock-out" mice) have improved glucoregulation, and studies conducted in humans with DPP-4 inhibitors showing lowering of plasma glucose concentrations. *Dpp4*$^{-/-}$ mice are healthy, fertile, and have improved metabolic function [8,9]. Specifically, these animals have improved glucose tolerance, which is accompanied by increased levels of insulin and active GLP-1, and decreased circulating glucagon concentrations. Similar effects have been observed in several animal models of diabetes with structurally distinct DPP-4 inhibitors [10–12]. In 1999, Novartis (with DPP728, **2**, Figure 2) and Probiodrug (with P32/98, **3**) independently launched Phase I clinical trials. In single dose studies, both compounds were well tolerated, increased active GLP-1, and reduced glycemic

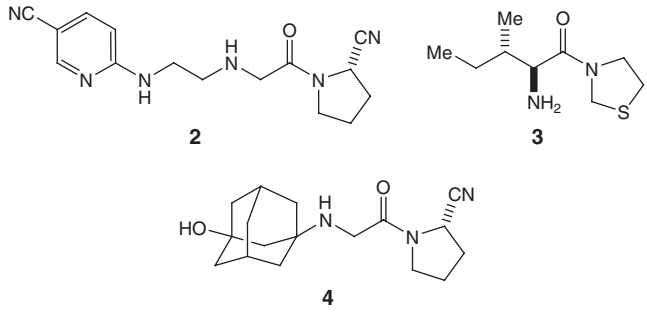


Figure 2 Structures of DPP-4 inhibitors.

excursion following food or glucose intake in normal volunteers [13–15]. Subsequently, Novartis reported the results of a 4-week phase II study with DPP728 in 93 patients at 100 mg T.I.D. or 150 mg B.I.D.: significant decreases were observed in maximal glucose excursion, fasting plasma glucose, and 24 h glucose [16]. Development of this compound was discontinued in favor of LAF237 (**4**), now known as GALVUS^{TM} (vildagliptin) [17].

As described above, the reductions in glucose levels that are observed with DPP-4 inhibitors are believed to be mediated primarily through stabilization of the incretin GLP-1 [7–36] amide, a ~3 kDa peptide hormone that is intimately involved in the regulation of glucose homeostasis. GLP-1 is efficiently hydrolyzed *in vitro* (k_{cat}/K_m ~1×10^6 M/s) by DPP-4 to generate an inactive product, GLP-1 [9–36] amide [18]. The most compelling evidence that DPP-4 is primarily responsible for the rapid regulation of GLP-1 *in vivo* ($t_{1/2}$ ~1 min) is provided by studies with specific DPP-4 inhibitors, which produce increased circulating concentrations of GLP-1 in both rodents and humans [13,14,19], and by the finding that DPP-4-deficient mice have increased (~3-fold) circulating levels of intact GLP-1 [9].

Although GLP-1 is believed to be the primary mechanism by which DPP-4 inhibitors lower glucose, other substrates may also be important. Several members of the glucagon peptide family are cleaved by DPP-4 *in vitro*, and the incretin glucose-dependent insulinotropic peptide (GIP), in particular, is clearly regulated by DPP-4 *in vivo* in both rodents and humans. Evidence that both GLP-1 and GIP are important for improved glucose AUC in rodents has been provided in studies with a DPP-4 inhibitor in GLP-1 and GIP receptor knockout mice, and mice which are deficient in both receptors [8,20]. The importance of GIP stabilization to improved glucose control in diabetic humans, who have a diminished response to exogenous GIP, has not been established.

4. MRL'S DPP-4 INHIBITOR PROGRAM: *THREO*- AND *ALLO*-ISOLEUCYL THIAZOLIDIDES

In order to jump-start internal efforts on the DPP-4 inhibitor program, L-*threo*-(2*S*,3*S*)-isoleucyl thiazolidide **3** (Figure 2) and its *allo* (2*S*,3*R*) stereoisomer were

licensed from Probiodrug in late 2000. Development of both compounds was discontinued in February 2001 due to unacceptable toxicity profiles in rats and dogs [21]. Available evidence suggested that the toxicity was not DPP-4 mediated, but more likely due to an off-target activity, in particular DPP-8, a closely related proline-specific protease. The *threo* and *allo* isomers had identical inhibitory activity against DPP-4, both *in vitro* and *in vivo*; however, although the toxicity profiles of these compounds are qualitatively similar in both rats and dogs, the *allo* isomer is significantly more toxic (approximately 10-fold) when compared on either a milligram per kilogram or plasma exposure basis. The toxicity profiles of these compounds include gastrointestinal toxicity characterized by bloody diarrhea and tenesmus in dogs, thrombocytopenia and anemia, and multiple organ pathology in both species.

In view of the comparable pharmacodynamic activity, the differences in the dose–response curves for the various toxic effects suggested that these toxicities were not mechanism-based. Evidence that they might be due to the inhibition of one or more proline-specific dipeptidyl peptidases was provided by studies with tissue extracts from DPP-4-deficient mice [21]. Detergent-solubilized extracts from the kidneys, liver, lung, and gastrointestinal tract of these animals were found to contain low levels of a Pro-specific dipeptidyl peptidase activity, detected using the fluorogenic substrate Gly-Pro-AMC. The Pro-selective dipeptidase activity in tissues isolated from *Dpp4*$^{-/-}$ mice was 10–25 fold lower than that measured in corresponding tissues of wild-type animals, and unlike DPP-4, was differentially inhibited by *threo*- and *allo*-isoleucyl thiazolidide ($IC_{50} = 726$ and 86 nM, respectively). The 8.5-fold greater potency of the *allo* diastereomer against this activity suggested that off-target inhibition of one or more DPP-4-like peptidases by this inhibitor could be responsible for preclinical toxicity.

In an effort to evaluate this hypothesis, the *allo* and *threo* isomers were screened for activity against a panel of related dipeptidases. A comparison of the inhibitory activities of the isomers revealed that although they had comparable activity against DPP-4, their activities against the related dipeptidase DPP-8 differed by about 10-fold. Activities against five other available related peptidases were similar. Since the differences against DPP-8 were consistent with the observed differences in dose necessary to produce toxicity, it was further hypothesized that inhibition of DPP-8 was responsible for the observed toxicities of these compounds. To evaluate this theory, a series of inhibitors with similar pharmacokinetic profiles in rats but with differing activities against DPP-4, DPP-8, and QPP (quiescent cell proline peptidase, aka DPP-II and DPP-7) were tested in exploratory 2-week rat oral toxicity studies. In addition, the potent and selective DPP-4 inhibitor desfluorositagliptin **27** (Figure 9) was also tested in parallel.

The results of these studies showed a remarkable similarity in the effects produced by the selective DPP-8 inhibitor and *allo*-isoleucyl thiazolidide [21]. Both compounds produced mortality and alopecia at the highest doses tested, and both also produced thrombocytopenia of similar magnitude at doses >30 mpk, and enlarged spleens and lymph nodes at all doses tested. The QPP selective inhibitor produced significant reductions in reticulocyte counts at the 100 mpk dose. No other changes were noted. In contrast to the above compounds,

the selective DPP-4 inhibitor did not produce any changes in physical appearance, body weight, hematology, clinical chemistry, and urinalysis, and histopathology was clean. Therefore, these results strongly support the hypothesis that the toxicities observed in rats with thiazolidide **3** and its *allo* (2*S*,3*R*) stereoisomer are not DPP-4 related but are consistent with inhibition of DPP-8.

This conclusion is also supported by studies in dogs. Acute oral and intravenous administration of both the *allo* and *threo* compounds resulted in bloody diarrhea, emesis, and tenesmus. These effects were reproduced in dogs treated with 10 mg/kg of the DPP-8 selective inhibitor; however, no acute effects were observed in dogs given desfluorositagliptin [21]. Since no toxicity was observed in dogs given single oral doses of the QPP selective inhibitor, these results also support the conclusion that the dog acute toxic effects are mediated by DPP-8.

During the course of these studies, another proline selective peptidase, DPP-9, closely related to DPP-8, was described [22]. The DPP-8 selective inhibitor was then found to be a dual DPP-8/9 inhibitor. Thus inhibition of one or both of these enzymes, or possibly another closely related enzyme, may be responsible for the observed toxicity. Efforts to identify potent and selective inhibitors of the individual enzymes in order to further deduce the mechanism of toxicity have thus far not succeeded.

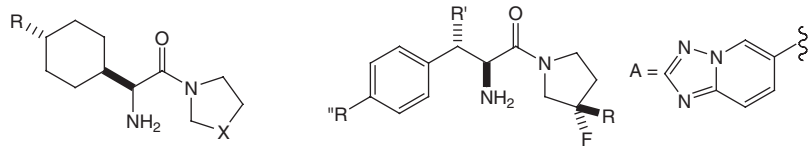
5. MEDICINAL CHEMISTRY EFFORTS LEADING TO SITAGLIPTIN

5.1 Program objectives

Following the completion of the studies described above, the objective of the internal program was to identify a potent DPP-4 inhibitor for development with >1000-fold selectivity over related proline peptidases, especially DPP-8 and DPP-9. A half-life suitable for once daily dosing was preferred, though a compound with twice daily dosing would still provide an advantage over DPP728, the only DPP-4 inhibitor known to be in development for diabetes at the time. To achieve a greater duration of action, we elected from the beginning to only consider structures lacking a reactive electrophile. Most of the known DPP-4 inhibitors contained such an electrophile, typically a nitrile, which forms a covalent (though reversible) bond with the alcohol of the active site serine [23]. Because these inhibitors also require a free amine five atoms away, the potential for intramolecular cyclization to form a six-membered ring exists. Chemical instability is seen with many of these inhibitors *in vitro* [23], and this may contribute to the short half-life often observed with these DPP-4 inhibitors *in vivo*.

5.2 α-Amino acid derived DPP-4 inhibitors

When the medicinal chemistry program began in late 1999, nearly all of the DPP-4 inhibitors known in the literature were derived from α-amino acids, and those lacking an electrophile such as the isoleucyl thiazolidides were considerably less potent than those containing a nitrile or boronic acid such as DPP728. One report suggested that cyclohexylglycine derived inhibitors showed improved potency



5 (R = H,X = S)
6 (R = 4-CF_3O-$PhSO_2NH$-, X = C[III]F)
7 (R = H,R' = Me,R" = 4-F-Ph)
8 (R = F,R' = $CONMe_2$,R" = A)

Figure 3 α-Amino acid derived DPP-4 inhibitors.

[24]. Indeed, cyclohexylglycyl thiazolidide **5** (Figure 3) has an IC_{50} of 89 nM in our assay. In order to develop a proprietary position in this series, substitutions on the cyclohexyl ring were explored [25,26]. In addition, SAR studies of the amine substituent indicated that the fluoropyrrolidine derivatives were nearly equipotent to the thiazolidine analogs [27]. Since the thiazolidine ring is prone to oxidation on sulfur, the former analogs are more metabolically stable. This work culminated in the identification of sulfonamide **6** (DPP-4 $IC_{50} = 36$ nM) which had good to excellent oral bioavailability and half-life of 4–12 h in preclinical species [27]. Once the toxicity of the isoleucyl thiazolidides was traced to probable inhibition of DPP-8 and/or DPP-9, development of this compound was halted due to its low micromolar affinity for these two enzymes. Efforts were then focused on two promising leads that emerged from screening.

Later, after the identification of sitagliptin, work in the α-amino acid series resumed. A new approach based on the *threo* isoleucyl thiazolidide lead gave a β-methyl phenylalanine series, typified by **7** (DPP-4 $IC_{50} = 64$ nM), with increased selectivity [28]. Substitution of the β-methyl group with a β-dimethylamido moiety provided increased selectivity against off-target activity, in particular binding to the hERG potassium channel [29]. The 4-heteroarylphenylalanine derivative **8** (DPP-4 $IC_{50} = 8.8$ nM) is among the most selective analogs made in this series, with >10,000-fold selectivity against other dipeptidyl peptidases, cytochrome P450 enzymes, and ion channels [30]. In addition, its pharmacokinetic profile was characterized by low clearance (1–5 mL/min/kg), good half-life (2–7 h), and excellent oral bioavailability (56–100%) across species. This compound was brought forward as an "insurance back-up", to be developed should sitagliptin have faltered.

5.3 High throughput screening hits

High throughput screening led to the identification of surprisingly few hits, and only two, β-aminoacyl amide **9** and piperazine **10** (Figure 4), were deemed worthy of extensive follow-up. Amide **9**, with a DPP-4 IC_{50} of 1.9 μM, was originally prepared in-house for the MRL thrombin inhibitor program. As such, amide **9** inhibited thrombin with an IC_{50} of 52 nM. SAR evident from screening and initial exploratory chemistry is shown in Figure 4. Both the benzyl and phenethyl analogs of **9** had DPP-4 inhibitory activity, and the (*R*) stereochemistry at the amino center was preferred. The second hit **10** (DPP-4 $IC_{50} = 11$ μM) was prepared as part of a proprietary screening library. A variety of groups was tolerated on the

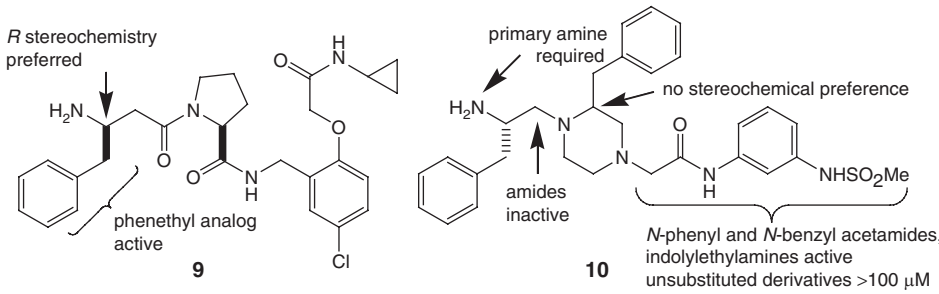


Figure 4 HTS hits β-aminoacyl amide **9** and piperazine **10**.

"right hand side" of the piperazine moiety, although unsubstituted derivatives were substantially less potent. The primary amine was strictly required, and conversion of the reduced phenylalanine to a phenylalanine amide led to complete loss of potency. Both of these hits were developed simultaneously, eventually merging into one lead series.

5.4 SAR in the β-aminoacyl amide series

Replacement of the β-aminoacyl moiety with an α-aminoacid derivative such as isoleucyl or cyclohexylglycyl led to a 2- to 4-fold decrease in potency. This was the first indication that SAR between this series and the α-amino acid series was distinct. Early on it was discovered that the "right hand side" amide could be replaced with an ester or acid moiety. This result led to a more systematic exploration of acid substitutions. *Ortho*-, *meta*- and *para*-substituted phenylacetic acid derivatives were prepared, and the latter analog (**11**, Figure 5) proved to be the first submicromolar inhibitor prepared in this series ($IC_{50} = 510$ nM). Gratifyingly, **11** was devoid of thrombin inhibitory activity [31].

Because the entire proline amide moiety could be replaced with a thiazolidine without significant loss of activity, much of the initial SAR studies on the β-aminoacyl portion of the molecule was done in the thiazolidide series [32]. Shortening or lengthening the distance between the primary amine and the phenyl ring led to decreased activity. Replacing the phenyl group with a heterocycle or saturated ring led to derivatives that were also much less potent. Substitutions on the phenyl ring were somewhat tolerated. The 2-fluorophenyl analog (**12**) had an IC_{50} of 931 nM, and the addition of a second and third fluorine led to further increases in potency. In particular, the 2,5-difluorophenyl and 2,4,5-trifluorophenyl analogs **13** and **14** inhibited DPP-4 with IC_{50}s of 270 and 119 nM, respectively.

Substitution with a 2-fluoro group in the fully elaborated phenylacetic acid series gave analog **15** ($IC_{50} = 54$ nM), with an even more dramatic boost in potency [31]. The acetic acid was readily replaced with (2*S*)-lactic acid to provide inhibitor **16** which had an IC_{50} of 12 nM (Figure 6). More lipophilic groups at the carbon α to the acid are preferred, with the (*S*)-isopropyl substitution optimal. SAR of 2,5-difluoro analog **17**, a subnanomolar DPP-4 inhibitor, is summarized in Figure 6.

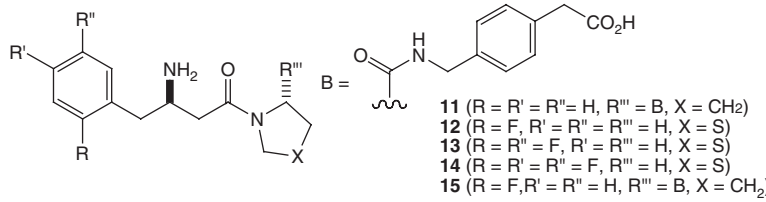


Figure 5 Analogs of β-aminoacyl amide hit **9**.

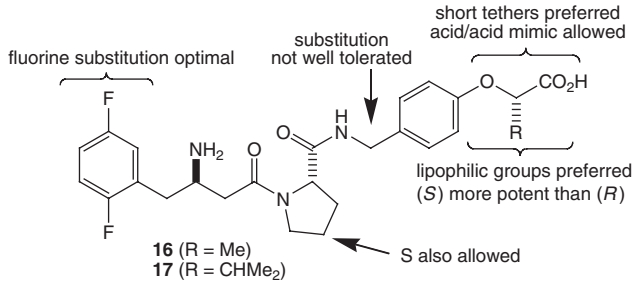


Figure 6 SAR of β-aminoacyl amide lead.

Aminoacyl lead **17** had several desirable properties. It was exquisitely potent (DPP-4 $IC_{50} = 0.48$ nM) and highly selective (IC_{50}s >100 μM at QPP, DPP-8 and DPP-9). In addition, in an increasingly competitive area of research, it represented a unique structural class. Unfortunately, oral bioavailability in rats of this compound and many others in this series was very low (~1% for **17**) due primarily to poor absorption, and clearance after IV administration was generally high (150 mL/min/kg for **17**). This lead was not progressed further, but rather SAR from this series was eventually incorporated into screening hit **10** to provide a hybrid lead series (*vide infra*).

5.5 SAR in the piperazine series

Initial SAR derived from screening suggested that substitution on the terminal nitrogen of the piperazine was tolerated; however, extensive systematic exploration of SAR in this region of the molecule did not lead to substantial increases in potency. Recognizing that both leads shared a common phenethylamine pharmacophore, substitution of the phenyl ring by fluorine, which led to increases in potency of the amide hit, was examined. In this series as well, fluorine substitution gave compounds with increased potency suggesting that these moieties bound in the same site of the enzyme. With this information in hand, a major breakthrough was achieved by replacing the reduced phenylalanine "left hand side" with the corresponding homophenylalanine, leading to a 100-fold increase in potency [33].

In this new β-aminoacyl amide series, the (*R*) benzyl isomer is ~50-fold more potent than the (*S*) isomer, and 2-fluoro substitution on the phenyl ring provides inhibitor **18** which had an IC_{50} of 14 nM (Figure 7). Truncation of the right-hand side to provide monosubstituted piperazine **19** ($IC_{50} = 140$ nM) led to only a 10-fold loss in potency. Morpholine analog **20** had similar potency, though the piperidine amide was much less active ($IC_{50} = 1040$ nM). Addition of fluorines to the phenyl ring gave a further boost in activity, with the 2,4,5-trifluoro derivative (**21**, $IC_{50} = 19$ nM) being the most potent analog in this series.

Despite the low molecular weight of many of these derivatives oral bioavailability in rats was low and variable. This was traced in part to extensive metabolism, in particular, on the piperazine ring. In order to stabilize this ring to oxidation, bicyclic derivatives were prepared [34,35]. A wide variety of heterobicyclic amides was evaluated. The initial compounds in this series included imidazolopiperazine **23** ($IC_{50} = 640$ nM) and triazolopiperazine **24** ($IC_{50} = 460$ nM). A comparison of these bicyclic analogs with the unsubstituted piperazine **22** ($IC_{50} = 3100$ nM) showed that cyclization led to an increase in potency. Initial work was done in the 3,4-difluorophenyl series as shown in Figure 8; the requisite β-amino acid starting material is commercially available, and much of the SAR of fluorine substitution was developed in parallel.

Substitution of hydrogen for ethyl in the triazolopiperazine lead gave analog **25** ($IC_{50} = 230$ nM), which was extensively profiled. This analog had weak activity at both DPP-8 and DPP-9 (IC_{50}s = 45 and 100 μM, respectively). It was very stable in incubations with rat hepatocytes suggesting that cyclization did indeed stabilize the molecule toward oxidative metabolism; however, oral bioavailability remained low (2%) in rats [34]. In dogs, oral bioavailability of **25**

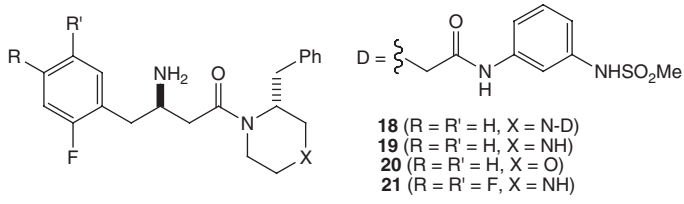


Figure 7 Analogs of piperazine hit **10**.

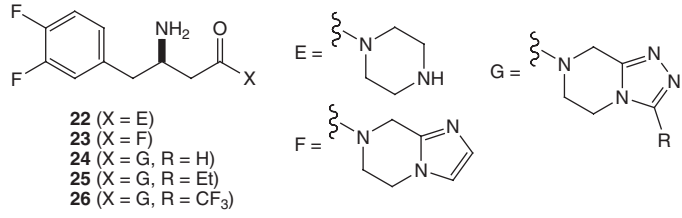
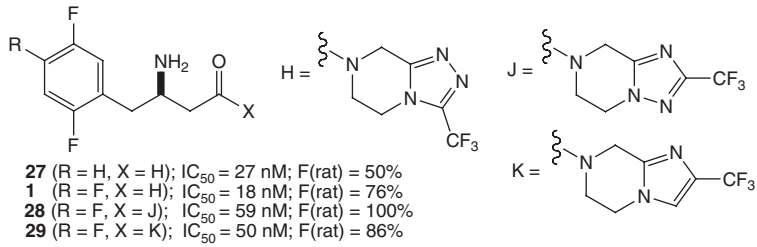


Figure 8 Bicycle piperazine analogs.



27 (R = H, X = H); IC_{50} = 27 nM; F(rat) = 50%
1 (R = F, X = H); IC_{50} = 18 nM; F(rat) = 76%
28 (R = F, X = J); IC_{50} = 59 nM; F(rat) = 100%
29 (R = F, X = K); IC_{50} = 50 nM; F(rat) = 86%

Figure 9 Trifluoromethyl substituted bicyclic DPP-4 inhibitors.

improved to 33% so a third species, rhesus monkeys, was studied. In monkeys, like rats, oral bioavailability was low. Low, variable absorption appeared to be the cause of the poor pharmacokinetics in rats based on results from *in vitro* permeability, intestinal loop, and portal vein cannulated rat studies. At the same time, hepatic extraction in rats was low (10–20%), suggesting that increased stability in rat hepatocytes translated to decreased metabolism *in vivo*.

Given these results, further exploration of the bicyclic series continued. Replacement of the ethyl side-chain with a methyl group did not provide an improvement in either potency or oral exposure; however, somewhat surprisingly, trifluoromethyl analog **26** ($IC_{50} = 130\,nM$) showed much improved oral bioavailability in rats (44%). Inhibitory activity was improved by adjusting the fluorine substitution on the phenyl ring. Both 2,5-difluoro analog **27** and 2,4,5-trifluoro analog **1** (sitagliptin) showed increased potency (IC_{50}s of 27 and 18 nM, respectively) while maintaining the weak DPP-8/9 affinity ($>45\,\mu M$) inherent to this series. In addition, both compounds had good oral bioavailability in rats (50% and 76%, respectively). Interestingly, a variety of trifluoromethyl-substituted heterocycles had similarly good pharmacokinetic properties [35]. These included the isomeric triazolopiperazine **28** and the imidazolopiperazine **29** (Figure 9); however, neither of these compounds was superior to **27** or **1** in terms of potency.

6. PROPERTIES OF ANALOG 27 AND SITAGLIPTIN

Because SAR on the phenyl ring and the amide substituent in this series developed simultaneously, difluoro derivative **27** was prepared several weeks before trifluoro analog **1**, and at the time analog **1** was first prepared, extensive profiling of **27** was well underway. Difluoro analog **27** is highly selective for DPP-4 over not only DPP-8 ($IC_{50} = 69\,\mu M$) and DPP-9 ($IC_{50} > 100\,\mu M$), but over QPP, FAP, PEP, APP, and prolidase as well (IC_{50}s $> 100\,\mu M$) [34]. It is clean against a variety of CYP450 enzymes (IC_{50}s $> 50\,\mu M$) and ion channels (IC_{50}s $> 35\,\mu M$). Oral bioavailability is high across preclinical species (51–95%). In mice, **27** induced dose-dependent decreases in glucose excursion accompanied by increases in plasma DPP-4 inhibition and active GLP-1 levels. Maximal efficacy was achieved at 3 mg/kg, corresponding to plasma levels of 700 nM, consistent with the decreased potency seen in the mouse enzyme ($IC_{50} = 100\,nM$). In a chronic efficacy

study in high fat diet, streptozotocin-treated (HFD-STZ) diabetic mice, treatment with **27** induced a dose-dependent decrease in glucose levels as assessed by glycosylated hemoglobin HbA_{1c} [36]. Histological examination of islets from these animals indicated that desfluorositagliptin treatment normalized β-cell mass, suggesting that DPP-4 inhibition may lead to improved β-cell function and ultimately alter the course of the disease.

Desfluorositagliptin was clean in a 2-week exploratory safety study in rats and in an acute dog tolerability study, as described above. This compound did show some effects in the anesthetized, vagotomized cardiovascular (CV) dog model [37] including decreased blood pressure and heart rate and increased PR interval at 10 mpk IV, where plasma levels reached 46 μM. The NOEL was 1 mg/kg or 6.5 μM. Because it was anticipated that the trifluoro analog, with similar ion channel binding, would have similar effects in the CV dog model, and because **27**, but not as yet **1**, had successfully completed a 2-week safety study, **27** was brought forward and approved as a preclinical candidate (PCC) for development.

Meanwhile, work up of **1** continued. This compound was also highly selective in the proline peptidase panel of assays (48 μM at DPP-8 and >100 μM at all others) and had similar off-target activity to **27**. Oral bioavailability of **1** was comparable to or better than that of **27** (61–100%), and it displayed a similar profile in *in vivo* efficacy models [34]. Surprisingly, **1** was much cleaner in the CV dog with a NOEL of 10 mg/kg, corresponding to maximal plasma levels of 59 μM. Given that this difference was not insignificant and could potentially translate into a cleaner profile in the clinic, **1** was quickly scaled up and submitted for an exploratory rat safety study. When results from this study showed a clean profile, **1** was approved for development only 2 months after **27**, and positioned as the lead development candidate. Preclinical development of both compounds proceeded in parallel, but only compound **1**, which came to be known as MK-0431 and later sitagliptin, was taken forward into the clinic.

7. CLINICAL STUDIES OF SITAGLIPTIN

In clinical studies sitagliptin showed a dose-dependent increase in plasma DPP-4 inhibition with greater than 80% inhibition for 24 h achieved at doses ≥ 100 mg [38]. This degree of inhibition correlated with maximal efficacy in preclinical models. Once daily dosing is supported by a half-life of 8–14 h. Using ^{14}C-labeled compound oral bioavailability was found to be 87% [39].

In patients with type 2 diabetes, administration of sitagliptin provided a decrease in glucose excursion following an oral glucose challenge that was accompanied by increases in active GLP-1 and GIP, insulin, and C-peptide levels and a decrease in glucagon levels [40]. In Phase III studies, as monotherapy, a 100-mg once daily dose in patients (baseline HbA_{1c} of 8.0%) gave a 0.6–0.8% decrease in HbA_{1c} vs. placebo following 24 weeks of treatment [41]. In patients with higher baseline HbA_{1c} values ($\geq 9\%$), greater reductions were achieved (–1.52%). Fasting plasma glucose and postprandial glucose levels were also significantly reduced. An increase in both HOMA-β, a measure of the pancreatic β cell's ability to secrete insulin in the fasting state, and the proinsulin/insulin ratio was consistent

with an improvement in β-cell function. Sitagliptin was well-tolerated with a very low incidence of hypoglycemia, and, unlike many oral anti-hyperglycemic agents, was weight neutral.

In addition to demonstration of efficacy in monotherapy trials, sitagliptin provided improved glycemic control when used in combination with pioglitazone [42] or metformin [43]. In a 52-week non-inferiority study, patients taking metformin ($\geq$1500 mg/day) were treated with either sitagliptin (100 mg) or glipizide (5 mg titrated to a maximum dose of 20 mg daily), a sulfonylurea [44]. Both treatments resulted in an overall HbA_{1c} decrease of 0.67%, demonstrating non-inferiority. A similar percentage of patients taking sitagliptin achieved HbA_{1c} levels of $<$7% (63% for the sitagliptin group and 59% for the glipizide group). While glucose lowering efficacy was similar, a much larger proportion of patients taking glipizide experienced hypoglycemic events (32% vs. 4.9% for those taking sitagliptin). In addition, sitagliptin-treated patients lost an average of 1.5 kg of body mass, while those taking glipizide gained 1.1 kg.

8. CONCLUSION

Simultaneous optimization of two screening hits led to a novel series of β-amino acyl amides. Further refinement of the resultant lead gave sitagliptin, a potent and highly selective DPP-4 inhibitor. Selective inhibition of DPP-4, in particular with respect to DPP-8 and/or DPP-9, provided an improved safety profile in preclinical species; and sitagliptin has been very well tolerated in pre-clinical toxicity studies and in human clinical trials. In patients with type 2 diabetes, a 100 mg once daily dose stabilizes active GLP-1 and GIP, reduces glucose excursion, enhances insulin levels, suppresses glucagon levels, and improves glycemic control. JANUVIA^{TM} (sitagliptin) was approved in October 2006 by the FDA as the first DPP-4 inhibitor for the treatment of type 2 diabetes, providing a novel, safe and effective option for patients with this disease.

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CHAPTER 8

Cathepsin K Inhibitors

W. Cameron Black and **M. David Percival**

Contents

1. INTRODUCTION

Cathepsin K (Cat K) is a member of the CA1 family of lysosomal cysteine proteases. This family is comprised of 11 human members (cathepsins B, C, F, H, K, L, O, S, V, W, Z) which share a common papain-like structural fold and a conserved active site Cys-Asn-His triad of residues [1–3]. These enzymes are synthesized as pre-pro-enzymes and are converted from the catalytically inactive zymogen into the active form in acidic lysosomal environment. In some cases, cathepsins are also secreted in the active form from cells. The sequence identity of

Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada H9H 3L1

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the mature enzymes ranges between 20% (cathepsin B) and 56% (Cathepsin S) versus Cat K. No new family members have been identified in the years since the mid to late 1990s, when six (including Cat K) were discovered through molecular biology screening [4].

Cat K was initially distinguished for its high and selective expression in the osteoclast, the hematopoietic-derived cells responsible for bone resorption. Bone is comprised of ~60% calcium hydroxyapatite and ~40% protein, the majority of the latter being comprised of type I collagen. The destruction of both components is required for the normal turnover of bone, the resorbed surface being subsequently replaced by new bone to maintain structural integrity. Under pathological conditions, such as in osteoporosis, bone resorption outpaces bone formation, leading to a loss of bone strength and an increased fracture risk. As was predicted on the basis of its high expression in osteoclasts, there is much evidence that Cat K plays a key role in bone resorption. Cat K is unique in its ability to cleave native collagen in both the proteolytically resistant helical and non-helical regions [5]. The autosomal recessive genetic disease pycnodysostosis is caused by Cat K gene mutations which result in the production of catalytically inactive enzyme [6]. These patients suffer from a pathologically increased bone mass and have a similar phenotype to that of the Cat K deficient mouse. In contrast, Cat K overexpressing transgenic mice display decreased bone mass and an increased bone turnover rate. The pharmacological inhibition of Cat K in both *in vitro* osteoclast-based bone resorption assays, as well as in animals and humans results in a reduction in bone resorption and an increase in bone mass, consistent with the premise that Cat K is a promising therapeutic target for the treatment of diseases involving inappropriately rapid bone turnover, such as osteoporosis, Paget's disease or metastatic bone disease.

The development of Cat K inhibitors, with an emphasis on the fundamental biology, pharmacology and human clinical trials has been recently reviewed [1–3]. The medicinal chemistry of Cat K inhibitors has also been the subject of recent reviews [7,8]. This article reviews recent publications and meeting abstracts on the design of Cat K inhibitors, as well as developments in Cat K-related biology including animal models for the prediction of inhibitor efficacy, the potential for the separation of bone resorption and formation by Cat K inhibitors, inhibitor selectivity considerations and other potential indications for Cat K inhibitors.

2. NEW BIOLOGY

2.1 New animal models

The standard model for the preclinical development of anti-osteoporosis therapies is the ovariectomized (OVX) rat. However, Cat K inhibitors developed specifically against the human enzyme are generally significantly less potent (~2-orders of magnitude) against the rat and mouse enzymes than against human Cat K [9]. This loss of potency towards the rodent enzymes, which is consistent with their low sequence homology, therefore restricts the use of

pharmacological inhibitors in rodent osteoporosis models. In some cases, rat models have found utility using inhibitors of picomolar potency against human Cat K, which were still relatively potent nanomolar inhibitors of the rat enzyme [10]. Nevertheless, selectivities versus off-target rat cathepsins are rarely presented.

The majority of published pharmacological studies of Cat K inhibitors have employed OVX or a gonadotropin-releasing hormone agonist to induce bone turnover in rhesus or cynomolgus monkeys [11–13]. These two species have identical mature Cat K amino acid sequences to that of human [14,15]. Most recently the rabbit has been identified as a suitable model for testing the anti-resorptive activities of Cat K inhibitors. The mature form of rabbit Cat K shares 96% sequence identity and 99% similarity with the mature human enzyme, with only two active site amino acid differences (Tyr/Asp^{61}, Val/Leu^{157}, respectively). In general, inhibitors of human Cat K suffer only a small loss of potency towards the rabbit enzyme [16]. The ''rabbit Schenk'' assay was patterned after the rat model of the same name [17]. This rabbit model measures the inhibition of the normal, ongoing bone resorption in rapidly growing young animals at both the periosteum and the distal metaphysis of the long bones. In this model, 7-week old female rabbits were treated with doses of 1–30 mg/kg of a number of aminoacetonitrile-containing Cat K inhibitors, or alendronate (ALN) for 10 days. Bone mineral density (BMD) analysis of the distal 3 cm of the long bone showed that Cat K inhibitors dose-dependently increased BMD and that the maximal increases were equivalent to that provided by ALN (14–22% compared to vehicle). The ''rabbit Schenk'' model can therefore serve as a rapid and cost-effective model to prescreen Cat K inhibitors prior to testing in non-human primates [18].

A more long-term rabbit model of estrogen deficiency has also been recently described in which adult OVX rabbits (7 months) were treated with the Cat K inhibitor L-006235 (**12**) at 0, 2, 10 mg/kg, or ALN (0.125 mg/kg, 3x/wk) for 27 weeks. OVX resulted in an 11.5% vertebral bone loss compared to sham-operated controls. Both the high dose of L-006235 and ALN completely prevented this bone loss, whereas the low dose of L-006235 produced a partial response [19].

A non-invasive method of monitoring osteoclast activity has been recently published that employs a Cat K-activated near-infrared optical substrate [20]. The long excitation and emission wavelengths of the activated fluorophore (~700 nm) allow tissue penetration thereby permitting the visualization of Cat K activity *in vivo*, as well as *in vitro*. OVX, sham operated mice and OVX with pamidronate (0.5 mg/kg) were treated with the probe (i.v.) 7 days following surgery. On day 8, the mice were imaged by 3-D fluorescence molecular tomography showing that Cat K activity in the proximal tibia was increased 38% over sham animals, and that this increase was completely blocked with pamidronate. This optical, non-invasive technique shows promise as a means to visualize and quantify Cat K and osteoclast activity during changes in bone metabolism. However, unless a humanized mouse or rabbit can be used, the utility of this technique to follow the activity of human Cat K inhibitors will be restricted.

2.2 Separation of bone resorption/formation

Under normal conditions, bone resorption and formation are tightly coupled events. Although bisphosphonates, calcitonin, SERMs and estrogen reduce bone resorption clinically, after months of treatment the rate of bone formation is also suppressed, thereby reducing the long-term therapeutic effect. Recent evidence suggests that Cat K inhibitors may have less of a negative effect on bone formation, thereby promising an additional anabolic effect for these agents. Histomorphometric analysis of cancellous regions of femoral bones from Cat K-deficient mice showed an increased bone formation rate versus that in wild-type littermates. An intermediate effect was observed for the female heterozygotes [21]. In the OVX rabbit model in which treatment with L-006235 and ALN for 27 weeks completely blocked the bone loss observed in vehicle-treated animals, ALN reduced the bone formation rate for both cancellous and endocortical bone. In contrast, no reduction in bone formation rate at either site was observed for the L-006235 group [19]. Treatment of OVX cynomolgus monkeys for 18 months with balicatib (AAE581, **13**) blocked the loss of vertebral and femoral bone mineral density (BMD) associated with OVX. Significantly, femoral BMD in the balicatib-treated animals was greater than that of sham-operated, vehicle-treated animals. Bone formation, as assessed by mineral apposition rate, was significantly increased by balicatib treatment compared to both OVX and sham-operated animals at the periosteal side of the femoral neck. In contrast, bone formation was reduced in cancellous bone of the femoral neck [22]. Under a similar paradigm with OVX cynomolgus monkeys, relacatib (SB-462795, **1**) treatment at three doses for 9 months dose-dependently reduced urinary CTx, a bone resorption marker, to a similar extent to ALN. The bone formation marker osteocalcin was increased at the mid and low doses of relacatib, but was reduced by ALN. Histomorphometric analysis showed a reduction in both bone formation and resorption at cancellous bone sites for relacatib and ALN. However, periosteal bone formation was increased by relacatib, but not ALN [23]. The findings of these monkey studies suggest that Cat K inhibitors may suppress both bone resorption and bone formation in cancellous bone, but may stimulate periosteal cortical bone deposition. Similar observations on bone formation markers have also been made clinically and will be discussed in a later section.

The mechanism of this effect was addressed in a study in which 2–5 days treatment of female cynomolgus monkeys with relacatib transiently caused a 2–3-fold increase in plasma levels of parathyroid hormone (PTH, a bone anabolic agent) post-dosing compared to vehicle-treated animals [24].

2.3 Other indications

The evidence for the utility of Cat K inhibition in the treatment of osteoporosis is compelling, but there is mounting evidence that this enzyme may also play a role in other pathologies. Although Cat K is highly expressed in osteoclasts, more recently its expression has been documented in a number of other tissues including, but not limited to, cartilage [25], atherosclerotic plaques [26], adipose

tissue [27,28], lung [29], skin [30] and brain [31]. However, levels in these tissues are generally orders of magnitude lower than that in osteoclasts.

A Cat K inhibitor may be beneficial for the treatment of osteoarthritis (OA) via either of the following two mechanisms [25]. Firstly, a case has been made that OA pathology can be triggered by inappropriate subchondral bone turnover. Increased bone resorption and microfracture could increase the stiffness of subchondral bone, transmitting increased stress to the cartilage that increases cartilage degradation [32]. Furthermore, the formation of osteophytes, or bone spurs, in the joints of OA patients is probably related to increased endochondral bone formation. Secondly, Cat K is powerful collagenolytic enzyme and may play a direct role, along with metalloproteinases, in the degradation of articular cartilage. Cat K expression has been shown to be increased in cartilage samples from human OA patients as well as in synovial tissue and articular chondrocytes [25]. Despite this evidence, the ability of Cat K inhibitors to prevent the progression of OA in preclinical models has not yet been reported.

Metastatic bone disease (MBD) is characterized by very high levels of bone turnover in regions proximal to the tumour [33]. Bone resorption inhibitors such as bisphosphonates represent the current standard of care for the treatment of bone metastases primarily due to breast or prostate cancer and multiple myeloma. It has been proposed that other strong anti-resorptives such as a Cat K inhibitor could be useful in the treatment of bone metastases. Evidence for this has been presented in the form of a preclinical MBD model in which human breast cancer cells are implanted into nude mice. Treatment with a Cat K inhibitor gave a significantly lower area of breast cancer-mediated osteolytic lesions in the tibia [34]. In a separate study, the efficacy of a Cat K inhibitor in the reduction in tumour-induced osteolysis was found to be enhanced in the presence of the bisphosphonate zolendronic acid [35,36]. When prostate cancer cells were injected into the tibia of SCID mice, treatment with a Cat K inhibitor both prevented and diminished the progression of cancer growth in bone [37].

Cat K inhibitor therapy may also result in protection against the development of atherosclerosis. Cat K-deficient mice show reduced atherosclerotic lesion number and size on an ApoE receptor-deficient background, compared to wild-type animals [38,39]. Cat K is also associated with increased adiposity in humans [27,28] and may also play a role as a kininase, suggesting a role in blood pressure regulation [40]. Cat K has also been postulated to play a role in the pathology of rheumatoid arthritis [41,42].

2.4 Selectivity considerations

A number of physiological and pathological roles for the off-target cysteine cathepsins have been identified [2,3]. Although some off-target activities may augment the potencies of Cat K inhibitors for the treatment of diseases such as cancer and rheumatoid arthritis, a high degree of Cat K selectivity is probably optimal to minimize the potential for adverse side effects during the long-term treatment of chronic diseases such as osteoporosis.

Several Cat K inhibitor series contain a basic nitrogen in the P3 position which interacts with an Asp residue in the S3 pocket of Cat K resulting in increased potency and selectivity. As a consequence of their basic, lipophilic nature, the Cat K inhibitors L-006235 and balicatib exhibit lysosomotropic properties [16,43]. This results in their concentration in acidic lysosomes within cells, where both Cat K and the off-target cathepsins are located. Enzyme occupancy assays of Cat K, B, L and S activity in whole cells (utilizing a non-selective activity-based probe) show that the activities of these basic compounds against these lysosomal enzymes are increased compared to non-basic analogues. This increased activity of basic Cat K inhibitors is maintained in a functional cell-based Cat S assay and was also observed in tissues using an enzyme occupancy-type *in vivo* assay against cathepsins B, L and S. Interestingly, the efficacies of the basic L-006235 and non-basic L-873724 (**14**), which have similar intrinsic Cat K potencies and exposures, were similar in both the ''rabbit Schenk'' and rhesus OVX models of osteoporosis, suggesting that lysosomotropism does not lead to enhanced Cat K inhibition. Thus, a basic Cat K inhibitor does not appear to afford any advantage over a non-basic inhibitor with the same intrinsic potency and selectivity, but may result in increased off-target activity and an increased potential for associated side effects [16,43].

3. RECENT ADVANCES IN INHIBITOR DESIGN

The most convenient way of categorizing the classes of cathepsin inhibitors is based on the nature of the electrophilic warhead that interacts with the sulfhydryl group of the active site cysteine residue. Since a large portion of the binding energy of a cysteine protease inhibitor comes from the covalent interaction with this thiol, the properties of the resulting molecules are largely derived from the electrophile. In broad terms, these inhibitors can be broken down into ketone and nitrile-based reversible covalent inhibitors, or the more recent non-covalent inhibitors based on an aminoaniline template.

3.1 Ketone-based inhibitors

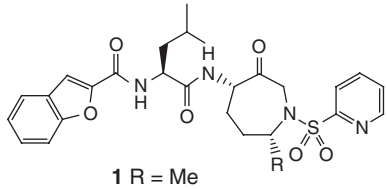
The majority of the research published in the past 2 years has centered on the use of an electrophilic ketone. Ketone warheads provide the advantage of accessing the prime side of the enzyme active site via the ketone substituent. Additionally, the electrophilicity of the ketone can be readily controlled by the substituents on the ketone. While an unactivated ketone generally does not have sufficient electrophilicity for optimal interaction with the active site cysteine, the introduction of an electron-withdrawing substituent (α-heteroatom, carbonyl or heterocycle) can generate potent inhibitors. Challenges include epimerization of chiral centers in the P1 position and high molecular weight associated with prime side substituents.

3.1.1 Azepanones

Cyclic ketones of various ring sizes have been described. Seven-membered cyclic α-amino ketones – azepanones – have been reported to give extremely potent inhibitors of Cat K. The larger ring size has the advantage of slowing the epimerization at the α-amido substituent [9]. The addition of a methyl group at the 7-position [44] provides relacatib with a 4-fold increase in potency over the corresponding des-methyl derivative SB-357114 (**2**). This C-7-methyl group interacts with the S1′ site of the enzyme, while the sulfonylpyridine binds in S2′. The methyl group also enhances the configurational stability at C-4. While the C-4 stereocenter rapidly equilibrates in pH 11 buffer, the desired 4-(*S*) isomer is thermodynamically favoured by a 9:1 ratio over the less active 4-(*R*) isomer. Relacatib has a K_i of 0.04 nM against Cat K. This molecule is effectively a pan-cathepsin inhibitor with little selectivity over other cathepsins (Cat L $K_i = 0.07$ nM; Cat V $K_i = 0.06$ nM; Cat S $K_i = 1.6$ nM; Cat B $K_i = 13$ nM). In a functional bone resorption assay using human osteoclasts, the potency of relacatib is shifted considerably ($IC_{50} = 22$ nM). Relacatib is orally bioavailable in the rat (F = 89%) and the monkey (F = 28%), with half-lives of 109 and 168 min, respectively. The ability of relacatib to suppress biochemical markers of bone resorption was evaluated in ovariectomized cynomolgus monkeys. A 54% reduction in serum NTx relative to baseline was observed 4–8 h after oral administration of relacatib at 10 mg/kg [13]. Significant reduction in NTx was observed within 1.5 h of dosing, a rapidity of response unprecedented with other antiresorptive mechanisms. Inhibition of NTx was observed at timepoints when circulating drug could no longer be detected, but returned to baseline within 48 h. A 9-month study of relacatib (10 mg/kg PO) in cynomolgus monkeys showed a 42 and 73% protection of ovariectomy-induced bone loss in the lumbar spine and distal femur respectively. This differed from the alendronate control which provided 100 and 23% protection at these respective bone sites [45] suggesting that this mechanism may provide a different efficacy profile compared to traditional anti-resorptives.

3.1.2 Cyclohexanones

Analogous six-membered cyclic ketone inhibitors have also been described. Among these, OST-4077 (**3**), which is a mixture of diastereomers at C-4, has an IC_{50} of 11 nM against Cat K and is selective over other cathepsins (Cat B $IC_{50} = 239$ nM; Cat L $IC_{50} = 981$ nM; Cat S $IC_{50} = 196$ nM) [46]. It is noteworthy that in this study SB-357114 was used as a control, and was found to be 20x less potent on Cat K ($IC_{50} = 3.5$ nM) than has been reported previously ($IC_{50} = 0.16$ nM), making it difficult to compare potencies between publications. Although OST-4077 has an IC_{50} of 427 nM against rat cathepsin K, the compound prevents bone resorption in the ovariectomized rat, as shown by inhibition of urinary DPD and significantly increased bone mineral density when administered orally at 100 mg/kg BID for 4 weeks.



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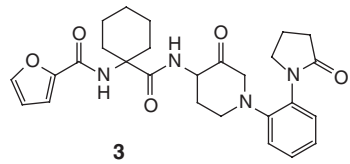
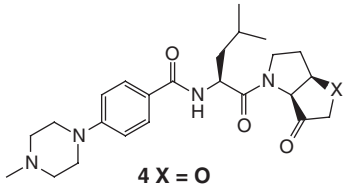
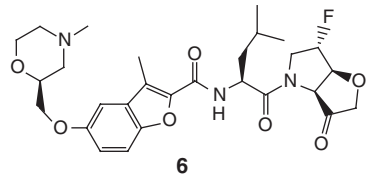
2 R = H



3



4 X = O
5 X = NBz



6

3.1.3 Dihydrofuranones

Five-membered cyclic ketones, particularly those with a ring oxygen atom in the α-position, have been previously reported to be configurationally unstable at the α-amido substituent [9]. By introducing a *cis*-fused bicyclic ring system, configurational stability was achieved due to the conformational restraint provided by the fused ring system. Compound **4** has a K_i of 9 nM against Cat K with >1000-fold selectivity over Cat L and S in enzyme assays [47]. This K_i represents a 4-fold increase in potency over the corresponding monocyclic ketone derivative, a somewhat surprising observation since **4** lacks the NH of the P2 amide bond which is known to interact with the enzyme active site. However, the ability of this structure to bind in a bioactive conformation appears to more than compensate for the loss of this hydrogen bond. The oxygen of the dihydrofuranone ring of **4** can be replaced by an *N*-benzoate, giving compound **5** with increased potency (Cat K $K_i = 5$ nM) and enhanced chemical stability, but with only 44-fold selectivity over Cat L (Cat B $K_i > 4000$ nM; Cat L $K_i = 221$ nM; Cat S $K_i = 1242$ nM) [48]. This change in properties could be due to binding in the prime side of the active site, but likely also reflects differences in ketone electrophilicity imparted by this β-substituent. The addition of a 7-*(S)*-fluorine atom on the pyrrolidine ring of this bicyclic system has also been reported to enhance Cat K potency [49]. The optimized structure **6** inhibits Cat K with a K_i of 2 nM and exhibits >250-fold selectivity over Cat L and S in enzyme assays. No data on Cat B inhibition was provided.

3.1.4 Ketoamides

The α-ketoamide group is a well-known electrophile for the inhibition of both serine and cysteine proteases. Introducing a pyrazole as the N-substituent of the ketoamide provides potent inhibitors of Cat K [50]. SAR exploration of the P2-P3 residues in a series of Cat K inhibitors led to the identification of the pyrrolidine

derivative **7**, which has an IC_{50} of 0.4 nM against Cat K, but has low selectivity over Cat S and Cat V (Cat B $IC_{50} > 250$ nM; Cat L $IC_{50} > 250$ nM; Cat S $IC_{50} = 2.8$ nM; Cat V $IC_{50} = 15$ nM) in recombinant enzyme assays [51]. Compound **7** has an oral bioavailability in rats of 26% with a half-life of 75 min. In this molecule, the gem dimethyl substituents of the pyrrolidine fill the S2 pocket, but the aromatic ring does not interact with the S3 binding site, extending into solvent instead.

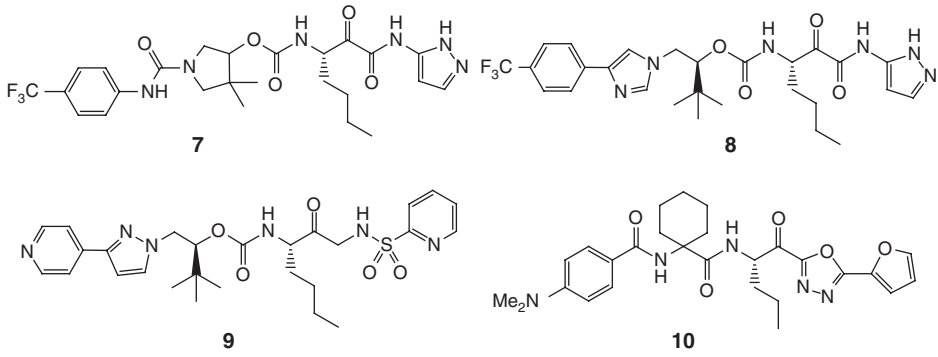
An alternate P2-P3 template has been disclosed wherein a *t*-butyl group fills S2 and an aryl ring attached to a five-membered heterocycle interacts with the S3 subsite giving highly potent compounds. A preferred example is the trifluoromethylphenyl-substituted imidazole **8**, which has an IC_{50} of 0.026 nM against Cat K and is orally bioavailable in rats (F = 55%, $t_{1/2} = 7.6$ h) [10]. Despite this high potency in the Cat K enzyme assay, the potency of this inhibitor was shifted over 20,000-fold in a functional bone resorption assay in human osteoclasts to give an IC_{50} of 570 nM. An additional liability of this compound is the poor selectivity over off-target cathepsins (Cat B $IC_{50} = 27$ nM; Cat L $IC_{50} = 10$ nM; Cat S $IC_{50} = 0.16$ nM; Cat V $IC_{50} = 0.13$ nM).

3.1.5 Sulfonamidoketones

A series of acyclic ketone-based inhibitors activated by a α-sulfonamido group was originally reported in 1997 [52]. While this ketone is less electrophilic and therefore may afford less potent inhibitors than the more electrophilic ketoamide inhibitors, it was thought that the lower reactivity of ketone-based inhibitors might lead to better tolerability in chronic therapy. By combining the α-sulfonamidoketone with the P2-P3 template illustrated by compound **8**, a new series of potent and selective inhibitors has been identified [53]. Representative compound **9** has an IC_{50} of 3.8 nM against Cat K, but has low selectivity over off-target cathepsins (Cat B $IC_{50} = 13$ nM; Cat L $IC_{50} = 38$ nM; Cat S $IC_{50} = 11$ nM). However **9** has excellent pharmacokinetics in the rat, with an oral bioavailability of 100% and a half-life of 2 h. In this series, the nature of the five-membered heterocycle appears to have little impact on potency, and it is proposed based on modelling efforts that this ring serves mainly to orient the pyridyl ring in S3 and has little interaction with the enzyme itself.

3.1.6 Ketooxadiazoles

A series of inhibitors containing an electrophilic keto-1,3,4-oxadiazole moiety as the warhead has been reported in which the substituent at the 5-position was varied resulting in the identification of furan as the optimal prime side substituent. Exploration of P3 substituents led to the identification of **10** with a K_i of 1 nM against Cat K with >700-fold selectivity over off-target cathepsins (Cat B $K_i = 730$ nM; Cat L $K_i = 960$ nM; Cat S $K_i = 700$ nM) [54]. The potency of this compound was shifted in a functional bone resorption assay (Cat K $IC_{50} = 132$ nM).



3.2 Nitrile-based inhibitors

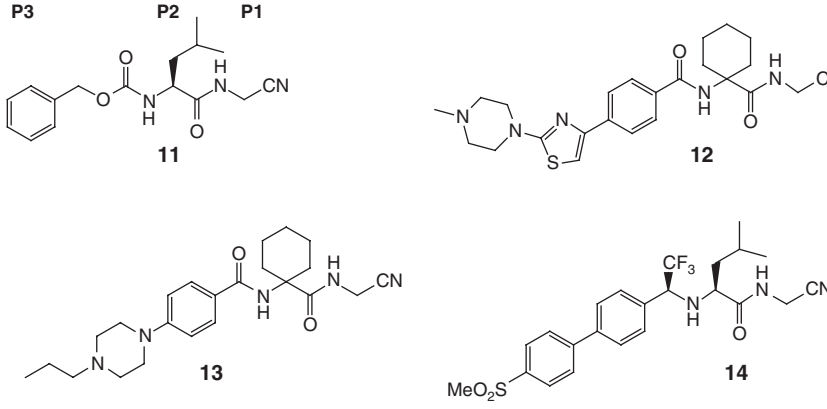
Nitrile-containing molecules have been known to inhibit cysteine proteases since 1971 [55], and are reported to selectively inhibit cysteine proteases over serine proteases [56], forming a covalent but reversible thioimidate intermediate with the enzyme [57]. The geometry of this sp^2 covalent intermediate makes P1 substituents bind poorly in the S1 pocket, and makes access to the S1′ pocket challenging. However, the resulting inhibitors tend to be of low molecular weight and often have fewer metabolism issues than ketone inhibitors. A direct comparison of warheads on a simple inhibitor template has shown the nitrile to be 50-fold less potent against human Cat K than the corresponding aldehyde and of similar potency to an unactivated methylketone [58].

3.2.1 Dipeptide nitriles

A study of the P1 position of Cbz-Leu-acetonitrile **11** has shown that lipophilic groups adjacent to the nitrile can provide additional binding potency, but the changes are relatively modest, with the preferred benzyloxymethyl substituent providing only a 4-fold increase in potency (9 nM vs 35 nM) [59]. A thorough study of P2 SAR based on the same starting peptide has shown that only Leu and 1,1-cyclohexyl glycine were tolerated in P2 [60]. The latter substituent was preferred due to greater metabolic stability and the elimination of a chiral centre. P3 SAR from the same study clearly indicated a preference for sp^2 hybridization adjacent to the P3 carbonyl and para-substituted benzamides gave a ~10-fold increase in potency relative to the Cbz group. The introduction of a basic amine in the P3 residue provided an additional 10-fold increase in Cat K potency, as well as providing an exceptional level of selectivity vs cathepsins B, L and S. The thiazolylpiperazine derivative L-006235 (**12**) has a K_i of 0.2 nM against Cat K with high selectivity (Cat B $K_i = 1\,\mu M$; Cat L $K_i = 6\,\mu M$; Cat S $K_i = 47\,\mu M$) [60]. As has been noted above, this high selectivity is eroded significantly in cell-based enzyme occupancy assays due to inhibitor lysosomotropism (Cat B $IC_{50} = 17$ nM; Cat L $IC_{50} = 340$ nM; Cat S $IC_{50} = 790$ nM) [16]. L-006235 has an IC_{50} of 5 nM in a functional bone resorption assay in rabbit osteoclasts and has 68% oral

bioavailability in rats with a 3.4 h half-life. It is active in an ovariectomized rhesus monkey model of osteoporosis, giving a 68% decrease in urinary NTx over 7 days at an oral dose of 3 mg/kg/day.

The structurally related dipeptide nitrile balicatib (**13**) has an IC_{50} of 1.4 nM against Cat K with high selectivity over off-target cathepsins in purified enzyme assays (Cat B $IC_{50} = 4800$ nM; Cat L $IC_{50} = 503$ nM; Cat S $IC_{50} = 65000$ nM). These off-target activities are increased 10- to 80-fold in cell-based enzyme occupancy assays (Cat B $IC_{50} = 61$ nM; Cat L $IC_{50} = 48$ nM; Cat S $IC_{50} = 2900$ nM) due to the lysosomotropism of the compound [16]. Balicatib was effective at preventing ovariectomy-induced bone loss in OVX cynomolgus monkeys at 30 mg/kg bid over 18 months. The increase in BMD relative to sham-operated animals was more pronounced in the femur (+18%) than in the lumbar spine (+4%). Mechanical testing of the lumbar vertebrae show a significant increase in maximum load, and a high correlation between BMD and maximum load is observed in both the vertebrae and the femur [22].



3.2.2 Trifluoroethylamine-containing peptidic nitriles

While dipeptide nitrile inhibitors provide excellent potency and selectivity when bearing basic amines in P3, much of this profile is lost when non-basic derivatives are prepared. However, the replacement of the P2 amide bond with an (*S*)-trifluoroethylamine residue provides a conformationally rigidified inhibitor that maintains the important P2 hydrogen bond and gives a 20-fold increase in potency relative to the amide derivatives [61]. Inhibitors in this series are sufficiently potent and selective such that the basic P3 moiety can be removed to generate inhibitors that lack the liability of lysosomotropism. L-873724 (**14**) has an IC_{50} of 0.2 nM inhibitor against Cat K and is >800-fold selective against off-target cathepsins (Cat B $IC_{50} = 5239$ nM; Cat L $IC_{50} = 264$ nM; Cat S $IC_{50} = 178$ nM) [62]. L-873724 is orally bioavailable in rats (F = 40%) with a half-life of 3 h. It is efficacious in an ovariectomized rhesus monkey model of osteoporosis, giving a 68% reduction in urinary NTx excretion over a 6-day dosing period at an oral dose of 3 mg/kg/day.

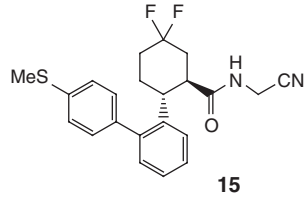
3.2.3 Di-substituted cyclohexane-based nitriles

The P2 amino acid in the dipeptide nitrile series has been replaced with a 1,2-disubstituted cyclohexane ring to give potent Cat K inhibitors [63]. The β-substituent on this ring does not make the important hydrogen bond to Gly^{66} in the Cat K active site that is common to most other Cat K inhibitors. Nevertheless, the resulting compounds have IC_{50}s of <5 nM against Cat K and are highly selective over off-target cathepsins. Extensive metabolism on the cyclohexyl ring resulted in poor pharmacokinetics in the prototype compounds, but this was improved dramatically by the preparation of gem-difluorinated cyclohexane derivatives. The preferred inhibitor **15** has an IC_{50} of 0.3 nM inhibitor against Cat K and is $>36{,}000$-fold selective against Cat B, 780-fold selective against Cat L, and 940-fold selective against Cat S. The pharmacokinetic profile is good in three species (rat F = 39%, $t_{1/2} = 3.5$ h; dog F = 55%, $t_{1/2} = 15$ h; squirrel monkey F = 78%, $t_{1/2} = 8.8$ h).

3.2.4 Aromatic nitrile inhibitors

A series of non-peptidic nitrile inhibitors has been described in which the electrophilicity of the nitrile is modulated by an electron-deficient aromatic ring instead of an α-amido group. The purine-derived inhibitor **16** is a 3 nM inhibitor of Cat K in which the cyclohexyl group binds in S2 and the purine ring lies against the hydrophobic wall of the active site [64]. Attempts were made to reach the S3 pocket by replacing the cyclohexyl ring with an *o*-substituted phenyl ring, but no improvement in potency was observed.

Replacing the purine template with a pyrimidine provides a series of inhibitors with an alternate means of reaching the S3 pocket. Compound **17** is a 1.5 nM inhibitor of Cat K, but no selectivity data has been disclosed [65]. A close analogue of this compound, **18**, has been given the generic name dutacatib [66]. The electrophilicity of these nitrile derivatives has been determined to be considerably greater than that of the α-amidoacetonitrile derivatives above. When incubated with cysteine for 30 min at pH 7.4, **17** forms 79% of the thiazoline adduct compared to only 5% adduct formation for the α-amidoacetonitrile L-873724 [67].



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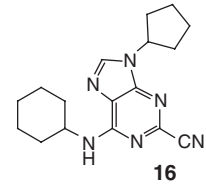

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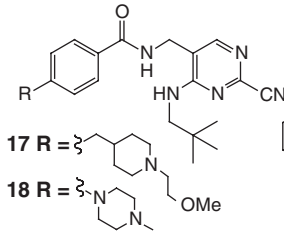

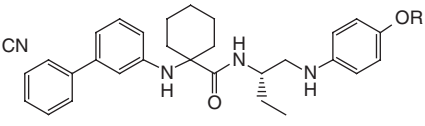

19 R = Me
20 R = CH_2COOH

3.3 Aminoethyl non-covalent inhibitors

The final class of inhibitor to be described contains no electrophilic warhead to interact with the sulfhydryl group of the active site cysteine. The binding affinity of these non-covalent, competitive inhibitors is partly achieved through lipophilic P1′ interactions of an aminoethylaniline moiety [68]. Electron-donating substituents on the aniline are required for potency against Cat K [7].

SAR studies have determined that an ethyl group is preferred at the P1 position, and either an isobutyl or 1,1-cyclohexylgroup is preferred at P2 to generate compound **19** which has an IC_{50} of 2.7 nM vs Cat K [69]. This compound has poor stability in rat microsomal incubations (35% recovery after 30 min incubation). However, it was found that incorporating an acidic group on the P1′ aniline improved microsomal stability while maintaining Cat K potency and improving selectivity. Compound **20** is a 5 nM inhibitor of Cat K with >2000-fold selectivity over cathepsins B, L and S [70]. In rat pharmacokinetic studies, a 50 mg/kg dose provided an AUC of 104 $\mu g \cdot h/ml$.

4. CLINICAL DATA

Clinical data for two Cat K inhibitors, balicatib and MK-0822, has been reported. Additionally, relacatib has been reported to be in clinical development [44], but no data have been disclosed.

Fourteen-day administration of 50 mg balicatib to post-menopausal Japanese women was safe and well-tolerated and showed an elimination half-life of 15.5 h [71]. A 12-week placebo-controlled dose-ranging study of balicatib in postmenopausal women at 5, 10, 25 and 50 mg daily ($n = 28$/group) showed a dose-dependent decrease in serum CTx, a biochemical marker of bone resorption. At the 50 mg dose, a ~70% reduction in sCTx was observed (22). A subsequent 1-year study at the same doses ($n = 135$/group) found a 61% decrease in sCTx at 50 mg qd and a 55% decrease in urinary NTx. Serum osteocalcin and bone-specific alkaline phosphatase, markers of bone formation, were similar to placebo after 1 year of dosing [72]. This apparent decoupling of bone resorption and bone formation, based on bone turnover markers, distinguishes Cat K inhibition from other anti-resorptives such as bisphosphonates, denosumab and SERMs, all of which suppress markers for both resorption and formation. Increases in bone mineral density of 2.2% at the hip and 4.5% at the lumbar spine were also observed at the 50 mg dose, providing the first clinical evidence that this mechanism is effective at increasing BMD in humans. However, drug-related skin AEs, mainly characterized as pruritis and sclerodema/morphea were observed, which may prove limiting for this compound, and the discontinuation of the development of balicatib was announced in a press release in late 2006 [73].

A 3-week study of MK-0822 (0.5, 2.5 and 10 mg qd) in post-menopausal women showed a 70–80% reduction in serum CTx at the top dose and an 80% reduction in urinary NTx at both the 2.5 and 10 mg doses [74]. The compound was safe and well-tolerated throughout the dosing period.

5. CONCLUSIONS

The past 3 years have seen tremendous advances in both the design of Cat K inhibitors and in our understanding of the effect of Cat K inhibition on bone remodeling. The structural diversity of Cat K inhibitors has expanded considerably from simple peptidomimetics to non-peptidic derivatives and even non-covalent inhibitors. The potency, selectivity and pharmacokinetic properties of key compounds are very attractive and seem well-suited to further development. The disclosure of clinical validation of the effect of Cat K inhibition on bone mineral density, plus the provocative data suggesting a decoupling of bone resorption and bone formation provides a compelling framework for further development of Cat K inhibitors for the treatment of osteoporosis.

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CHAPTER 9

New Nonpeptide-Binding GPCRs as Targets for Diabetes and the Metabolic Syndrome

Matthew C. T. Fyfe, Hilary A. Overton, Martin J. Procter, Christine Reynet and **Jonathan R. White**

Contents

1. INTRODUCTION

Obesity is becoming evermore prevalent in society as more sedentary lifestyles become commonplace and high-energy foods become more widely available [1]. The obesity epidemic [2] has been accompanied by a dramatic rise in the incidence of type 2 diabetes (T2D), leading to the introduction of the term ''diabesity'' to describe obesity-induced diabetes [3]. The diabesic condition is often supplemented by a range of closely linked maladies, such as insulin resistance, dyslipidemia, microalbuminuria, and hypertension. Taken together, these ailments comprise a much broader underlying disorder known as the ''metabolic syndrome'' [4], an affliction correlated with a markedly increased

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incidence of cardiovascular disease. At present, no single drug can alleviate all of the risk factors of the metabolic syndrome [5]. Furthermore, the existing oral pharmacotherapies available to the physician for treating the individual components of the metabolic syndrome have limitations. Thus, the current range of oral antidiabetic agents suffer from inadequate efficacy, limited tolerability, and marked mechanism-related side effects [6,7]. By the same token, there are only two therapies approved for the long-term treatment of obesity in the United States, both of which are limited by poor efficacy and adverse side effects [8]. It is also recognized [9] that there is still a need to develop new therapies for dyslipidemia in order to lessen the burden of atherosclerotic cardiovascular disease.

G-protein-coupled receptors (GPCRs) [10] represent the most successful target class in drug discovery [11,12]. As a result, it comes as no surprise that modulators of these receptors have been identified as prime candidates for novel treatments of diabetes and the metabolic syndrome [13–15]. Here, we describe research aimed at discovering modulators of new nonpeptide-binding GPCRs, considered more druggable than their counterparts possessing peptidic ligands [16], as potential therapies for T2D, obesity, and related metabolic disorders. In particular, we focus on GPCRs that have been deorphanized [17–20], i.e., for which physiological ligands have been discovered, in the past few years.

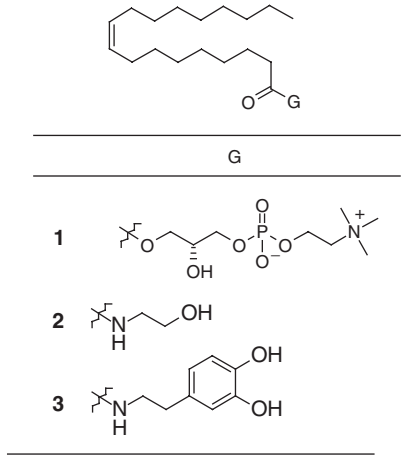
2. GPR119

The GPCR now called GPR119 [21] was identified independently by several research groups. As a result, this receptor appears in the literature under various names, including SNORF25 [22], RUP3 [23], 19AJ [24], GPCR2 [25], PFI-007 [26], OSGPR116 [27], and the glucose-dependent insulinotropic receptor (GDIR [28,29]). Isoforms of the receptor have been identified in a number of mammalian species, including rats, mice, hamsters, chimpanzees, rhesus monkeys, cattle, and dogs.

In human and rodent tissues, GPR119 mRNA is principally localized in the pancreas and gastrointestinal tract [22,26]. Many areas of the rodent brain also show GPR119 expression, a phenomenon that has not been demonstrated in humans. Within the rodent pancreas, immunofluorescent staining has implicated the islet β-cells as the main site of expression [23], a premise supported by evidence of GPR119 expression in β-cell derived insulinoma cell lines, such as HIT-T15 [23,30], NIT-1, MIN6, and RIN5 [31]. Recently, however, contrary evidence has been presented [32], in which immunofluorescence indicated that pancreatic polypeptide-secreting cells were the only site of GPR119 expression in islets.

Deorphanization of GPR119 has produced two classes of possible endogenous ligands, viz., phospholipids [31,33], such as 1-oleoyllysophosphatidylcholine (**1**), and fatty acid amides [27]. The fatty acid amides oleoylethanolamide (OEA, **2**) and endovanilloid *N*-oleoyldopamine (**3**) are the most active potential endogenous ligands described to date [34,35]. Both classes of ligand raise intracellular cyclic AMP (cAMP) levels in GPR119-expressing cells, as do small-molecule GPR119 agonists [23,26,30,34,36]. The enhanced cAMP levels produced by GPR119 agonists are a consequence of adenylate cyclase stimulation through $G\alpha_S$ coupling [30], a process which also occurs [37] with the β-cell receptors for the

incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). Since GPR119 is a $G\alpha_S$-coupled receptor expressed in the insulin-secreting β-cells of the pancreas, it is conceivable that, like the incretin receptors [38,39], this protein could play a role in the stimulation of glucose-dependent insulin release [31]. Importantly, GPR119 belongs to the family of class A (rhodopsin-like) GPCRs for which small molecule agonists have been discovered previously. In contrast, small molecule agonists for the class B (secretin-like) GPCRs, which include the GLP-1 and GIP receptors, have proven elusive until recently [40]. From this perspective, GPR119 appears a more tractable target than the incretin receptors. Indeed, numerous GPR119 agonists have been discovered that elicit glucose-dependent insulin release in cultured cells and in isolated rat islets [26,30,31,41,42]. Furthermore, these compounds have been shown to reduce the glucose excursion in rodent oral glucose tolerance tests (OGTTs) [23,26,30,43].

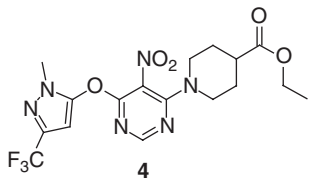


Interest in GPR119 as a therapeutic target has been further stimulated by the finding that the endogenous ligand OEA reduces food intake and body weight gain when administered to rodents [44]. The anorectic actions of OEA have been attributed to PPARα activation [45], but GPR119 agonism may also play a role. In support of this latter assertion, a selective, orally available, small-molecule GPR119 agonist has been shown to mirror the effects of OEA in reducing feeding and body weight gain in rodent models, actions that were accompanied by reductions in white adipose tissue deposits and plasma leptin levels [34]. However, it should also be noted that OEA was reported to suppress food intake similarly in both wild-type and GPR119 knockout mice [42].

GPR119 is expressed in the GLUTag (GLP-1 secreting mouse enteroendocrine) cell line. GLP-1 release is stimulated upon exposure of these cells to GPR119 agonists, and acute administration of agonists to rats raises plasma GLP-1 levels [43,46]. GPR119 agonism also leads to stimulation of GIP secretion in mice [28]. The fact that GPR119 agonists can stimulate incretin secretion is consistent with the observations that, although GPR119 agonists are able to improve oral glucose tolerance, they have markedly reduced antihyperglycemic actions in

intraperitoneal [30] or intravenous [43] glucose tolerance tests. The reduction in food intake seen upon administration of GPR119 agonists could also be a consequence of increased GLP-1 release, as GLP-1 is known to reduce caloric intake and body weight in experimental animals [47]. Like GLP-1 [48], GPR119 agonists inhibit gastric emptying in rodents [43]. The combined antidiabetic and anorectic effects of GPR119 agonists suggest a possible role for such molecules in the treatment of diabesity and associated metabolic disorders.

The GPR119 agonist on which most data has been disclosed is AR231453 (**5**). This compound was obtained through optimization of **4**, an inverse agonist found in a HTS campaign. AR231453 is highly selective for GPR119, having no affinity for more than 230 other GPCRs [30] or enzymes, such as dipeptidyl peptidase-4. This compound displayed good PK in mice [$T_{1/2} = 3.4$ h, $C_{max} = 9.8$ μM after a 10 mg/kg p.o. dose], but its bioavailability was low in rats [28]. In HIT-T15 cells expressing GPR119, AR231453 dose-dependently increased insulin release with an EC_{50} of 3.5 nM, an EC_{50} similar to that obtained for stimulating cAMP accumulation (4.7 nM). Insulin secretion was also stimulated from isolated rat islets, where, at 15 mM glucose, 300 nM AR231453 induced comparable increases in insulin secretion to 100 nM GLP-1. Notably, no effects on insulin secretion were observed in isolated islets at 5 mM glucose, indicating that the insulinotropic effects of AR231453 are glucose-dependent. In an oral glucose tolerance test in C57Bl/6J mice [30], AR231453 (20 mg/kg p.o.) elicited antihyperglycemic effects comparable to those of the sulfonylurea glyburide (30 mg/kg p.o.), except that, unlike the sulfonylurea, it did not exhibit hypoglycemic effects prior to administration of the glucose load. Elevated insulin levels were observed during this experiment, indicating that AR231453 enhances glucose-dependent insulin secretion from pancreatic β-cells *in vivo*. Moreover, this GPR119 agonist exhibited significant antihyperglycemic effects when dosed orally at 3 and 10 mg/kg to diabetic *KK/A*^{y} mice [30]. In contrast to the situation in their wild-type littermates, in GPR119 knockout mice, AR231453 displayed no antihyperglycemic effects and did not stimulate insulin secretion [30]. Interestingly, it has also been demonstrated [28] that AR231453 (10 mg/kg p.o.) stimulates both GLP-1 and GIP release during OGTTs in C57BL/6J mice, while no effects were seen on GIP release in GPR119 knockout mice. Moreover, it has been claimed that coadministration of AR231453 with an incretin-protecting dipeptidyl peptidase-4 inhibitor (DP4I, [49]) leads to synergistic increases in active GLP-1 levels, a phenomenon that results in the AR231453–DP4I combination inhibiting the glucose excursion during an OGTT much more than its constituents dosed alone. The potential of GPR119 agonists to exert β-cell protective effects through increased cAMP levels has also been demonstrated. In MIN6 pancreatic β-cells expressing GPR119, AR231453 induced Akt phosphorylation and IRS-2 expression [28], key measures of islet mass protection.



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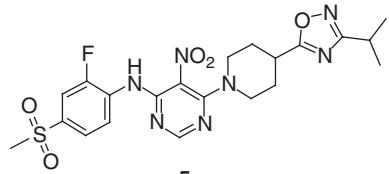

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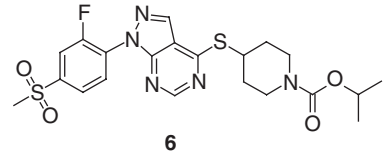
Several other GPR119 agonists have been reported to show antihyperglycemic effects in rodents. By way of illustration, carbamates **6** and **7** reduced the glucose excursion by >30% in OGTTs in Sprague–Dawley rats following oral dosing at 30 and 10 mg/kg, respectively [50,51]. Similarly, the related carbamates **8** and **9** attenuated the glucose excursion by >30% in OGTTs in C57Bl/6J mice when administered at 10 mg/kg p.o. [52,53], while the thioether **10** lowered fed blood glucose levels in diabetic *db/db* mice [23]. Separately, it was reported [26] that the thioester **11**, which raised intracellular cAMP levels with an EC_{50} of 3.2 μM in 293-EBNA cells, stimulated insulin secretion and improved glucose tolerance when administered at 100 mg/kg i.p. to Sprague–Dawley rats in OGTTs. GPR119 agonist **11** also demonstrated antihyperglycemic effects in diabetic Goto–Kakizaki rats following oral administration at 100 mg/kg.



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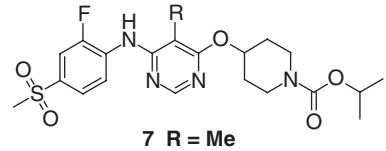

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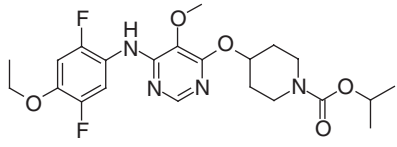

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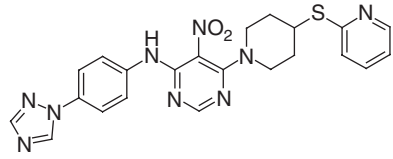

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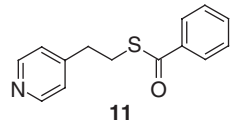

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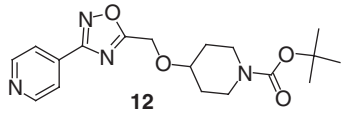
The oxadiazole-containing carbamate PSN632408 (**12**), obtained through optimization of the HTS hit PSN375963 (**13**), underscores the potential for GPR119 agonists to reduce body weight [34]. Like OEA (EC_{50} = 2.9 μM), PSN632408 produced concentration-dependent increases in intracellular cAMP levels in a HEK cell line expressing GPR119 with an EC_{50} of 1.9 μM. However, in contrast to the natural ligand, PSN632408 has physicochemical properties associated with good oral absorption (cLog $P = 2.2$, molecular weight = 360.4). When administered orally to rats at 100 mg/kg, PSN632408 reduced 24 h cumulative food intake. This reduction in food intake was not associated with drug-induced malaise, as no effects were seen on locomotor activity or in conditioned taste aversion and kaolin consumption tests. The anorectic effects observed in an acute setting translated into chronic effects on body weight. In both diet-induced obese mice and growing, high-fat diet-fed Sprague–Dawley rats [36], attenuations of body weight produced by PSN632408 (100 mg/kg/day p.o.) were comparable to those

of the prescribed anorectic drug sibutramine hydrochloride hydrate (5 mg/kg/day p.o.).



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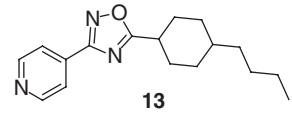

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In summary, initial results with prototypical GPR119 agonists indicate that these compounds could be potential therapies for diabetes and related metabolic disorders by: (1) Stimulating glucose-dependent insulin secretion, (2) Inducing incretin release, (3) Reducing food intake and body weight, and (4) Protecting pancreatic β-cells through raised cAMP levels. As a result, interest in this target is increasing rapidly, especially following the entry of the GPR119 agonist APD668 into the clinic [29].

3. GPR40 FAMILY

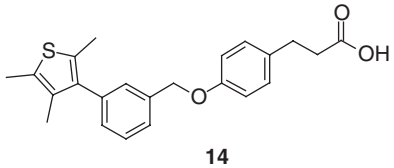
The proteins of the GPR40 family, comprising GPR40, GPR41, and GPR43, have attracted attention recently as potential targets for diabetes and the metabolic syndrome due to their function as receptors for plasma-free fatty acids (FFAs) in cell types central to these conditions. The actions of plasma FFAs are thought to underlie several mechanisms of the pathophysiology of T2D and the metabolic syndrome [54]. The GPR40 family proteins are encoded by tandemly located genes, sharing 30–40% sequence homology, and are differentiated by specificity for fatty acid ligands (chain length and saturation) and tissue localization [55].

3.1 GPR40

GPR40 is a class A, $G\alpha_q$-coupled receptor, localized in insulin-producing pancreatic islet β-cells, which is activated by medium and long-chain (C_{10}–C_{22}) saturated and unsaturated fatty acids [56–58]. Long-chain fatty acids (LCFAs) have several effects on pancreatic β-cells, where they amplify glucose-dependent insulin secretion and prime the cells to respond to glucose following fasting [59]. The effects of LCFAs on insulin secretion in pancreatic β-cells are dependent on GPR40, as shown by siRNA gene silencing, genetic deletion of GPR40, and a selective GPR40 antagonist [56,60–62]. Agonists specific to GPR40 may therefore have therapeutic utility as insulin secretagogues, a property that has been demonstrated *in vitro* for one such selective synthetic GPR40 agonist [62]. In opposition, data from GPR40 knockout mice appear to indicate that GPR40 antagonists may have potential for the treatment of diabetes, since these mice are protected from the effects of a high-fat diet, which include insulin resistance, hyperinsulinemia, and glucose intolerance [61]. This observation may reflect the effects of chronic exposure to elevated FFAs, which leads to impaired pancreatic β-cell

function, and suggests a key role for GPR40 in the development of the metabolic syndrome in obesity.

Since the putative natural ligands for the GPR40 receptor are LCFAs, it comes as no surprise to find that the majority of synthetic agonists reported to date are lipophilic carboxylic acids. Thus, GPR40 agonists **14–18** have all been reported to have EC_{50}s of 20 nM or less in various patent applications [63–67]. Recently, the first two papers detailing the SAR associated with a series of GPR40 agonists appeared. Arylpropionic acid **19** (EC_{50} 500 nM) was identified as a moderately potent agonist in an HTS. The low molecular weight, simple structure and good aqueous solubility (0.83 mg/mL) of this compound made it an ideal starting point for exploration [68]. Initial optimization identified GW9805 (**20**), a compound with an EC_{50} of 80 nM that displayed good oral PK properties in rats ($F_{po} = 65\%$, $T_{1/2} = 5.3$ h). GW9805 was further optimized to the cyclopropyl-containing analogues **21** and **22**, which had EC_{50}s of 12 nM and 5 nM, respectively [69]. The *trans*-(*S*,*S*)-cyclopropyl ring was shown to be optimal for potency. Interestingly, the activity of a number of amide analogues related to **21** and **22** was also described. These amides tended to retain binding potency, but did not activate the receptor to the same extent as the acid analogues. It is noteworthy that the potency of these synthetic ligands is orders of magnitude greater than that reported for the endogenous fatty acid ligands (for example, the EC_{50} of linoleic acid is 2.2 μM). Unfortunately, however, no pharmacodynamic data have been described for any of these GPR40 agonists.



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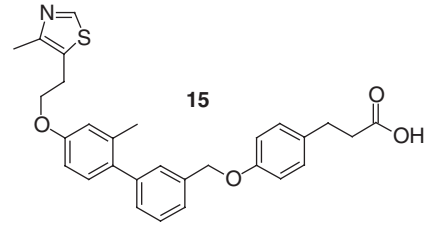

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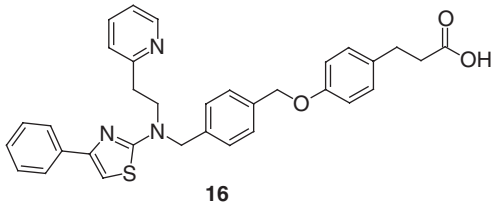

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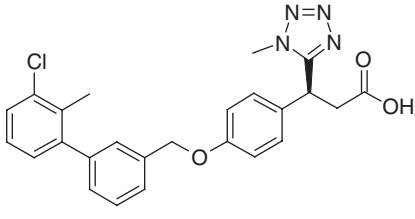

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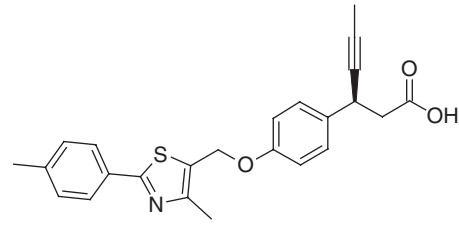

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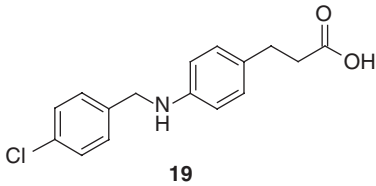

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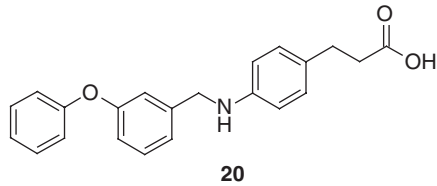

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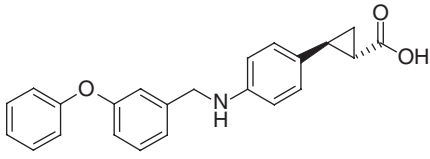

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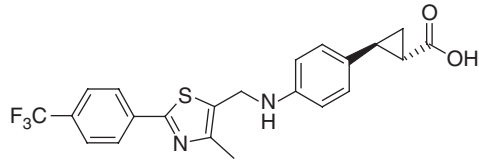

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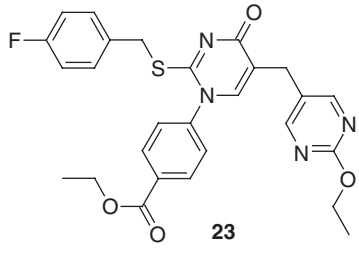
The *in vitro* pharmacology of GW9805 has been described in some detail. This compound is described as a partial agonist of GPR40 and a full agonist of GPR120 (vide infra) with EC_{50}s of 48 nM and 3.5 μM, respectively [62]. Furthermore, GW9805 is at least 100-fold selective versus 220 other GPCRs (including receptor family members GPR41 and 43) and many other targets, including the fatty acid binding PPAR receptors α, δ, and γ. GW9805 was shown to augment insulin secretion from the MIN6 mouse insulinoma cell line with an EC_{50} of 720 nM in the presence of 25 mM glucose. Importantly, this compound did not stimulate insulin secretion in the presence of low glucose concentrations (5 mM). The selective GPR40 antagonist GW1100 (**23**) was employed to demonstrate that the enhanced insulin secretion arose through the GPR40, not the GPR120, receptor.



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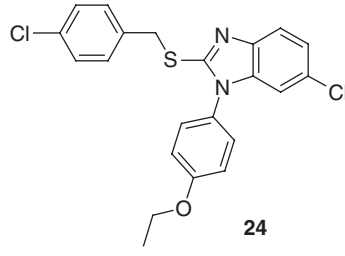

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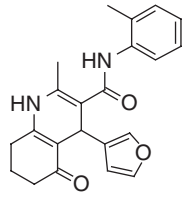
Data on the pharmacology and SAR of GPR40 antagonists are sparse and only recently have the first GPR40 antagonists been reported in the literature. GW1100 displays micromolar potency versus a range of natural agonists of the receptor, such as linoleic, α-linoleic, palmitoleic and *cis*-5,8,11,14,17-eicosapentaenoic acids [62]. At 10 μM, the benzimidazole antagonist **24** has been reported to reduce 1 mM palmitic acid-induced insulin secretion from rat islets from 930 to 369 pg/ng [70]. As is the case with GPR40 agonists, to date, no supporting *in vivo* efficacy data have been tendered to demonstrate the potential therapeutic

benefits of GPR40 antagonists in diabetes. Thus, at the time of writing, it is still unclear which of GPR40 agonists and/or antagonists represent the best potential antidiabetic therapies.

3.2 GPR41 and GPR43

GPR41 and GPR43 are receptors for short-chain (C_2–C_6) fatty acids (SCFAs), which are coupled primarily to $G_{i/o}$ and G_q family G-proteins, respectively [71,72]. Both receptors are expressed prominently in adipose tissue, although GPR43 is present at higher levels in white blood cells. The endogenous ligands, i.e., SCFAs, are produced by bacterial fermentation in the lower gut and are present in blood. Administration of propionate has been shown to reduce food intake in both sheep and chickens and to lower blood glucose concentrations in obese hyperinsulinemic *fa/fa* rats [73–75]. Propionic acid stimulates secretion of the anorexigenic hormone leptin by adipocytes *in vitro* and increases leptin levels when administered to mice [76]. siRNA gene knockdown experiments and examination of ligand and G-protein specificities suggest that SCFA-stimulated leptin secretion is mediated through GPR41. Expression of GPR43 in enteroendocrine cells expressing the satiety hormone peptide-YY (PYY) has been reported, suggesting a possible mechanism for the hypophagic effects of SCFAs [77]. However, gut expression of GPR43 is at much lower levels than in immune cells, and the effects of SCFAs on intestinal mobility have been shown to be independent of GPR43 [72,78]. Thus, it appears that GPR41 has more potential as a target for therapies for T2D and the metabolic syndrome. Although GPR43 also remains a possible therapeutic target for metabolic diseases, its prominent expression in immune cells makes it less attractive for drug discovery.

The first small molecule ligands for GPR41 appeared recently in a patent application [79], in which it was claimed that agonists of the receptor are useful in reducing insulin secretion. At micromolar concentrations, the GPR41 agonist cyclopropanecarboxylic acid reduces insulin secretion *in vitro* from the mouse MIN6 β-cell line at high glucose concentrations (300 mg/dL). Subsequently, in an OGTT in mice, a 30 mg/kg dose of GPR41 agonist **25** is reported to partially reverse the glucose lowering effects of a 10 mg/kg dose of a GPR119 agonist. As a result, it is claimed that this indicates that GPR41 antagonists/inverse agonists, such as **26**, may have glucose lowering effects, although no data are available to support these claims directly.



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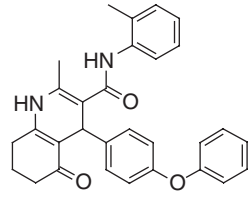

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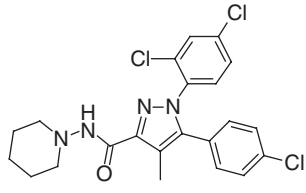
4. GPR120

GPR120 is a receptor for unsaturated LCFAs whose ligand specificity overlaps that of GPR40. This receptor has been shown to mediate the effects of unsaturated LCFAs in stimulating GLP-1 secretion in the enteroendocrine cell line STC-1, acting via a $G\alpha_q$-coupled mechanism [80]. Moreover, GPR120 was reported to localize to GLP-1 secreting L-cells in gut tissue sections, indicating a potential function in modulating GLP-1 secretion *in vivo*. It should be noted, however, that the STC-1 cell line is regarded as a poor model of L-cells since they are derived from a secretin-producing intestinal endocrine tumor [81]. In contrast, in the GLUTag cell line, which is a closer model of primary L-cells, GPR120 is unlikely to mediate the effects of unsaturated LCFAs on GLP-1 secretion [82,83], since oleic acid-induced GLP-1 secretion occurs in the absence of $G\alpha_q$-coupled receptor signaling and there are differences in the specificity of LCFAs that induce GLP-1 secretion. Furthermore, other studies indicate that GPR120 is widely expressed in gut epithelial cells, rather than specifically in L-cells, as initially thought [84]. These findings raise questions regarding the validity of GPR120 as a diabetes target involved in modulating GLP-1 secretion *in vivo*. On the other hand, the potential for GPR120 antagonists to be antiobesity therapies has been suggested by the upregulation of receptor expression in diet-induced obese rats, as well as by a recent demonstration that GPR120 is required for adipogenesis *in vitro* [84,85]. Nonetheless, further work will be required to validate GPR120 as an obesity target.

GW9805 is the first reported small molecule full agonist of the GPR120 receptor (*vide supra*). The EC_{50} (3.5 μM) of this compound is similar to that of the natural ligands of this receptor, e.g., linolenic acid has an EC_{50} of 1.3 μM.

5. GPR55 AND GPR35

Interest in the cannabinoid receptors CB_1 and CB_2, class A GPCRs that mediate the effects of cannabinoids, as targets for metabolic diseases has intensified lately [86]. Indeed, the CB_1 inverse agonist rimonabant (**27**) has recently been approved as an antiobesity therapy by the European Medicines Agency [87]. Recent publications have indicated that GPR55 responds to ligands, both natural and synthetic, that are similar to those of CB_1 and CB_2 [88]. The nonselective cannabinoid receptor agonist CP55940 (**28**) apparently binds strongly to GPR55, but whether it functions as an agonist or as an inverse agonist is controversial.



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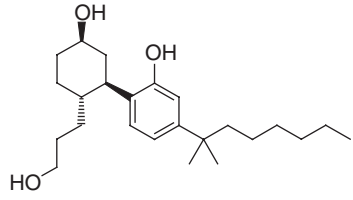

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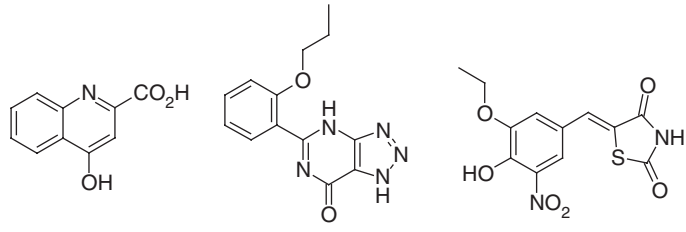
The role of GPR55 is unknown at present. Transcripts have been identified in various human tissues, including brain, spleen, ileum and omental (but not subcutaneous) adipose tissue. This last observation may point to a role in regulation of central adiposity, but blood pressure control has also been suggested as a possible function [88]. Specific ligands and/or knockout animals will be required to probe further the role of this receptor.

GPR55 has low overall sequence homology with CB_1 and CB_2. This receptor does not appear to possess a classical cannabinoid binding pocket, based on analysis of the key amino acid residues, identified by mutagenesis or molecular modeling, required for interaction with cannabinoid ligands [89]. In terms of sequence identity, GPR55's closest relative is GPR35 (37%), a GPCR predominantly detected in pancreatic islets, the gastrointestinal tract and immune cells. This receptor has been shown to be activated by kynurenic acid (**29**), an intermediate in the metabolism of tryptophan to kynurenine [90], and by the well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor zaprinast (**30**), which activated rat and human receptors with respective EC_{50}s of 16 and 840 nM [91]. The potential for GPR35 agonists as antidiabesic agents is highlighted by the thiazolidinedione **31**, which is reported to improve glucose tolerance and reduce free fatty acid levels in mice [92].



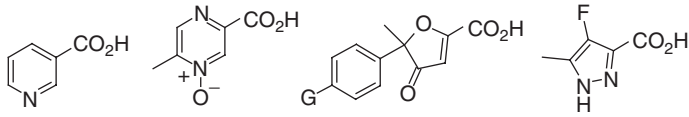
29 **30** **31**

6. GPR109A AND GPR109B

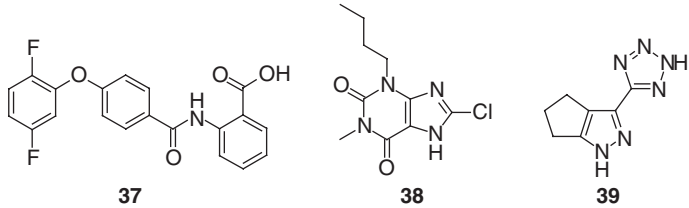
Interest in GPR109a [93] was stimulated by the discovery that it is a receptor for niacin (**32**), a vitamin which, since the 1950s, has been known to exhibit antidyslipidemic effects. When taken in high doses, niacin reduces serum triglycerides and low-density lipoprotein cholesterol, while simultaneously increasing high-density lipoprotein (HDL) cholesterol. GPR109a, which is expressed mainly in adipocytes and immune cells, is also known as HM74A [94] or RUP25 [95], while its mouse orthologue has been termed PUMA-G. In adipocytes, agonists interacting with this GPCR cause a reduction in triglyceride hydrolysis via $G\alpha_i$-mediated inhibition of adenylate cyclase. This inhibition results in a rapid decrease in serum-free fatty acids, not seen in mice lacking PUMA-G, which leads subsequently to raised HDL levels. GPR109b (HM74) is a closely related receptor to GPR109a (96% homologous), which appears to have arisen from a very late gene duplication and has no known rodent orthologue. Niacin is a much

weaker ligand for GPR109b (millimolar EC_{50}) than for GPR109a, which is activated by the vitamin with an EC_{50} of 250 nM in a GTPγS assay [96]. The chief disadvantage of niacin as a lipid-lowering therapy is its tendency to elicit a strong cutaneous flushing reaction, which limits patient compliance. Unfortunately, this effect appears to be mechanism-based and originates from activation of GPR109a in epidermal Langerhans cells [97], which results in the release of prostaglandin D_2 [98]. The flushing associated with niacin can be attenuated by pre-treatment with cyclooxygenase inhibitors, while its frequency and severity can be diminished through the use of extended-release formulations of niacin [99].

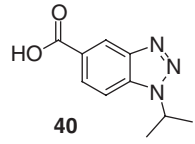
Acipimox (**33**) is another drug for dyslipidemia that binds to GPR109a, albeit with reduced potency ($EC_{50} = 5.1\ \mu M$). This compound seems to have fewer side-effects than niacin, but may also be less effective in its lipid-modulating capabilities. Acifran (**34**), another compound that has been shown to raise HDL in humans [100], has similar potencies at both GPR109a and GPR109b. The corresponding 4-fluoro analogue **35** displayed significant anti-dyslipidemic properties in fructose-fed rats [101], where it reduced serum triglycerides by up to 69% when administered orally at 15 mg/kg. In a whole cell assay, the pyrazole carboxylic acid **36** activated GPR109a with an EC_{50} of 0.2 μM, while no activity was found at GPR109b at up to 50 μM [102]. When administered to Sprague–Dawley rats, a 1 mg/kg dose of this compound inhibited free fatty production more effectively than the equivalent dose of niacin. Moreover, the cutaneous flushing provoked by **36** in mice was significantly lower than that generated by niacin, perhaps indicating that it is possible to achieve a safety margin greater than niacin with regard to flushing.



Several anthranilic acid derivatives have been reported to be GPR109a agonists in various patent applications [103,104]. For instance, **37** had an EC_{50} of 101 nM in a fluorescent imaging plate reader (FLIPR) assay evaluating GPR109a agonistic activity in transformed HEK293 cells [105]. Xanthines have also featured prominently in the patent literature [106]. The rise in ear temperature in anesthetized guinea-pigs, used as a surrogate of the flushing response, produced by xanthine **38** at a dose of 10 mg/kg i.v. was 85% less than that of an equivalent dose of niacin [107]. The carboxylic acid moiety seen in other GPR109a agonists was replaced [108] with the isosteric tetrazole moiety in **39**.



It has been postulated that, since niacin causes vasodilation in rodents, where only the GPR109a orthologue PUMA-G is present, selective GPR109b agonists could provide the beneficial effects of niacin on lipid levels without the flushing response associated with GPR109a activation. Accordingly, the selective GPR109b agonist **40** ($pEC_{50} = 6.4$) has been identified [109]. This compound shows no activity at GPR109a and, like niacin, dose-dependently inhibits isoproterenol-stimulated lipolysis in human subcutaneous adipocytes.



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7. CONCLUSIONS

GPCRs represent extremely attractive targets for developing new pharmaceuticals [110], but, despite this, only a fraction of the ca. 800 GPCRs encoded in the human genome are targeted by marketed therapeutics at present [11]. The remaining GPCR family members symbolize a huge untapped resource for the discovery of next generation drugs for treating T2D and related metabolic disorders [13]. The pharmaceutical industry is now starting to exploit this resource, and modulators of recently deorphanized nonpeptide-binding GPCRs are demonstrating their potential as medicaments for metabolic diseases. In this regard, GPR119 agonists could represent a rare opportunity to achieve blood glucose control with concomitant body weight loss using an oral agent, an outcome only possible currently with injectable GLP-1 receptor agonists [111], while agonists of the GPR109 receptors may be able to raise HDL levels meaningfully with a reduced flushing response. Nonetheless, the very fact that these receptors were orphans until recently means that much still remains to be discovered about their pharmacology, as highlighted by the agonist versus antagonist quandary that exists for GPR40 modulators.

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CHAPTER 10

Novel Selective Estrogen Receptor Modulators (SERMs)

Jeffrey A. Dodge and **Timothy I. Richardson**

Contents

1. INTRODUCTION

Selective estrogen receptor modulators (SERMs) such as tamoxifen **1** and raloxifene **2** have demonstrated the ability to mimic estrogen (E2) in some tissues, such as bone, while suppressing its effects in other tissues, such as the breast and uterus. This unique tissue selectivity has proven beneficial for the prevention and treatment of diseases such as breast cancer and osteoporosis. The origin of the tissue selectivity demonstrated by SERMs is complex [1–4]. Two estrogen receptor (ER) subtypes have been described, ERα and ERβ, and some of the observed selectivity may be derived from differential activation of these subtypes [5,6]. Crystal structures of ligands bound to the estrogen receptors indicate that small molecules can induce a spectrum of receptor conformations [7]. In turn, these conformational states interact uniquely with co-regulators, as well as target gene promoters, thus making the interplay between the ligand and the receptor a key molecular determinant of tissue selectivity. Herein, we review recent progress made since 2003 in the development of structurally novel SERMs that have

Lilly Research Laboratories, Indianapolis, IN 46285, USA

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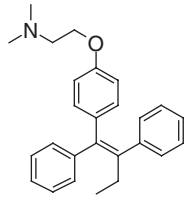
demonstrated tissue-selective pharmacology in pre-clinical models. Previous reviews have appeared in this journal and elsewhere [8–12].



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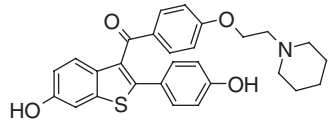

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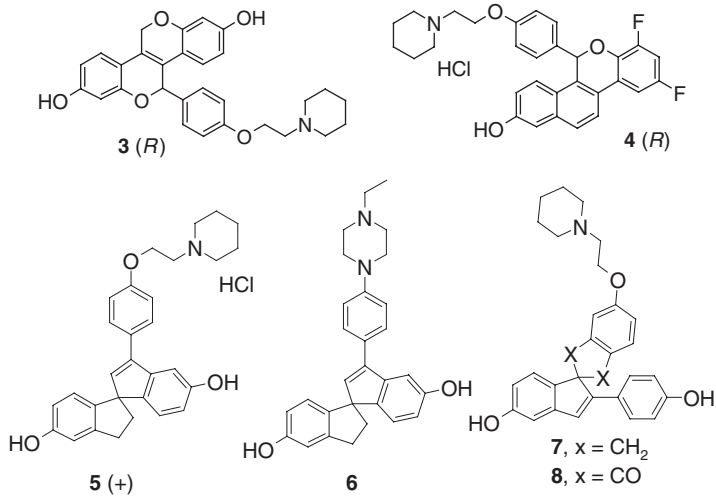
2. SERMS FOR HOT FLUSHES

Hot flushes (flashes) are characterized by a warming sensation that begins in the chest and moves towards the neck and head, and are often accompanied by sweating, palpitations and cutaneous flushing. The episodes generally last from 30 s to 10 min. The majority of postmenopausal women experience hot flushes, with a significant percentage of these women continuing to suffer symptoms for more than 5 years [13,14]. The hot flush event is thought to be centrally mediated, resulting from a transient lowering of the thermoregulatory set point in the hypothalamus [15]. Regulation of the thermoregulatory process may involve hormones or neurotransmitters such as catecholamines, estrogen, testosterone, opioids and serotonin, among others [16]. In fact, compounds which modulate the signaling pathways of each of these have been evaluated clinically for the treatment of hot flushes. Unfortunately, all of the therapies investigated to date suffer from poor efficacy, are associated with unacceptable side effects or are contraindicated for certain patient populations [3]. Tamoxifen (**1**) is reported to induce hot flushes in more than 50% of patients [17] and raloxifene (**2**) is also reported to increase the incidence of hot flushes in clinical trials [18,19]. Estrogen or hormone replacement therapy (ERT or HRT) is currently the treatment of choice and is effective in $>80\%$ of women who initiate treatment. However, estrogen replacement therapy is not recommended for women with a history of breast cancer, uterine cancer, ovarian cancer, or venous thromboembolism.

Because current SERMs are largely ineffective at treating hot flushes, significant pre-clinical efforts have been directed towards the identification of ligands that could potentially alleviate this prevalent symptom in post-menopausal women. Along these lines, the chromene-derived SERM **3** [20] has been reported to exhibit traditional SERM estrogen agonist properties on bone and lipid parameters in ovariectomized (ovx) rats while having estrogen antagonist properties on breast and uterine tissues. In a rodent model of hot flushes, **3** suppresses the rise in tail skin temperature of morphine-dependent ovx rats following morphine withdrawal. In addition, the amount of vaginal fluid in ovx rats treated with **3** is comparable to that of the positive control, ethinyl estradiol, while tamoxifen showed no effect. Overall, the unique pharmacological profile of this compound is

well-suited for clinical evaluation for the treatment of hot flushes and vaginal dryness in post-menopausal women.

Benzopyran SERM **4** displays dose-dependent activity in the morphine-withdrawal rat model of hot flush efficacy [21]. This compound also increases bone mineral density, lowers serum cholesterol, and exhibits minimal uterine agonist activity in ovx rats. SERM **4** binds with high affinity to both ERα and ERβ and is an antagonist in the breast and uterine cancer cell lines, MCF-7, and Ishikawa, respectively. Spiroindane **5** has shown estrogen-like effects on thermoregulation, bone, and lipids in ovx rats. Additionally, **5** had minimal stimulatory activity toward the uterus in ovx rats. Distinct from **3** and **4**, this compound acts as a weak estrogen agonist in the uterus of immature rats and has modest stimulatory effects on breast cancer cell proliferation. Further SAR studies show that the piperazin-1-yl basic side chain in **6** is bioequivalent to the traditional piperidinyl side chain in **5** [22]. The spirocyclic SERM **7** has been reported [23] to bind with high affinity to both ERα and ERβ, while the analogous spiroindenedione **8** is considerably less potent. No data regarding tissue-selective pharmacology have been reported for either of these compounds.



3. OVARIAN SELECTIVE SERMS

Uterine leiomyomas, or fibroids, are the most common type of solid tumors in adult women, clinically apparent in at least 25% of those of reproductive age [24–26]. Abnormal menstrual bleeding, pelvic pain, and infertility are the most commonly experienced symptoms in these women. Uterine fibroids are the leading cause of hysterectomies performed in the United States, accounting for over 200,000 of these procedures each year. Other invasive surgical interventions for the treatment of uterine fibroids include myomectomy and uterine artery embolization. Leiomyomas are estrogen-responsive tumors that can be treated

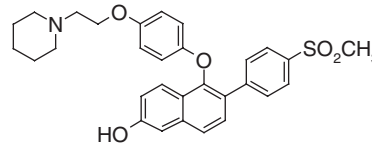
with gonadotropin-releasing hormone (GnRH) agonists. These injectable peptides inhibit estrogen synthesis and result in the reduction of both uterine volume and fibroid size [27]. However, GnRH therapy can result in hot flushes and osteoporosis, a side-effect that restricts use for chronic treatment. Because leiomyomas are dependent on estrogen for growth, antagonism of this steroid hormone receptor is a viable therapeutic approach.

Along these lines, SERMs such as tamoxifen **1** and raloxifene **2** have been clinically evaluated for the treatment of leiomyoma. Tamoxifen lacks sufficient efficacy to reduce tumor size in pre-menopausal women due, in part, to the uterine agonist characteristics exhibited by this SERM [28,29]. In addition, treatment with tamoxifen has resulted in ovarian cysts, an undesired side-effect that severely limits the use of this compound for the treatment of fibroids in ovulatory women. These stimulatory effects on the ovaries have been attributed to the inhibitory properties that tamoxifen has on the hypothalamic–pituitary–ovarian (HPO) axis, i.e., this SERM acts as an estrogen antagonist at the hypothalamus resulting in increased gonadotropin levels (luteinizing hormone, follicle-stimulating hormone) and, ultimately, in hyperstimulation of the ovaries. In fact, inhibition of the HPO axis by SERMs such as clomiphene has been clinically exploited to induce ovulation in women [30].

Raloxifene, a more complete uterine antagonist than tamoxifen or clomiphene, significantly reduces leiomyoma size in post-menopausal women [31], yet it is less efficacious at reducing tumor volume in pre-menopausal women [32]. This result has been attributed to the poor pharmacokinetic properties of this compound in which extensive conjugative metabolism of the phenol(s) limits the circulating levels of the parent drug. In addition, clinical outcomes in pre-menopausal women treated with raloxifene suggest that this compound, like tamoxifen and clomiphene, can affect the ovaries via the HPO axis [33]. These data, taken collectively, indicate that current SERMs lack the efficacy, pharmacokinetic, and ovarian safety properties needed to treat leiomyoma in ovulatory women.

Recently, a SERM with improved selectivity for uterus and ovary in rats has been identified [34]. Naphthalene sulfone **9** binds with high affinity to both ERα and ERβ and is a potent inhibitor of breast cancer cell proliferation. The effects on uterine tissue were assessed at the *in vitro* level in Ishikawa cells in the presence (antagonism) and absence (agonism) of E2. In the antagonist mode, this SERM blocks the effects of 1 nM E2 by $> 90\%$ with an IC_{50} of 10.7 nM. When tested in rodents, this compound proved to be a highly potent, orally active uterine antagonist, blocking estrogen-induced uterine hypertrophy in immature, ovary-intact rats. In addition, it does not have agonist properties in the uterus when administered to ovx rats. The effects on the uterus and ovaries were studied in 6-month-old ovary-intact female rats [35]. Oral administration of **9** for 35 days results in a dose-dependent decrease in uterine weight. The effects on the ovaries were determined by measuring serum E2 levels and histologic evaluation of ovarian cross-sections. Treatment with **9** results in serum E2 levels that are similar to those of vehicle-treated animals, while histological evaluation of the ovaries indicates minimal ovarian stimulation relative to untreated controls. These data

collectively indicate that **9** is a potent uterine antagonist with minimal ovarian stimulation in rats.



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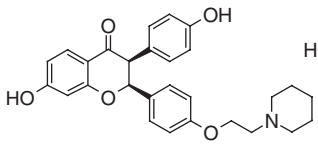
4. ERα SELECTIVE SERMS

The discovery of a second estrogen receptor, ERβ, in 1996 has generated considerable interest in the development of sub-type selective SERMs for ERα and ERβ, including a series of ligands that are highly selective for ERα such as flavanone **10** [36], dihydrobenzoxathiin **11** [37], and chromane **12** [38]. Flavanoid **10** was found to be >50-fold selective for ERα in binding. The selectivity was postulated to lie in the differential interaction of the carbonyl moiety of **10** with the two residues which vary in the respective binding pockets of the receptor subtypes: Leu384 in ERα and Met354 in ERβ. Further evidence that receptor subtype selectivity results from the heterocyclic core is found when point modifications of **1** and other SERMs (bazedoxifene, lasofoxifene) are compared with the analogous modifications in the benzoxathiin core [39]. In all cases, the ERβ/ERα binding ratio is significantly enhanced with the latter scaffold. Compound **11** is a potent inhibitor of estradiol-induced uterine growth in immature rats after subcutaneous administration, and prevents ovariectomy-induced bone loss in rats [40]. Modification of the pendant 3-aryl ring of **11** with 3-alkyl, -cycloalklyl, or -heteroaryl substitution retains ERα selectivity. Repositioning of the phenolic hydroxyl group in **11** to the 3′ position, such as found in analog **13**, results in 40-fold selectivity while exhibiting SERM pharmacology on bone and uterus in rodent models [41]. Analogs of **12** in which the basic side chain is modified with bicyclic amines, heteroatoms, or alkyl-substituted pyrrolidines leads to ERα selective ligands with a diverse spectrum of activity on uterine tissue in rodents. Compound **14** is a full uterine antagonist while **15** is a partial agonist. Subtle changes in structure that result in dramatic pharmacological effects on uterine tissue have been investigated by crystallographic analyses of these compounds bound to ERα. It has been postulated that the less favorable interactions of the pyrrolidine methyl groups on **15**, relative to **14** in the ligand binding domain of ER destabilize the antagonist conformation of helix 12 leading to the partial agonist effects observed for **15** [37,42,43]. Optimization of the dihydrobenzoxathiin scaffold for uterine and breast antagonism led to the discovery of **16** which is superior to tamoxifen and fulvestrant in the compound's ability to lower tumor growth rates and tumor burden in MCF-7 human breast cancer xenografts in athymic mice. The activity of compounds such as **16** against breast cancer cells may result from their ability to downregulate the estrogen receptor, leading the authors to classify these

particular analogs as selective ERα downregulators, or SERADs [44].



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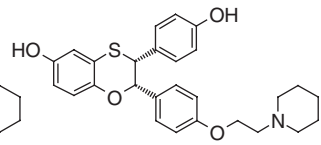

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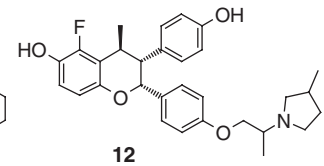

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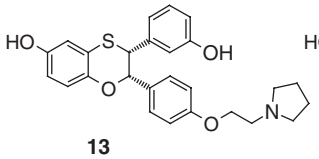

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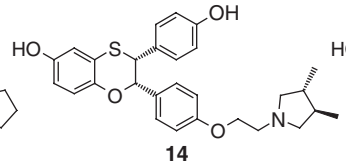

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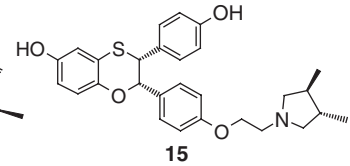

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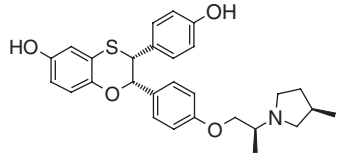

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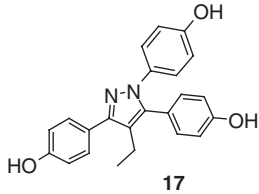
A successful strategy for designing ERα SERMs has been to transform ERα agonists into modulators by appending a basic side chain at the appropriate position. Pyrazole agonist **17** was converted into an antagonist by attaching the piperidinylethyl side chain shown in **18**, which displays high affinity for ERα (relative binding affinity (RBA) = 11.5%) and is ~18-fold selective for ERα [45]. The addition of the basic side chain has a significant effect on transcriptional activity in that **18** is a full antagonist on ERα (IC_{50} ~20 nM). The potency, selectivity, and efficacy in the functional assay parallels the binding selectivity. In a similar fashion, ERα agonist **19** was converted into antagonist **20** by the addition of the ethoxypiperidine side chain. Furan **20** was almost 25-fold selective for ERα (RBA ERα = 75%; RBA ERβ = 3.1%) [46].



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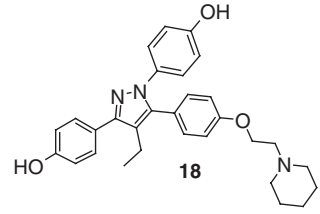

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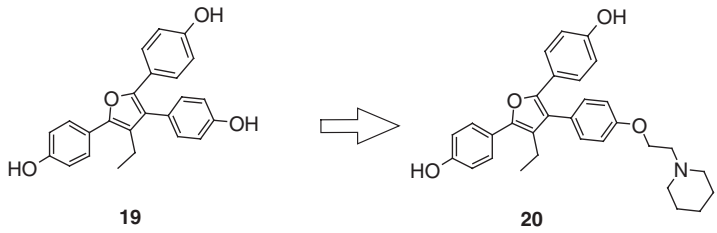
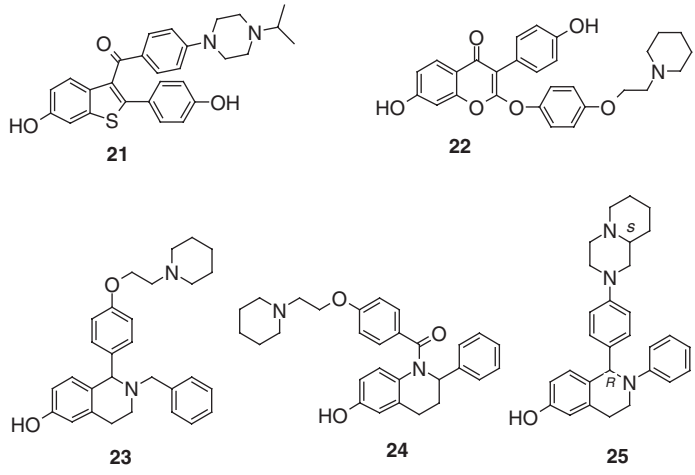


19 20

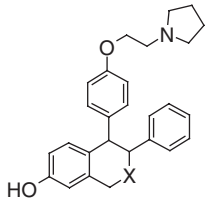
Other scaffolds that have demonstrated selectivity for ERα are shown below. Benzothiophene **21** has high affinity for ERα (0.23 nM) with >100 fold selectivity [47]. This compound is a potent estrogen agonist on bone and an antagonist in uterine tissue. Other ERα SERMs include isoflavone **22** [48], tetrahydroisoquinoline **23** (~20-fold) [49], and tetrahydroquinoline **24** (>10-fold) [50]. A series of tetrahydroisoquinoline ligands incorporate novel conformationally restricted side chains as a replacement for the traditional aminoethoxy functionality [51]. For example, inclusion of the diazadecalin side chain found in **25** provides a compound which demonstrates reduced agonism in MCF-7 cell proliferation relative to the corresponding compound bearing a piperidine side-chain. Substitution of the *N*-phenyl moiety with a phenolic hydroxyl group at the 3-position enhances selectivity for ERα.



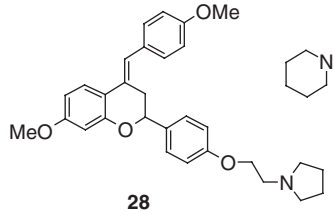
5. OTHER NOVEL SERM SCAFFOLDS

Despite the discovery of ERα selective SERMs and their subsequent use as chemical tools to evaluate the pharmacological significance of this receptor subtype, the respective roles that ERα and ERβ play in regulating tissue selectivity is not yet clear. As a result, the identification of non-receptor subtype selective SERMs remains of considerable interest. Along these lines, isochroman **26** and

isothiochroman **27** have been prepared based on structural analogy to lasofoxifene [52]. Compound **27** demonstrates potent affinity to both ERs but is a mixed agonist/antagonist on the uterus of immature rats. A series of benzopyrans, such as **28**, demonstrate partial estrogen antagonist activity in rats [53], and a benzopyranone SERM **29** that binds with good affinity to ERα, is a functional antagonist of breast cancer cell proliferation and inhibits IL6 production in an osteosarcoma cell line transfected with ERα [54]. A related class of analogs, benzopyran **30** [55], binds with high affinity to ERα and ERβ, is a uterine antagonist, and prevents bone loss in rats [56]. Benzothienoindole SERM **31** shows high affinity to both ERs and significantly increases the bone mineral density of ovx mice while being less uterotrophic than **2** [57]. Novel quinoline-derived SERMs have been identified through the use of co-factor recruitment assays thus providing a complementary method for discovering SERM chemotypes [58]. In this context, quinoline **32** exhibited a profile distinct from 17β-estradiol and 4-hydroxytamoxifen. Imidazoline **33** provides an ERα antagonist profile after structural modifications to the central core scaffold which included the attachment of a basic side-chain and an ethyl group at C2 [59]. Phenanthrenes with basic amine side-chains such as **34** show anti-proliferative activity against breast cancer cells and anti-tumor efficacy in DMBA-induced hormone-dependent mammary tumors in rats [60]. Direct binding interactions with the ER have not been reported.



26, X = O
27, X = S



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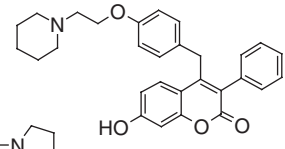

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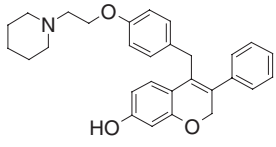

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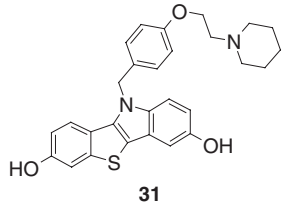

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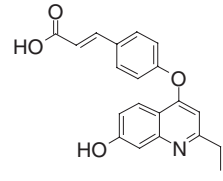

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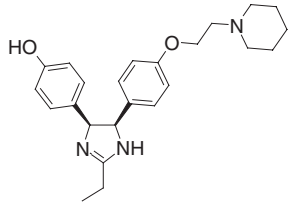

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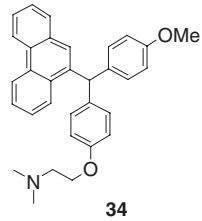
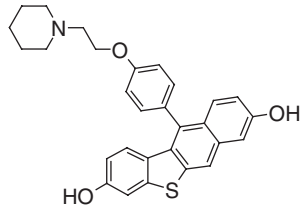


34

The orientation of the pendant aryl ring that connects the basic side to the embedded stilbene core has also been studied [61]. The relative position of the side chain was hypothesized to have a significant impact on functional activity depending on whether this bond is sp2 or sp3 hybridized. For example, while compounds **35** and **36** bind with similar affinity to the ERs, the analog that has sp2 geometry, i.e., **35**, is significantly less potent than **36** in its ability to inhibit uterine cell proliferation in Ishikawa cells. Likewise, naphthalene SERM **37** is less potent than **38** in functional assays. These data are consistent with previous studies showing that the orientation of the side-chain with respect to the stilbene core plays an important role in regulating functional activity [62].



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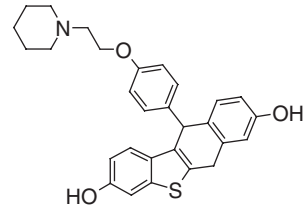

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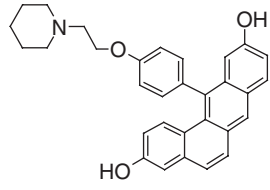

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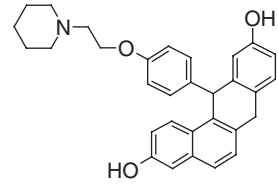
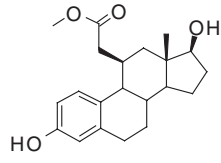


38

6. STEROID-DERIVED SCAFFOLDS

An agonist/antagonist switch has been reported when comparing **39** and **40**, i.e., the extension of the ester functionality by a single methylene unit converts a potent estrogen agonist (methyl ester **39**) into an antagonist (ethyl ester **40**) in Ishikawa cells. In estrogen-dependent rat models, **40** displays SERM-like pharmacology on uterus (antagonism) and bone (agonism) [63]. While **40** was administered subcutaneously in rodents to avoid hydrolysis of the ester to the corresponding carboxylic acid *in vivo*, this potential metabolic conversion cannot be ruled out and may complicate interpretation of these studies.



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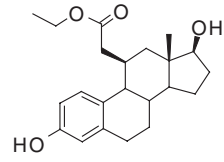

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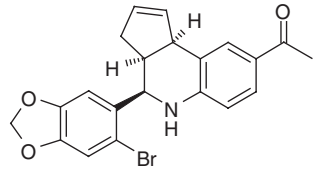
7. NONCLASSICAL ESTROGEN SIGNALING

The classical model of E2 signaling involves binding to ERs within the cell nucleus, followed by receptor dimerization and interaction of ligand-bound receptor and coactivators with estrogen response elements (EREs) located in the promoter region of target genes. Measurable responses can be expected within hours of E2 exposure. In contrast to these gene regulatory effects, other rapid, and presumably nongenomic, effects of E2 are also observed. Since evidence for nongenomic pathways is controversial and still emerging, we will restrict our discussion to those pathways for which novel ligands have been reported.

An estrogen-dendrimer conjugate (EDC) was synthesized in order to provide evidence for nongenomic pathways of estrogen activity [64]. A large, abiotic, and nondegradable poly(amindo)amine (PAMAM) dendrimer was conjugated to estrogen through the 17α-position, which preserved ER affinity, and provided a fluorophore in order to trace cellular localization. Results show that this EDC is localized at the membrane/cytoplasm of cells and is excluded from the nucleus. It effectively stimulates extra-nuclear, nongenomic activities (ERK, Shc, and Src phosphorylation in MCF-7 breast cancer cells) at low concentrations, and is ineffective in stimulating classic ERE-dependent gene expression.

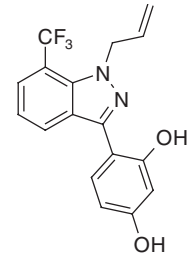
Evidence suggests that one candidate for a membrane-bound ER is the orphan G protein-coupled receptor GPR30 [65]. It has been described as a transmembrane intracellular estrogen receptor located in the endoplasmic reticulum where it binds E2 with high affinity and activates multiple signaling pathways [66–68]. Compound **41** has been disclosed as a ligand for GPR30 [69,70]. It was discovered by virtually screening a library of GPCR-biased compounds for similarity to E2 and then assaying the top 100 compounds in transiently transfected COS7 cells using a fluorescently labeled E2 derivative that binds to both ERs as well as GPR30. Compound **41** has a K_i in GPR30 expressing cells of 11 nM (compared to 5.7 nM for E2) and no substantial binding to either ERα or ERβ up to 1 μM, and also effects mobilization of intracellular Ca^{2+} with an EC_{50} of 2 nM (compared to 0.3 nM for E2) in GPR30 expressing COS7 cells. These data indicate that **41** is an agonist with potency similar to E2 but with complete selectivity for GPR30 over ERα and ERβ.



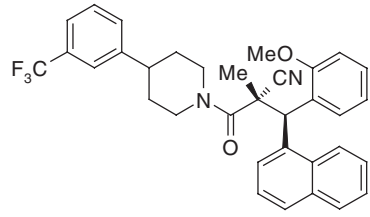
41

Pathway selective ER ligands have been reported that selectively inhibit nuclear factor κB (NF-κB) mediated gene expression [71]. Since NF-κB is a pivotal regulator of pro-inflammatory gene expression, ligands that selectively inhibit the NF-kB pathway could be developed for the treatment of chronic inflammatory diseases such as arthritis, atherosclerosis, sepsis, and inflammatory bowel disease

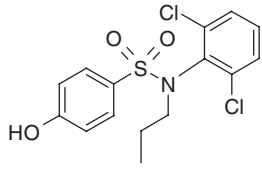
(IBS) [72]. WAY-169916 (**42**) has been described as the first representative of a new class of pathway selective-ER ligands for the treatment of rheumatoid arthritis (RA) [73]. This series of 4-(indazole-3-yl)phenols was discovered by screening for *in vitro* activity using an assay developed in HAECT-1 cells transfected with human ERα and a reporter gene NF-κB-luciferase [74]. WAY-169916 blocks the transcription of NF-kB induced by IL-1β with an IC_{50} of 93 nM and 93% inhibition compared to E2. Since **42** is active only when ER is coexpressed with NF-κB-luc, the inhibition is ER dependent. WAY-169916 has demonstrated inhibition of NF-kB target genes *in vivo*, but does not increase uterine wet weight after five days of dosing in female mice. This result indicates it does not inhibit transcriptional pathways classically associated with ERα in uterine tissues. It is orally active in an adjuvant-induced model of RA [75] and in models of IBS [74]. It also demonstrated activity after SC administration in an ischemia–reperfusion injury model [76]. Since this first report, several more pathway selective-ER ligands have been reported. WAY-204688 has an IC_{50} of 122 nM and 93% inhibition of NF-kB induction with only slight elevation of creatine kinase (CK), a measure of classical E2 effects *in vitro*. It is also highly effective in a neutropenic rat model of *Pseudomonas* sepsis [77]. The 4-hydroxyphenyl sulfonamide **44** has an IC_{50} of 16 nM (96% inhib.) and is 23-fold selective over CK expression [78]. The hydroxybenzoyl-3, 4-dihydroquinoxalin-2(1H)-one **45** has an IC_{50} of 52 nM (95% inhib.) and is a completely selective inhibitor of NF-κB over CK induction [79].



WAY-169916 (**42**)



WAY-204688 (**43**)



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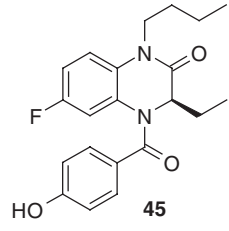

45

8. CONCLUSION

The discovery of structurally novel SERMs continues to provide insights into the complexities of ER signaling. Chemical tools have been developed to test clinical hypotheses that SERMs may be used to treat a variety of diseases ranging from

hot flashes in post-menopausal women to uterine fibroids in pre-menopausal women. Clarity around the role of ERα and ERβ has been aided by the identification of ERα sub-type selective ligands. Lastly, the study of rapid, non-genomic effects of E2 will be enhanced by the availability of ligands that interact with membrane estrogen receptors and pathway-selective ER ligands.

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CHAPTER 11

Lipid-Metabolizing Enzymes as Targets for Dyslipidemia and Insulin Resistance

Vince Yeh, Andrew S. Judd and **Andrew J. Souers**

Contents

1. INTRODUCTION

Obesity is one of the most significant public health concerns in the western world and is associated with numerous co-morbidities, including type 2 diabetes, insulin resistance, dyslipidemia, hypertension, coronary heart disease, stroke, and cancer, among others [1]. The prevalence of obesity in the United States has grown to epidemic proportions. Results from the 2000 National Health and Nutrition Examination Survey III (NHANES III) indicate that over 64% of US adults are overweight or obese, which is roughly double the percentage of 20 years ago [2]. Obesity can be present in different forms that vary by fat distribution, with abdominal fat carrying the highest association with the risk factors involved in cardiovascular disease (CD) [3]. The major risk factors of CD

Metabolic Disease Research, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60064, USA

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include hypercholesterolemia, hypertension, and hyperglycemia, while emerging risk factors include atherogenic dyslipidemia, insulin resistance, and proinflammatory- and prothrombotic states. The relationship of obesity to major and emerging risk factors depends upon the genetic and amassed characteristics of individuals, and is thus variable. The majority of obese persons who develop cardiovascular disease typically have a clustering of these risk factors, which are collectively known as the Metabolic Syndrome [3].

Dyslipidemia is one of the hallmarks of Metabolic Syndrome, and refers to any abnormality in circulating lipid levels including the four subfractions of low-density lipoprotein (LDL), high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), and triglyceride (TG)-rich chylomicrons. LDL is the major atherogenic lipoprotein and has long been targeted for cholesterol-lowering drug therapy. In contrast to LDL, HDL cholesterol levels are inversely correlated with coronary heart disease (CHD) risk. VLDLs are TG-rich lipoproteins secreted from the liver and contain 10–15% of the total serum cholesterol. Partial degradation of these particles affords VLDL remnants, which have been linked to the promotion of atherogenesis, similar to LDL [4]. Finally, chylomicrons are TG-rich lipoproteins that are formed in the intestine from dietary fat and appear in the blood after a fat-containing meal. While abnormalities in chylomicron metabolism are associated with increased risk of coronary events in patients with insulin resistance [5], increased post-prandial levels of triglycerides and apoB-48, both of which are components of chylomicrons and their associated remnants, have been found to predict the presence of coronary heart disease [6].

In 2003, the NHANES database estimated there to be approximately 160 million dyslipidemic subjects in the US. Approximately 101 million of these patients (63%) had only lipid disorder while 50 million (31%) had lipid disorder plus Metabolic Syndrome, and 9 million (6%) had lipid disorder plus diabetes [2]. For sufferers of these maladies, several therapeutics are available for controlling LDL, HDL, and plasma triglyceride levels, including statins, niacin, and fenofibrate. Although these drugs have gained widespread use, a significant number of patients have an inadequate response to these treatments and do not obtain the desired lipid profile. Consequently, several enzymes in the lipid synthesis and metabolism pathways have been investigated recently as potential targets for small molecule intervention. This review provides a synopsis of these efforts, along with representative examples of state of the art small molecule ligands. Some of these have entered clinical trials with varying results, while others are at the pre-clinical stage. Although most or all new therapeutic agents may ultimately be used in combination regimens [7], there still remains a large unmet medical need for both treating patients in the context of obesity, type 2 diabetes or Metabolic Syndrome and halting the progression of mild-moderate dyslipidemia into these more pronounced diseased states.

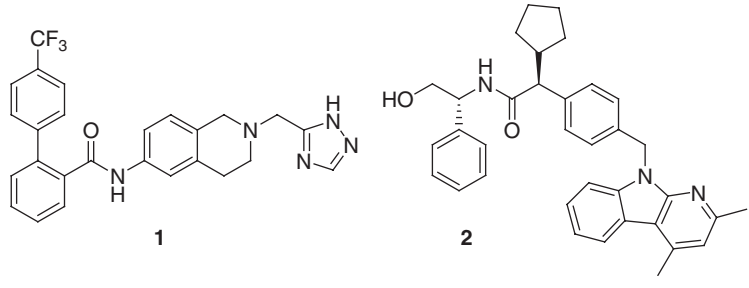
2. LIPID ABSORPTION AND MOBILIZATION

2.1 Microsomal triglyceride-transfer protein (MTP)

Microsomal triglyceride-transfer protein (MTP) plays a pivotal role in the assembly of TG-rich apolipoprotein B-containing VLDL in the liver and

chylomicrons in intestine. It is located within the lumen of the endoplasmic reticulum in hepatocytes and absorptive enterocytes. Its main function is to transfer lipids and cholesterol esters from the endoplasmic reticulum to lipoprotein particles for secretion into the circulation [8]. MTP is a heterodimer composed of a unique large subunit and a ubiquitous multifunctional subunit protein disulfide isomerase [9]. Mutations in the large subunit of MTP cause a rare lipid disorder called abetalipoproteinaemia. The disease is characterized by very low plasma concentrations of cholesterol and TG [10]. MTP is considered a potential target for the treatment of dyslipidemia. However, there are concerns that MTP inhibition could induce symptoms of abetalipoproteinaemia, such as fat-soluble vitamin malabsorption, steatorrhea, and hepatic steatosis.

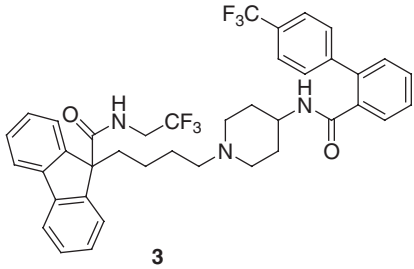
A number of diverse structural types have been reported as MTP inhibitors, and some clinical data have started to appear [8]. CP-346086 **1** was identified as a potent MTP inhibitor (IC_{50} 2.0 nM) via high-throughput screening and robotic-assisted parallel synthesis [11]. Compound **1** has shown robust dose-dependent serum TG lowering in a number of rodent studies. However, MTP inhibition by **1** caused an increase in liver and intestinal TG content when dosed with food. When dosed away from meals, only hepatic TG was increased. In a trial of healthy human volunteers treated with compound **1** (30 mg/day for 2 weeks), significant reductions in total cholesterol (47%), LDL cholesterol (72%), and TG (75%) were observed compared to the placebo group, while no changes were observed in HDL levels in either the drug- or placebo-treated groups [11]. In the presence of a high fat meal, all subjects receiving a single 100 mg dose of **1** experienced diarrhea and abdominal discomfort. These symptoms are consistent with previous observations of patients having an increase in intraintestinal TG concentration. A similar clinical efficacy and adverse-event profile was reported for another MTP inhibitor, implitapide (BAY-13-9952, **2**). In a clinical trial, **2** produced a dose-dependent decrease in total cholesterol (45%), LDL cholesterol (55%), and serum TG (29%) after 4 weeks of treatment at an oral dose of 160 mg/day [12]. Some of the adverse events included gastrointestinal toleration issues associated with steatorrhea and an elevation in the liver enzyme alanine aminotransferase due to liver lipid accumulation.



The structure activity relationship (SAR) and animal model studies of biaryl benzamide MTP inhibitors **3** and **4** have also been reported. Compound **3** has an IC_{50} of 0.5 nM against human MTP in an *in vitro* assay and showed normalization of plasma lipoprotein levels in Watanabe-heritable hyperlipidemic rabbits,

a model for human homozygous familial hypercholesterolemia [13]. Compound **3** was examined in a small clinical trial at four different doses (0.03, 0.1, 0.3, and 1.0 mg/kg) in six subjects. At the highest dose, the reduction in LDL levels was 50.9%. The adverse events were the increase in liver aminotransferase levels and the accumulation of hepatic fat [14].

Diaminoindane **4** has an IC_{50} of 0.7 nM in the *in vitro* MTP inhibition assay, and has shown *in vivo* efficacy in various animal model studies [15]. No clinical trials of **4** have been reported.



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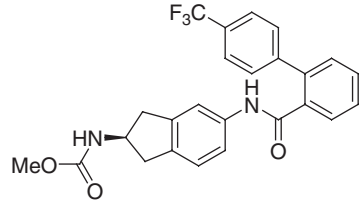

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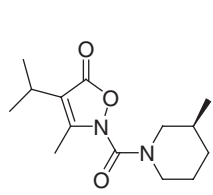
Unlike the previously discussed compounds, which inhibit MTP in both liver and intestine, an intestine-selective orally-active MTP inhibitor JTT-130 (structure not yet disclosed) has been reported to decrease plasma cholesterol and TG in guinea pigs with no hepatic lipid accumulation [16]. Although further studies in human are needed, inhibitors that selectively target intestinal MTP might be a safer alternative as a treatment for hyperlipidemia than the liver-targeting MTP inhibitors.

2.2 Hormone sensitive lipase (HSL)

Increased levels of free fatty acids in plasma are positively linked with the development of insulin resistance and diabetes [17]. Hormone sensitive lipase (HSL) is highly expressed in white and brown adipose tissues where it catalyzes the hydrolysis of stored triglycerides into monoglycerides and fatty acids [18]. The activity of HSL is stimulated by adrenalin/noradrenalin and inhibited by insulin [19]. Suppression of HSL activity by insulin is diminished under diabetic and obese conditions, which leads to excessive circulating fatty acid levels. A number of HSL inhibitors have been described in the literature, however, most of the reports are limited to *in vitro* enzymatic inhibition activities. The 5-(2*H*)-isoxazolonyl urea **5** is a selective HSL inhibitor with an IC_{50} of 5 nM and no activity against other mechanistically related lipases. Compound **5** exhibited robust inhibition of adipocyte lipolysis up to 8 h post dose (30 mg/kg) in *ex vivo* rat studies. In streptozotocin-induced diabetic rats, **5** lowered plasma glucose and free fatty acid levels [20]. The preliminary SAR studies of carbamoyl triazole-based inhibitors, such as **6** ($IC_{50} = 0.17\ \mu M$), which

are proposed to inhibit HSL through a pseudosubstrate mechanism, have also been reported [21].



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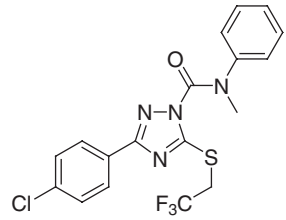

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3. TRIGLYCERIDE SYNTHESIS

3.1 AcylCoA: diacylglyceride *O*-acyltransferase 1 (DGAT-1)

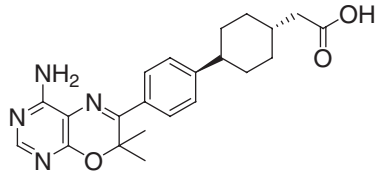
The small intestine is the site of significant triglyceride synthesis in humans, as >95% of the body's lipid is derived from dietary fat [22]. Through the monoacylglycerol pathway, dietary lipids entering the small intestine are hydrolyzed by lipases to free fatty acids and monoacylglycerols in the lumen. These are absorbed by enterocytes, the cells that form the luminal lining of the small intestine. Within enterocytes, fatty acids and monoacylglycerols are recombined by a series of sequential esterification steps involving acylCoA:monoacyl glycerol acyltransferase (MGAT) and DGAT. The triglycerides are then incorporated into nascent chylomicrons, which are secreted from the enterocytes into the lymphatic system. DGAT is required for the production of triglycerides upon absorption of nutrients from the gut, as well as triglycerides produced via *de novo* lipogenesis, and thus represents the sole enzyme common to both pathways [23].

Two enzymes that display DGAT activity have been characterized to date: DGAT-1 (acylCoA:diacylglycerol *O*-acyltransferase type 1) [24] and DGAT-2 (acylCoA:diacylglycerol *O*-acyltransferase type 2) [25]. DGAT-1 and DGAT-2, which share only 12% sequence identity, are both ubiquitously expressed, but the highest expression levels are in tissues typically associated with triglyceride synthesis and storage. DGAT-1 is predominantly expressed in small intestine, liver, and white adipose, while DGAT-2 is most highly expressed in liver and white adipose tissue, and, to a lesser extent, in the small intestine. Significant impetus for pursuing small molecule inhibitors of DGAT-1 was provided by the phenotype of DGAT-$1^{-/-}$ mice. These animals are resistant to diet-induced obesity and have increased sensitivity to insulin and leptin [26,27]. Additionally, DGAT-1 deficient mice are protected against hepatic steatosis, demonstrate increased energy expenditure, and decreased levels of tissue triacylglycerides. In addition to improved triacylglyceride metabolism, DGAT-1 deficient mice also have improved glucose metabolism, with lower glucose and insulin levels following a glucose load in comparison to wild-type mice. Finally, partial DGAT-1

deficiency in heterozygous DGAT-1$^{+/-}$ animals is sufficient to deliver an intermediate phenotype on body weight, adiposity, and insulin and glucose metabolism when compared to wild-type and homozygous littermates [28].

The effort to identify and characterize small molecule inhibitors of DGAT-1 has intensified over the last several years, and several diverse chemotypes have appeared in the literature. The first examples of small molecule inhibitors of DGAT-1 were disclosed in 2004 in the patent literature [29]. The enzymatic activities of well over 100 cyclohexyl acetic acid-based compounds were reported in ranges, and multiple compounds with IC_{50} values <10 nM against recombinant human DGAT-1 were disclosed (exemplified by structure **7**). A patent application from the same year disclosed the evaluation of compounds' effects on food consumption in Sprague–Dawley rats that had been pre-conditioned to a high fat diet (35% fat by weight) [30]. Several compounds were reported to inhibit food intake, with compound **7** conferring a decrease by as much as 24, 30, and 19% at the three time points, respectively.

A second patent application appeared in 2005, in which a series of pyrrolo[1,2-b]pyridazine-5,6-dicarboxylates was disclosed [31]. Several of the described compounds (exemplified by structure **8**) were reported to have IC_{50} values of $<0.5\,\mu$M against DGAT-1. Various hydrophobic and amino substitutions at positions 4 and 7, and 2, respectively, were tolerated on the heterocyclic ring, along with heterocyclic replacements for the 6-substituted ethyl carboxylate.



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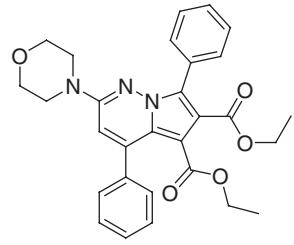

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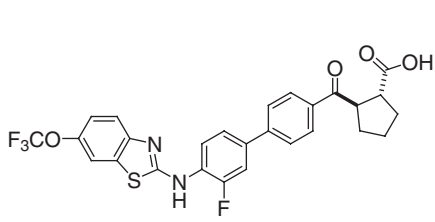
A series of biphenyl ketoacid-based inhibitors of DGAT-1 reported in 2004 is exemplified by structure **9** [32]. This disclosure described assays for both enzymatic potency and inhibition of cellular triglyceride synthesis, although data for specific compounds was not included. Additionally, several compounds were reportedly evaluated for effects on weight loss in DIO mice (C57BL/J6 background) fed on a high fat diet *ad libitum* for 10 weeks. Animals were orally gavaged daily before the dark phase of the light/dark cycle for 8–14 days, and compounds were considered to be active if a statistically significant reduction in body weight was observed after a treatment period of at least 7 days. Similar to the *in vitro* data, specific values were not reported.

A series of thiazole-based DGAT-1 inhibitors typified by the highly lipophilic compound **10** was disclosed [33]. This report described a tail-vein injection assay wherein a test compound (10, 30, and 100 mg/kg) and 20% emulsion containing a fatty acid mix were orally administered to male C57BL/6N mouse at 0.3 ml/mouse. After 1 h following test compound administration, mice were anaesthetized and

intestinal lymph was collected. Fatty acid concentration was measured, and 6 compounds, including **10**, were reported to show more than 60% inhibition of fatty acid excursion in this model at the two highest doses. Interestingly, no difference in fatty acid excursion was observed between the 30 and 100 mg/kg doses.



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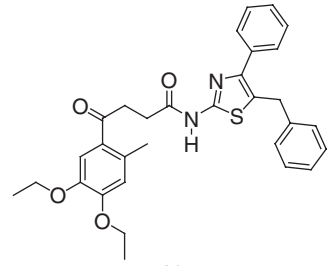
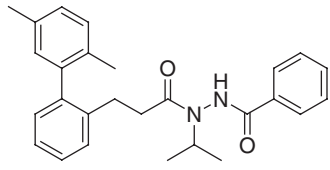


10

Several other diverse structural classes of DGAT-1 inhibitors have appeared in the literature since the seminal disclosure. Two patent applications published in 2006 disclosed related series of benzoic acid hydrazide-based inhibitors. Compound **11** showed an IC_{50} of 48 nM against recombinant human DGAT-1 [34], while oxygenated analog **12** was reported to inhibit cellular triglyceride synthesis with an EC_{50} of 0.23 μM [35]. Compound **13** exemplifies a class of oxadiazole-based inhibitors and was reported to have an IC_{50} of 120 nM against DGAT-1 [36].



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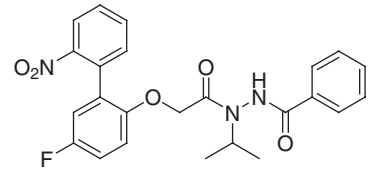

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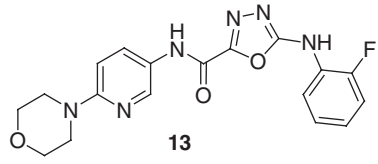


13

Other related classes of small molecule DGAT-1 inhibitors have appeared [37,38], as well as a set of structures that showed modest pan-inhibition of DGAT-1, DGAT-2, and ACAT2 [39]

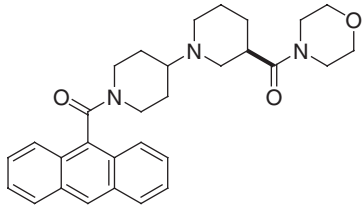
4. FATTY ACID SYNTHESIS AND METABOLISM

4.1 Acetyl-CoA carboxylase (ACC)

Acetyl-CoA carboxylase (ACC) catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the rate limiting, first committed step in fatty

acid biosynthesis [40]. ACC activity is tightly regulated by citrate, long chain fatty acids, and AMP kinases (AMPK) [41]. Malonyl-CoA not only serves as a key substrate in fatty acid synthesis, it also plays an important regulatory role in controlling mitochondrial fatty acid uptake through allosteric inhibition of carnitine palmitoyltransferase I (CPT-I), a mitochondrial outer membrane protein that shuttles long-chain fatty acyl-CoAs into the mitochondria for oxidation [42]. Inhibition of ACC is expected to lower malonyl-CoA levels, resulting in reduction of *de novo* fatty acid synthesis. Stimulation of fatty acid oxidation is also expected, which can consequently improve insulin sensitivity. In mammals, ACC exists in two isoforms encoded by two distinct genes. ACC1 is a 265 kDa cytosolic protein highly expressed in lipogenic tissues (liver and adipose), whereas the 280 kDa ACC2 is associated with the mitochondrial membrane and is primarily expressed in oxidative tissues (muscle, heart, and liver) [43,44].

ACC1 homozygous knockout mice are embryonically lethal [45], while ACC2 homozygous knockout mice are healthy and exhibit favorable metabolic phenotypes, such as increased fatty acid oxidation, reduced hepatic triglyceride content, and decreased body weight despite increased food intake [46]. Studies using liver-selective antisense oligonucleotides targeting ACC1 and ACC2 in a diet-induced fatty liver rat model have demonstrated that selective suppression of either ACC1 or ACC2 had moderate or no effect on lipogenesis, whereas suppression of both ACC1 and ACC2 stimulated fatty acid oxidation, lowered hepatic triglycerides, and improved insulin sensitivity [47]. Bipiperidylcarboxamide CP-640186 (**14**) is an isoform non-selective ACC inhibitor (IC_{50} ~60 nM). In Hep-2G cells (hepatocytes), **14** lowered fatty acid synthesis, TG synthesis, TG secretion, and apo-B secretion without affecting cholesterol synthesis. In addition, **14** increased fatty acid oxidation in mouse muscle cells. Treatment of *ob*/*ob* mice with **14** reduced malonyl-CoA in liver and muscle, reduced adipose and liver fatty acid synthesis, and increased whole body fatty acid oxidation [48]. The polyketide natural product soraphen A (**15**) is one of the most potent isoform non-selective ACC inhibitors known (IC_{50} 1–5 nM) [49]. It was shown that **15** stimulated fatty acid oxidation in Hep-G2 cells and in Wistar rats. Compound **15** also increased total lipid oxidation in Wistar rats at doses of 10 and 30 mg/kg [50].



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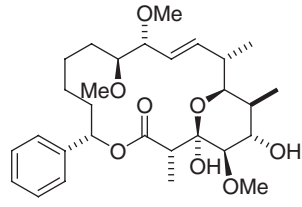
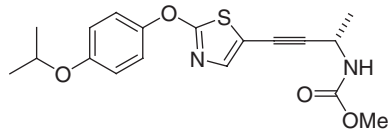


15

ACC2 selective inhibitors exemplified by **16** (ACC1 IC_{50} >30 μM, ACC2 IC_{50} 38 nM) have also been reported and were shown to reduce malonyl CoA levels in muscle tissues of Sprague–Dawley rats [51]. Although ACC inhibitors have

shown promising results in experimental animals, clinical efficacy has yet to be demonstrated.



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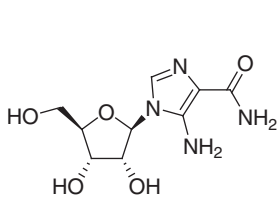
4.2 AMP-activated protein kinase (AMPK)

AMPK plays a central role in cellular metabolism as a key fuel gauge and regulator of energy consumption and storage [52,53]. AMPK is a serine–threonine kinase that consists of heterotrimeric α, β, and γ subunits. The α subunit possesses the catalytic site; the β subunit contains a glycogen-binding domain; and the γ subunit has the AMP-binding site [54]. AMPK is ubiquitously expressed in various tissues and is found in cytosol. Under conditions of energy depletion (low ATP vs. AMP ratio), AMPK is activated by AMP and leads to phosphorylation of a number of target molecules that result in increases in ATP-generating processes, such as fatty acid oxidation, muscle glucose intake, and cardiac glycolysis. Simultaneously, AMPK also inhibits ATP-consuming pathways, such as fatty acid synthesis, cholesterol synthesis, and gluconeogenesis, thus restoring overall cellular energy homeostasis [55,56]. In terms of lipid metabolism, enzymes that are inhibited by AMPK phosphorylation include the carboxylase ACC, glycerol-3-phosphate acyltransferase (GPAT), fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) [57,58]. AMPK α2 subunit specific knockout mice exhibit glucose intolerance, chronically elevated free fatty acid levels, and increase in body weight [59]. In contrast, overexpression of the α2 subunit results in reduced blood glucose and increased hepatic fatty acid oxidation [60].

AMPK is directly activated by physical exercise [61] and by the adipokines adiponectin and leptin [62,63]. AMPK is also activated by treatment with antidiabetic drugs metformin [64] and thiazolidinediones (TZDs) [65]. However, these compounds may not act on AMPK directly. The AMP analog 5-aminoimidazole-4-carboxamide 1β-D-ribofuranoside (AICAR, **17**) is phosphorylated within cells to yield a potent but non-specific activator of AMPK. Chronic treatment of obese Zucker *fa/fa* rats with AICAR produces effects very similar to those of metformin [66]. The biological effects of AICAR are confounded by the dual ability of AICAR to stimulate glycogen phosphorylase (GPPase) and to inhibit fructose-1,6-bisphosphatase (FBPase), both of which can lead to the lowering of blood glucose levels and an increase in insulin sensitivity [67,68]. Despite the intense interest in this target, there are very few publications describing direct activators of AMPK. A series of thienopyridones has been identified as non-nucleoside direct activators of AMPK [69]. A-769662 (**18**) directly stimulated partially purified rat liver AMPK ($EC_{50} = 0.7\,\mu M$). The compound inhibited fatty

acid synthesis in primary rat hepatocytes ($IC_{50} = 3.2\,\mu M$) and increased ACC2 phosphorylation, hence confirming a functional consequence of AMPK activation. Treatment of *ob/ob* mice with **18** (30 mg/kg, bid) resulted in lowering of plasma glucose by 40%, lowering of body weight gain, and significant lowering of both plasma and liver TG levels. Due to poor oral bioavailability of this compound, the studies were carried out by i.p. injection. Further improvements in the physiochemical properties of **18** or identification of other chemotypes would greatly stimulate interest in AMPK as a target for diabetes and dyslipidemia.



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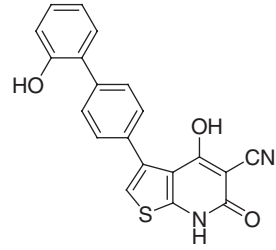

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4.3 Stearoyl-CoA desaturase (SCD)

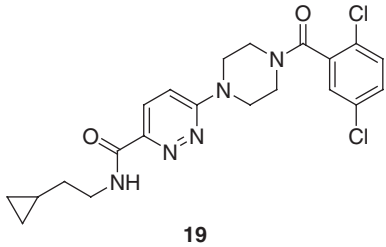
Stearoyl-CoA desaturase (SCD) is a key lipogenic enzyme involved in the biosynthesis of mono-unsaturated fatty acids. Its preferred substrates are long-chain acyl-CoAs, such as palmitoyl (16:0)-CoA and stearoyl (18:0)-CoA, which are desaturated to give palmitoleoyl (16:1)-CoA and oleoyl (18:1)-CoA, respectively [70]. These products, in turn, are the predominant mono-unsaturated fatty acid components of triglycerides, phospholipids, and cholesteryl- and wax-esters [71]. Mono-unsaturated fatty acids also serve as components of signal transduction through effects on protein kinases and transcription factor activation. Therefore, changes in the activity or levels of SCD would be expected to alter lipoprotein metabolism, adiposity, membrane fluidity, and signal transduction.

To date, four murine isoforms of SCD (SCD1–4) have been identified [72–75]. Although the four isoforms share considerable sequence homology (>80% amino acid sequence identity) and catalyze the same biochemical transform, their tissue distribution varies. For example, SCD1 is expressed in lipogenic tissues, such as liver, adipose, and sebaceous glands [76]. SCD2 is ubiquitously expressed in most tissues, with the exception of liver [76], while SCD3 is found in the Harderian gland [74] and SCD4 primarily in the heart [75]. Additionally, two human SCD genes with >85% homology to murine SCD1 have been identified [77,78].

SCD1-deficient asebia mice bred onto a leptin-deficient (*ob/ob*) background show reduced adiposity despite higher food intake and have a corrected hypometabolic phenotype, suggesting that down-regulation of SCD1 is an important component of leptin's metabolic actions [79]. SCD1 knockout mice are viable and

are resistant to diet-induced obesity, have lowered plasma VLDL and TG levels, and show increased energy expenditure and insulin sensitivity [80–82]. However, in addition to these favorable phenotypic components, SCD1-deficient mice develop cutaneous abnormalities including alopecia and narrow eye fissure [83]. More recently, pharmacological inhibition of SCD1 in mice with SCD1 antisense oligonucleotides has recapitulated characteristics of the KO animal, such as the improved insulin sensitivity and resistance to diet-induced obesity, in the *absence* of alopecia [84].

A number of patent applications that describe small molecule inhibitors of SCD-1 have recently published. For example, a series of patent applications describe related piperazine-based inhibitors of SCD-1 (exemplified by **19**) [85]. Piperazine replacements, such as piperidine, 4-aminopiperidine, and 3-aminoazetidine are tolerated, as are thiadiazole and pyridine surrogates of the 1,2-diazine [86–90].



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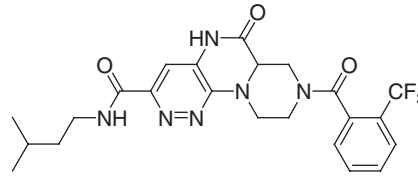

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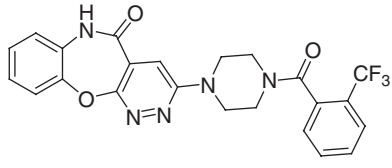
Constrained variants, such as **20** and **21** have also been reported [91,92]. Interestingly, the diazine-amide portion of **19** can be mimicked by the imidazo[1,2-b]pyridazine in **22** [93]. Presently, work in this field remains at a pre-clinical stage.



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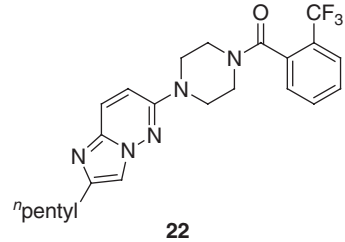

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5. CONCLUSION

The targeting of the lipid absorption and metabolism pathways has yielded several promising venues for the treatment of dyslipidemia and insulin resistance. Small molecule inhibitors of MTP have conferred significant reductions in total and LDL cholesterol, as well as plasma TG, in human subjects. While some mechanism-related side effects due to increased hepatic and intraintestinal TG

concentration have been observed, the selective targeting of intestinal MTP may confer significant advantages with respect to the side effect profile. Hormone sensitive lipase is still in the very early stages of drug development as a target, while efforts targeting enzymes involved with triglyceride and fatty acid synthesis are primarily in the pre-clinical stages. Results from genetically altered animals of DGAT-1 and SCD-1 provide significant impetus for the development of small molecule drugs. Effects on organs, such as skin and mammary glands will have to be considered going forward; however, the partial- or tissue-selective inhibition of these enzymes could yield novel and effective drugs with suitable therapeutic windows. Finally, inhibition and activation of ACC and AMPK, respectively, via small molecules represent intriguing possibilities to both mobilize fat oxidation as well as limit fatty acid synthesis.

Because of the polyfactorial nature of disease states, such as obesity, type 2 diabetes, and Metabolic Syndrome, it is expected that drugs targeting the lipid synthesis and metabolism pathways will be used in the context of combination therapy [7]. Pre-clinical and clinical results to date indicate that pronounced efficacy could be achieved toward the management of associated lipid levels and insulin resistance, and thus, investigation in these areas provides significant promise.

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CHAPTER 12

Recent Trends in HDL Modulating Therapies ☆

Dibyendu De and **Ish Khanna**

Contents

1. INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in both men and women in developed countries. Elevated levels of LDL-cholesterol (LDL-C), triglycerides (TG) and low levels of HDL are the key risk factors that are associated with atherosclerotic cardiovascular disease (CVD). Recent and ongoing clinical trials continue to demonstrate that aggressive lowering of LDL with statins yields significant benefits and a marked reduction in cardiovascular events. However, despite aggressive statin therapy, a significant number of patients ($>50\%$) continue to demonstrate a high risk of cardiovascular events [1,2]. These studies

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Reddy US Therapeutics, Inc. 3065 Northwoods Circle, Norcross, GA 30071-1542, USA

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suggest the limitations of statin monotherapy in preventing progression of atherosclerotic disease. Additional therapeutic approaches that modulate other lipid-related risk factors are needed to reduce and prevent atherosclerotic lesions.

Low levels of HDL have a strong genetic component, and are recognized as an important risk factor for CVD. Low HDL is a common lipid abnormality in patients with coronary heart disease (CHD), and is the primary abnormality in about half of these patients [3,4]. Epidemiological studies suggest that a 1 mg/dl increase in plasma HDL levels reduces the risk of CVD by 2–3% [5–7]. As reviewed below, treatment for low levels of HDL is emerging as an important paradigm to prevent atherosclerotic CVD. The pharmaceutical industry is exploring a number of attractive approaches and strategies directed at regulating the catabolism and function of HDL.

2. HDL AND ATHEROSCLEROSIS

HDL plays an important role in removing cholesterol from macrophages in the arterial wall and transporting it to the liver for excretion through bile and feces. This process is termed ''reverse cholesterol transport'' (RCT). HDL is synthesized in the liver and intestine as a small, nascent apolipoprotein (apoA-1) and secreted into plasma where it is lipidated through ABCA1 participation. The lipidated apoA-I is cardio-protective and critical to the efflux of cholesterol from macrophages through ABCA1 mediation. The cholesterol-rich, discoidal HDL is esterified by the enzyme lecithin cholesterol acyltransferase (LCAT) to give mature, spherical HDL and is responsible for moving cholesterol to the liver for uptake by the scavenger receptor (SR-B1). Cholesterol from mature HDL particles may also be transferred to LDL/VLDL by the cholesterol ester transfer protein (CETP) in exchange for TG from VLDL. Therefore, CETP-mediated cholesterol transfer could lead to low HDL levels and may potentially be atherogenic. Inhibitors of CETP are being clinically evaluated for their ability to raise HDL levels and reduce atherosclerotic lesions (*vide infra*).

Besides cholesterol efflux from arterial wall and its role in RCT, additional properties of HDL have been proposed for its protective anti-atherogenic activities. HDL protects vascular function by a number of potential alternative mechanisms, including inhibition of LDL oxidation [8,9], platelet aggregation and coagulation [10], and endothelial monocyte adhesion [11], as well as promotion of endothelial nitric oxide synthase (eNOS) [12], and prostacyclin synthesis [13–15]. The proposed alternate protective mechanisms for HDL are attractive but many of them lack validation under *in vivo* conditions.

3. SCOPE AND LIMITATIONS OF MARKETED THERAPIES

Despite a large body of evidence supporting the atheroprotective benefits of HDL, drugs available on the market that raise HDL are limited. Amongst the marketed drugs, the statins offer only marginal increases (5–10%), while fibrates elevate HDL levels by 10–15%. As indicated in Table 1, agents belonging to these classes have primarily been used to modulate LDL and TGs respectively. Of the

Table 1 Lipid profile and side effects of commercially available drugs

Drugs	% LDL↓	% TG↓	% HDL↑	Side effects
Statins [3,16]	20–60	20–40	2–10	Muscle toxicity, liver enzyme elevation
Fibrates [17]	8–13	18–48	10–15	Gastrointestinal, renal toxicity
Niacin ER [17,18]	10–22	11–40	10–30	Skin flushing, gastrointestinal, hepatic toxicity
Niacin ER/ lovastatin [19]	25–45	16–42	30–41	Flushing, pruritis, gastro-intestinal symptoms
Ezetimibe [20,21]	23–26	2–8	1.3–6	
Colesevelam [22,23]	14–28	–	2–3	Caution in patients with fat-soluble vitamin deficiency

commercially available drugs, niacin offers the best profile and increases HDL by 20–30%. A summary of the commercially available options and their lipid profiles is presented in Table 1.

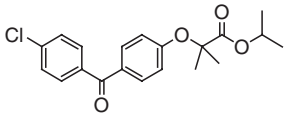
3.1 Statins

As indicated in Table 1, statins, which block cholesterol biosynthesis by inhibition of hepatic HMGCoA reductase, have been used extensively to reduce LDL-C levels. At most therapeutic doses, statins marginally increase HDL levels by 5–10% [3,16]. The HDL elevation observed with statins has been highly variable and not easily extrapolated from the effects on LDL. A recent study (STELLAR) demonstrated increased HDL elevation with the use of rosuvastatin compared to simvastatin, pravastatin or atorvastatin (10% vs. 2–6%) [16,24]. Although the mechanism of HDL elevation by statins is not clearly understood, it is proposed that statins enhance hepatic apoA-I synthesis [25] and decrease apoB-containing lipoproteins [26]. A number of clinical trials have demonstrated that statins reduce the risk of major coronary events. However, it is not clear if the statin-induced rise in HDL levels is an independent contributor to the reduced risk of coronary events. The observed small increase in HDL and adverse side effect profile related to liver function abnormalities and muscle toxicity limits the use of statins as monotherapy for HDL elevation [27].

3.2 Fibrates

Fibrates, such as fenofibrate (**1**), gemfibrozil (**2**), bezafibrate (**3**), clofibrate (**4**) and ciprofibrate (**5**) moderately enhance HDL levels by 10–15% [17]. Fibrates are

weak activators of PPARα and, as indicated in Table 1, decrease TG levels and increase HDL [28]. Different mechanisms have been proposed by which fibrates elevate plasma HDL levels [29]. Induction of PPARα in the liver leads to increased synthesis of apoA-I, resulting in enhancement of new HDL particles [30]. Studies on mouse hepatocytes indicate down-regulation of SR-B1 protein levels upon treatment with fibrates, possibly influencing clearance of HDL [31]. A meta-analysis of data from 53 clinical studies using fibrates **1**, **3**, **4** and **5** demonstrated a 25% reduction in the risk of coronary events [17]. A number of clinical studies have demonstrated the beneficial CV-related effects of gemfibrozil treatment. In the Helsinki Heart Study, CVD risk reduction upon gemfibrozil treatment was most pronounced in patients with atherogenic dyslipidemia, metabolic syndrome and diabetes [32,33]. In the veterans affairs high-density lipoprotein cholesterol intervention trial (VA-HIT) study, a 5% increase of plasma HDL resulted in an 11% risk reduction in the hazard ratio for CHD events [34,35]. These observations indicate that fibrates are useful to treat the CV risk in patients with dyslipidemia and insulin-resistant diabetes. The FIELD study with fenofibrate treatment indicated a non-significant decrease in deaths from CHD or non-fatal myocardial infarction (MI) [36,37]. However, recent clinical studies with compound **1** showed an elevation in serum creatinine and creatinine phosphokinase, suggesting potential safety issues associated with fenofibrate treatment [28].



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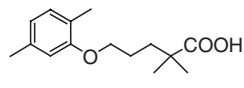

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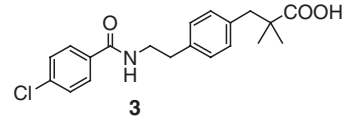

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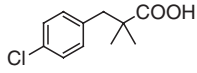

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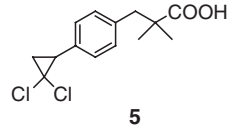

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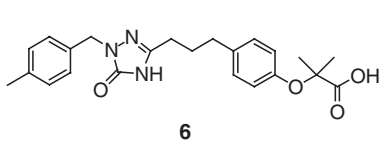
The search for more potent, selective and safe PPARα agonists has been challenging and only a limited number of compounds have progressed into the clinic. A number of phenoxyacetic acid derivatives and other diverse structures have emerged recently. Oral administration of LY-518674 (**6**) produced a 208% elevation in HDL and a 96% decrease in serum TG in apoA-I transgenic mice [38,39]. Recent clinical studies with compound **6** revealed a decrease in TG and an increase in HDL similar to fenofibrate. However, compound **6** also raised LDL-C in a dose-dependent fashion, and to a much higher level than seen with fenofibrate [28]. Both agents also raised serum creatinine levels above the upper limits of normal in 35–38% of patients [28].



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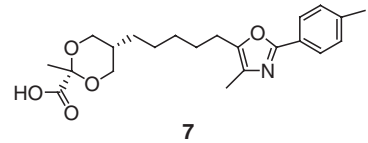

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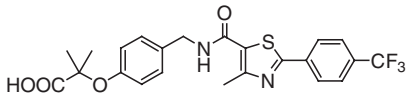

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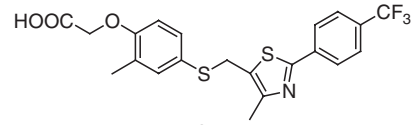

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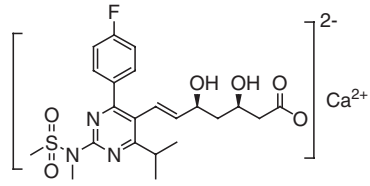

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A novel compound, NS 220 (**7**), from a 1,3-dioxane-2-carboxylic acid series, is being developed for the treatment of lipid metabolic disorder. Compound **7** is a highly selective PPARα agonist and oral administration to mice resulted in a substantial increase in HDL [40]. A Phase I clinical trial of **7** was completed in December 2006. A fibric acid derivative, GW 590735 (**8**), is also a highly potent and selective PPARα agonist [41]. In an apoA-I transgenic mouse model, GW 590735 afforded a 51% increase in HDL and a 48% decrease in TG at an oral dose of 1 mg/kg. Interestingly, GW 590735 was developed by structural modifications of GW 501516 (**9**), a potent PPARδ agonist [41]. Another alkyl carboxylic acid derivative, K-111 (formerly BM-170744), has also shown improved selectivity for PPARα over other PPAR-subtypes and this compound has been under Phase II clinical investigation [42].

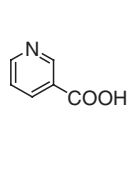
Fibrates are being combined with statins to expand their potential in the dyslipidemia market. A recent clinical study examined the effects of rosuvastatin (**10**) and fenofibrate as mono and combination therapy in hyperlipidemic diabetic patients [43]. In late 2006, large scale Phase III clinical trials of rosuvastatin in combination with a next-generation fibrate, ABT 335, were initiated for evaluation of safety and efficacy in patients with mixed dyslipidemia.

3.3 Niacin

Niacin (**11**) is the most potent HDL-raising drug (20–30%) on the market, and also provides some reduction in TG and LDL-C levels. Clinical studies with niacin as monotherapy or in combination with statins have yielded marked reductions in

cardiovascular events and enhanced angiographic plaque regression. Niacin's utility has, however, been limited by its adverse effects, in particular cutaneous vasodilation and hepatotoxicity. More than 90% of patients experience flushing to the face, neck and upper body during the initial stages of niacin treatment but seem to develop some tolerance after multiple dosing. The therapeutic dose of the immediate release (IR) niacin formulation is very high, 1–3 g/day. The sustained release (SR) niacin formulation reduces flushing but seems less effective than the IR formulation in raising HDL. The extended release (ER) formulation offers a better modulation of efficacy and cutaneous vasodilation, but tolerability continues to limit its use.

The flushing effects seen with niacin (**12**) are believed to be related to release of inflammatory prostaglandins from dendritic cells. Pretreatment with NSAIDs such as aspirin and ibuprofen reduces the incidence of flushing. MK-524A is under evaluation in Phase III trials as a combination of niacin ER and the DP-1 antagonist MK-524 (**12**) [44,45]. The results from clinical trials suggest that MK-524A suppresses niacin-induced flushing by >80% while retaining the lipid-modifying efficacy of niacin ER [46]. The residual flushing appears to be tempered by treatment with aspirin. A triple combination pill, MK-524B, which combines MK-524A, and simvastatin (**13**), is also under development as an oral lipid modulator for the prevention of CHD and atherosclerosis [47].



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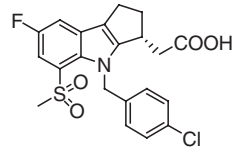

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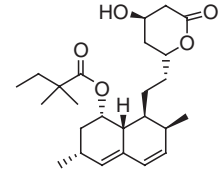

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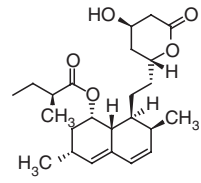
Niacin inhibits cAMP-mediated lipolysis in adipose tissues. More recently, two niacin receptors, GPR109a (HM74A) and GPR109b (HM74B), have been identified [48,49]. Niacin binds to GPR109a with high affinity and selectivity over GPR109b [50]. It has also been established that the anti-dyslipidemic effects of niacin as well as the observed flushing are mediated through GPR109a. Selective GPR109b agonists also inhibit lipolysis in human adipocytes. The hypothesis that selective niacin receptor agonists may offer an improved therapeutic index is being pursued by a number of companies as evidenced from recent literature reports [51–54]. Selective niacin receptor agonists such as MK-354, and GSK-256073 have entered clinical trials; however MK-354 appears to have been discontinued for atherosclerosis.

In clinical trials, the combination of niacin with lovastatin (**14**) afforded significant HDL elevation (30%) and reduced LDL-C (47%) and TG (41%) after 16 weeks of treatment [19]. At 52 weeks of treatment, HDL increased by 41% with this combination therapy [19].



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4. EMERGING CLINICAL COMPOUNDS

A brief summary of different clinical approaches including their profile and status is discussed below.

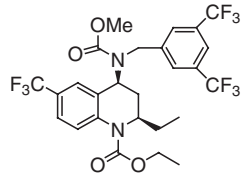
4.1 CETP inhibitors

CETP is a hydrophobic glycoprotein of hepatic origin that circulates in plasma and is bound mainly to HDL. It mediates the transfer of cholesterol ester (CE) from HDL to LDL and VLDL, and of TGs from VLDL to LDL and HDL. This results in a net reduction in HDL and an increase in LDL-C plasma levels, implicating CETP in the pathogenesis of atherosclerosis [55–57]. The crystal structure of CETP has been published recently [58]. It reveals a 60 Å long tunnel filled with two hydrophobic CEs and plugged by an amphiphilic phosphatidylcholine at each end. The topography of this tunnel suits the transfer of neutral lipids such that CETP admits a neutral lipid from the opening at one end, and deposits a bound lipid from the opposite end.

Studies of CETP polymorphism and genetic deficiency suggest direct links between CETP and HDL levels [58]. The precise role of CETP in promoting atherosclerosis has been under debate [59,60]. It is potentially atherogenic because it transfers CE from HDL to VLDL and LDL. In contrast, CETP may potentially be anti-atherogenic because it facilitates cholesterol removal by the RCT mechanism [61–63]. During recent years, several pharmaceutical companies have been evaluating small molecule inhibitors of CETP as potential therapeutic agents for the treatment of atherosclerosis.

4.1.1 CETP inhibitors in the clinic

Torcetrapib (CP-529414, **15**) is an inhibitor of CETP with an IC_{50} of 0.05 μM. Its history of development has been reviewed extensively [64–69].



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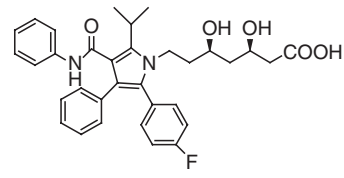

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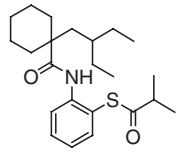
In preclinical studies in the rabbit, torcetrapib shows excellent HDL elevation and inhibition of aortic atherosclerosis [70]. The results from Phase II studies of **15** in combination with the HMGCoA reductase inhibitor atorvastain (**16**) showed excellent lipid control (~50% increase in HDL and ~30–50% decrease in LDL-C) [71]. However, Phase II studies also detected an increase (1–2 mm Hg) in systolic blood pressure (BP) in a small number of patients [71]. Moreover, approximately 4% of the subjects in Phase II studies experienced BP elevation in excess of 15 mm Hg [72]. In Phase III studies (ILLUMINATE), slightly higher BP elevation, an average of 3–4 mm Hg, was observed in patients [72]. The ILLUMINATE study was terminated in December 2006 because of statistically significant cardiovascular events in the treatment group. An ongoing analysis of clinical data from ILLUMINATE study is awaited and should provide greater insights into the reason(s) for the failure of **15**. Furthermore, results from the most recent Phase III trials (ILLUSTRATE and RADIANCE) showed that **15** did not affect atherosclerotic plaque volume, despite significant increases in HDL levels over 2 years [73,74]. This has raised an interesting debate on the quality of HDL being raised by CETP inhibition and the influence of BP increase on clinical outcome. It has also raised questions as to whether the observed clinical outcome is target-mediated or torcetrapib-specific.

JTT-705 (**17**) is an irreversible inhibitor of CETP ($IC_{50} = 6\,\mu M$) and is believed to affect the function of CETP protein by binding to its Cys^{13} residue. In rabbits, **17** dose-dependently reduced CETP activity, raised HDL and reduced atherosclerotic lesions [75]. The results from Phase-II clinical trials on **17** show good HDL elevation (26–37% at 600–900 mg/day) during 4 weeks of treatment [64,76–78]. Compound **17** is also reported to lack hypertensive effects.



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MK-0859 is another CETP inhibitor that has advanced into Phase II clinical trials [79,80]. In addition, a number of novel inhibitors of CETP have emerged from different pharmaceutical companies but their current status is unclear [81–88].

4.2 HDL mimetics

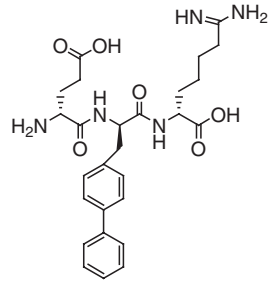
ApoA-1 is the major structural lipoprotein component of HDL particles. Transgenic over-expression of apoA-1 has been well documented to correlate very strongly with antiatherogenic effects seen in a number of animal models [89–91]. The genetic deficiency of apoA-1 in humans has also been linked to low levels of HDL and premature atherosclerosis [90–92]. It is believed that infusion of apoA-1 enhances the ABCA1-mediated cholesterol efflux from macrophages [93]. During the last decade, significant efforts have been spent to find small

molecule agents such as fenofibrate, gemfibrozil that either induce the apoA-1 gene or mimic the effects of apoA-1 upregulation by delivery of exogenous apoA-1 variants. Despite extensive research, success in obtaining selective and safe small molecules that upregulate apoA-1 has been limited. This has led to an increased pursuit of novel mimetics of apoA-1.

A recent study on recombinant apoA-1 Milano-phosphatidylcholine complex (ETC-216) has infused renewed interest in the field [94]. This agent differs from native protein by an arginine in place of cysteine at position 173. In a placebo-controlled Phase II study, 5 weekly infusions of ETC-216 produced significant regression in plaque size compared to baseline, whereas in the placebo group there was no detectable change in atherosclerosis, as measured by IVUS [94]. However, this study was conducted on a small number of patients and may have lacked statistical power. There were also observations such as stroke and cholelithiasis in the treatment group that might need to be monitored in larger trials.

In addition, peptides with amino acid sequences mimicking the structure of apoA-1 are being developed [95,96]. One such peptide in Phase I development is D-4F (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2), an 18 amino acid peptide designed to contain a class A amphipathic helix [96]. It is not recognized by gut peptidases and is partially absorbed. Some data suggests that D-4F enhances the anti-inflammatory function of HDL without increasing plasma HDL levels [96]. D-4F (APP018) is in clinical trials as an oral apoA-1 mimetic for the treatment of atherosclerosis. A reverse D-4F analog is believed to be as active as D-4F in apoE-knock-out mice [97,98]. Reverse D-4F showed potent anti-inflammatory properties *in vitro* and reduced atherosclerotic lesions in apoE-knock-out mice [98].

AVP-26452 (**18**) is an orally active small molecule apoA-1 mimetic. It reduces atherosclerotic plaques via RCT in apoE-knock-out mice [99]. The compound entered Phase I clinical trials but its current status is unknown.



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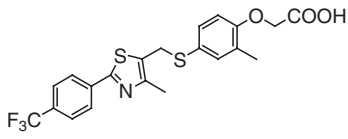
4.3 PPARδ agonists

PPARδ activation in animal models has been shown to elevate HDL levels. Using structure-guided studies, a high-affinity PPARδ specific-ligand, GW-501516 (**19**) (EC_{50} = 8 nM), was discovered, which recently advanced to Phase II

clinical trials [100]. In preclinical studies, the treatment of insulin-resistant, obese rhesus monkeys with **19** resulted in a 79% increase in HDL, a 56% decrease in TG, and a 29% decrease in LDL-C [101,102]. This result suggests that PPARδ agonists may have exceptional potential to raise low HDL levels. The significant increase in HDL levels correlates with an increase in plasma apoA-I, apoA-II, and apoC-III. PPARδ activation by compound **19** increases cholesterol efflux from peripheral cells via elevation of ABCA1 expression and also reduces intestinal cholesterol absorption via down-regulation of the *Niemann-Pick C1-like gene*, a key mediator of intestinal cholesterol absorption [101,103]. A number of novel compounds with high specificity for PPARδ have been reported in the literature recently, including **20** ($EC_{50} = 4$ nM) [104] and **21** ($EC_{50} = 53$ nM) [105]. However, none, other than **19**, have advanced into the clinic. Unlike PPARα agonists, no reports of PPARδ-induced myopathy have appeared, despite its abundance in muscle fibres, although *in vitro* experiments did not rule out this possibility [106].



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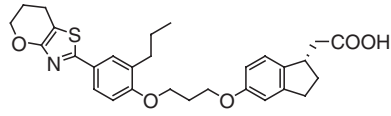

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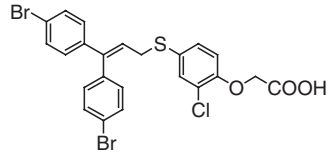

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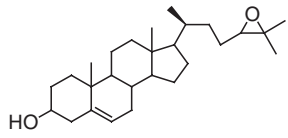
5. PRE-CLINICAL DEVELOPMENTS

5.1 LXR-modulators

The liver X receptor belongs to the nuclear hormone super family of ligand-activated transcription factors and has two isoforms, LXRα and LXRβ, which act as central regulators of genes that are involved in lipid metabolism [107]. A number of genes in mammals, including ABCA1, ABCG1, ABCG5, ABCG8, CETP, PLTP and SREBP-1c (sterol response element binding protein), are regulated by LXRs [108,109]. Agonists of LXR modulate RCT, cholesterol homeostasis and lipogenesis. LXR agonists such as the natural ligand 24-(S), 25-epoxycholesterol (**22**), and synthetic non-steroidal ligands such as GW-3965 (**23**), TO-901317 (**24**), and **25** have been shown to increase expression of ABCA1 and raise HDL levels in animal models [110]. Preclinical studies with compound **25** suggested good efficacy towards reducing atherosclerotic lesions in mice [110].

Treating mice with **23** led to the inhibition of atherosclerotic progression, whereas macrophage-specific knockout of LXR exacerbates atherosclerosis [111]. *In vivo* activation of LXR leads to increased fatty acid synthesis, accumulation of TG and the development of hepatic steatosis [109]. Successful LXR agonists will show desirable HDL elevation without these side effects [112].



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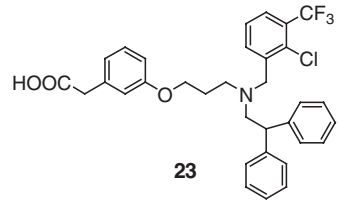

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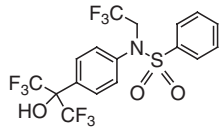

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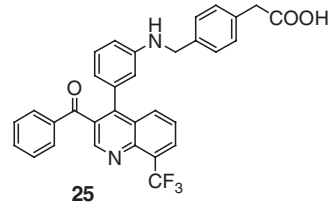

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6. CONCLUSION

HDL is an important and attractive target to reduce atherosclerotic progression or induce its regression. The therapeutic options currently available in the market place to raise HDL are inadequate. Despite its modest efficacy and irritating side effects, niacin continues to be the best choice with proven benefit for reducing cardiovascular risk. The results from the torcetrapib clinical trials have introduced some uncertainty to the field and, at the least, have delayed the availability of new HDL-elevating agents in the market. This has led to increased interest in the combination of extended release niacin with statins and/or anti-flushing agents to maximize the benefits of niacin therapy.

As discussed above, a number of approaches are being evaluated to slow catabolism of HDL or improve its function. The potential of apoA-1 Milano, or its orally viable synthetic congeners, for acute coronary syndrome are appealing. New research on the mechanism of HDL regulation and improved understanding of genes that regulate the RCT pathway are likely to yield new targets. For new HDL therapies, the challenge will also be to address functional or qualitative aspects of HDL relatively early in preclinical and clinical studies. Raising HDL levels without addressing these aspects may not be adequate for successful anti-atherosclerosis therapies.

The clinical validation of new approaches to raise functional HDL will potentially lead to a paradigm shift in the treatment of atherosclerosis, dyslipidemia and metabolic syndrome.

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CHAPTER 13

Case History on Tegaserod (Zelnorm®/Zelmac®)

Rudolf Giger, Henri Mattes and **Hans-Jürgen Pfannkuche**

Contents

1. INTRODUCTION

Tegaserod (HTF919; Zelnorm®/Zelmac®) is an innovative and potent partial agonist at 5-HT_4 receptors, which mediate multiple physiological functions in the gastrointestinal (GI) tract. The compound is a representative of a new class of 5-HT_4 receptor agonists, with regard to both chemistry and pharmacology. As a non-benzamide drug, it lacks 5-HT_3 receptor as well as dopamine D_2 receptor-blocking properties. Tegaserod has been shown to stimulate the coordinated

Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland

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release of neurotransmitters such as calcitonin gene-related peptide, substance P, vasoactive intestinal peptide (VIP) and acetylcholine from enteric nerves. As a result, tegaserod enhances basal motor activity and normalizes impaired motility throughout the GI tract. Preclinical investigations revealed that tegaserod can stimulate intestinal chloride/water secretion, in line with reports on functions of intestinal 5-HT_4 receptors. Importantly, preclinical as well as clinical studies demonstrated an attenuation of visceral sensitivity during colorectal distension following treatment with tegaserod. In conclusion, tegaserod restores GI homoeostasis via stimulation of 5-HT_4 receptors. At the same time, the drug does not cause exaggerated effects associated with full agonism at 5-HT_4 receptors and minimizes receptor desensitization. Tegaserod secures a balanced modulation of 5-HT_4 receptors expressed in the GI tract and, thereby, provides a mechanism to treat functional GI disorders (FGIDs).

FGIDs present with variable combinations of chronic or recurring GI symptoms not explained by overt biochemical or structural abnormalities and encompass conditions like functional dyspepsia (FD), irritable bowel syndrome (IBS) and chronic idiopathic constipation [1]. A variety of mechanisms that could explain the etiology of FGIDs are currently under investigation and include, but are not limited to altered GI motility, visceral hypersensitivity and post-infectious abnormalities [2].

In clinical phase III studies in predominantly female patients suffering from irritable bowel syndrome with constipation (IBS-C), tegaserod (6 mg b.i.d., 12 weeks) demonstrated significant improvements in self-reported symptom scores including abdominal pain, bloating and bowel habits. Phase III data in patients with idiopathic chronic constipation (CC) indicated that tegaserod (2 or 6 mg b.i.d., 12 weeks) significantly increased the number and quality of spontaneous bowel movements. In 2002, the FDA approved tegaserod for treatment of IBS-C in women and in 2004 expanded the indications to include treatment of idiopathic CC in men and women younger than age 65. Following an FDA request in March 2007 to review cardiovascular safety data marketing of tegaserod has been suspended in the US and over 20 other countries. In July 2007, the FDA announced that it is permitting the restricted use of Zelnorm under a treatment investigational new drug (IND) protocol to treat IBS-C and chronic idiopathic constipation in women younger than 55 who meet specific guidelines; the drug will remain off the market for general use.

The current case history will focus on the discovery of tegaserod and will summarize the pharmacodynamic effects in the GI tract and its therapeutic efficacy in IBS-C and CC.

2. DISCOVERY OF THE 5-HT_4 RECEPTOR IN THE GI TRACT

The 5-HT_4 receptor is a member of the seven transmembrane-spanning G protein-coupled family of receptors (GPCR) and constitutes an important subtype of the class of serotonin (5-HT) receptors. Initially, the 5-HT_4 receptor was characterized in the neuronal cell culture [3] of mouse colliculi and was shown to be positively coupled to adenylyl cyclase. The effect of serotonin was mimicked by the 5-HT_4 receptor agonists, BIMU 1 and BIMU 8, and was blocked by the 5-HT_4

receptor antagonist, DAU 6215. The discovery of serotonin type-4 receptors in the gut provided an explanation/mechanism responsible for the pharmacological activity of several gastro-prokinetic benzamide compounds, such as metoclopramide, zacopride and renzapride [4,5]. Saturable binding of [^{3}H]GR113808 was determined in the longitudinal muscle and myenteric plexus of the guinea-pig, with a larger number of sites in the upper part of the intestine: duodenum > jejunum > ileum > colon > rectum.

In the central nervous system (CNS) of guinea-pigs and rats, 5-HT$_4$ receptors are expressed in two anatomical and functional structures: the extrapyramidal motor system and the mesolimbic system [6,7]. In human brain, the presence of 5-HT$_4$ receptors has been shown in basal ganglia and in the caudate putamen nuclei, where the density is the highest [8].

5-HT$_4$ receptors are also present in the pig and human hearts, primarily located in the atrium [9]. Experiments showed that stimulation of these receptors can result in tachycardia and can trigger positive inotropic effects. Moreover, it has been demonstrated that the 5-HT$_4$ receptor is present in the human detrusor muscle and facilitates a cholinergic mechanism which may lead to increased bladder contractions [10]. Finally, acute (but not repeated) stimulation of 5-HT$_4$ receptors present on the human adrenal cortex has been reported to trigger the release of corticosterone and aldosterone [11].

In almost all tissues where 5-HT$_4$ receptors are present, 5-HT or any other agonists increase intracellular cAMP synthesis [12], as has been shown for hippocampus, atrium, esophagus, intestinal tissue and adrenal cortex. A number of processes can be triggered by an increase in intracellular cAMP. For instance in the intestine, an increase in intracellular cAMP concentrations following activation of 5-HT$_4$ receptors can trigger a relaxation of the smooth muscle. However, activation of 5-HT$_4$ receptors present on intestinal inter- and motor-neurons leads to a facilitation of acetylcholine release and, thereby, to increased contractions of intestinal smooth muscle [13].

As has been shown for other GPCRs, 5-HT$_4$ receptors are susceptible to desensitization. For instance, exposure of 5-HT$_4$ receptors on colliculi neurons to 5-methoxytryptamine or other agonists led to a rapid and long-lasting inactivation [14]. The intensity of receptor desensitization was dependent on the agonist concentration and exposure duration, and was related to the potency and efficacy of the agonist used. The desensitization was not dependent on the cAMP formed; it was due to phosphorylation of the occupied receptor by a specific receptor-dependent protein kinase (i.e., homologous but not cAMP-mediated heterologous desensitization).

3. TEST SYSTEMS ADDRESSING 5-HT$_4$ RECEPTOR AGONIST ACTIVITY

3.1 *In vitro* assays

3.1.1 Electrically stimulated longitudinal muscle/myenteric plexus preparations from guinea-pig ileum (LMMP-GPI)

The LMMP-GPI represents a tissue that allows studying enteric nerve – smooth muscle interactions. Low-frequency field stimulation of the LMMP-GPI results in

a stable twitch contraction that can be modulated through changes in neurotransmitter release, e.g., acetylcholine. Stimulation of presynaptic 5-HT_4 receptors facilitates the release of acetylcholine and thus enhances smooth muscle contractions. The electrically-stimulated LMMP-GPI was applied as a suitable model for the pharmacological characterization of 5-HT_4 receptor agonists *in vitro* [15,16]. Both potency and efficacy of 5-HT_4 receptor agonists were determined in this robust *in vitro* model prior to any investigations *in vivo*.

3.2 *In vivo* models

3.2.1 Gastric emptying in rats

Measurement of gastric motility and emptying rate is regarded as an integral part of an adequate characterization of drug candidates with promotile activity. The effects of the test compounds on gastric emptying of solids were therefore determined in conscious rats with the stomach made atonic by fasting. The passage of lead glass spheroids from the stomach into the duodenum was measured by an X-ray technique and quantified accordingly [17].

3.2.2 Small intestinal transit in guinea-pigs

Mechanisms and kinetics of gastric emptying of solids and liquids show differences and are triggered by different parts of the stomach. In general, liquid emptying is more rapid than emptying of solids and is mainly regulated by the corpus of the stomach, whereas emptying of solids is produced by contractions of the antrum. The effects of test compounds on liquid gastric emptying were determined in conscious guinea-pigs by measuring the distribution in the upper gastrointestinal tract of orally administered radiolabeled chromate (^{51}Cr) solution. This test system allowed the simultaneous determination of any effects on liquid gastric emptying and intestinal motility (intestinal transit) [18].

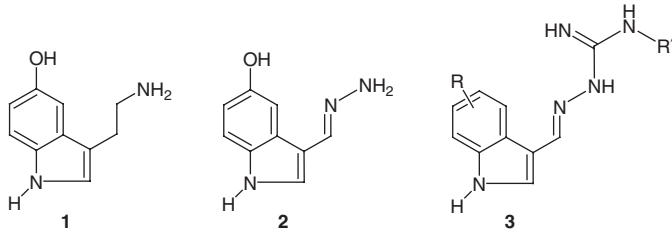
3.2.3 Electromyography studies in conscious dogs

Gastrointestinal motor activity in conscious dogs (and humans) is characterized by the postprandial/digestive state with rhythmic activity in the stomach and irregular contractile activity in the small intestine [19], and by the fasted state with interdigestive migrating contractions (IMCs). These IMCs are initiated at regular intervals in the stomach and duodenum and migrate through the small intestine. The IMCs are part of the Migrating Motor Complex (MMC) that consists predominantly of a quiescent period, which is then followed by the IMCs. The MMC is strictly cyclic. However, it becomes interrupted by food intake. The MMC pattern in dogs is similar to that in humans [20]. The effects of test compounds on gut motility, primarily the MMC (fasted state) or the postprandial phase, can be measured through electromyography using electrodes sutured to the serosa of different gut segments. Cisapride, a 5-HT_4 agonist/5-HT_3 antagonist with gastro-prokinetic properties has been characterized with respect to effects on MMCs in dogs and humans [21].

4. FROM SEROTONIN TO TEGASEROD

4.1 Design of indole carbazimidamides

At the time we embarked on the search for a 5-HT_4 agonist for development in gastrointestinal indications, high throughput screening facilities for identifying hits with novel structures were not yet available. Therefore, we took 5-HT **1** as a lead to design potential drug candidates [22]. We assumed that the conformation of the ethylamine side chain of 5-HT **1** would be trans-antiperiplanar for interaction with the 5-HT_4 receptor subtype. Thus, the first step toward designing a rigid analogue with the side chain in an extended conformation was to replace the ethyl moiety of **1** by an iminomethyl group. The resulting hydrazonomethyl-indolol **2** possesses the targeted conformation and is chemically stable.



The subsequent designing step, namely elongation of the primary amino group of **2** with an amidino moiety to the guanidino-iminomethyl-indolol (or indolol-carbazimidamide) **3** (R = 5-OH, R′ = H), was performed to introduce a functional group with multiple interaction potential to the putative receptor as well as to make this portion of the molecule resistant toward oxidative metabolic attack. In addition, the presence of a guanidinium cation in **3** at physiological pH should limit crossing the blood–brain barrier, thereby potentially avoiding any CNS side effects of the drug. Furthermore, the guanidino moiety offered the possibility to introduce various side chains for optimizing physico-chemical properties and interaction with the target receptor [23–25].

4.2 SAR of indole carbazimidamides and tautomerism

The activities of the indole carbazimidamide derivatives **5** at the 5-HT_4 receptor were measured *in vitro* using the field-stimulated LMMP-GPI preparation (Table 1) followed by *in vivo* investigations applying the gastric emptying and intestinal transit models in the guinea-pig and rat.

We first tried to optimize the environment around the charged guanidine using **5a** (R_5 = OH) as a reference compound for structure-activity relationship (SAR) determination.

Among aliphatic substituents, the pentyl side chain was found to be optimal for both *in vitro* potency and efficacy. Derivative **5b** (R_5 = OH, R_9 = *n*-pentyl) is a full agonist in the LMMP-GPI model (90% of the maximum efficacy of serotonin;

Table 1 5-HT_4 receptor agonism of carbazimidamides

No.	R_5	R_8	R_9	R_{10}	R_{11}	pD_2[a]	IA[b]
5a	OH	H	H	H	H	8.8	1.5
5b	OH	H	*n*-pentyl	H	H	9.3	0.9
5c	OH	H	CH_2CH_2Ph	H	H	9.1	1.0
5d	OH	H	$(CH_2)_2$-3,4-di-Cl-Ph	H	H	>10	0.1
5e	OH	H	H	Me	*n*-pentyl	7.7	0.3
5f	OH	Me	H	H	H	5.8	0.5
5g	H	H	*n*-pentyl	H	H	<6.0	0.1
5h	OMe	H	*n*-pentyl	H	H	6.9	0.2
5i	$OCH_2CH{=}CMe_2$	H	*n*-pentyl	H	H	8.15	2.2
5j	$NHSO_2Me$	H	pentyl	H	H	<4	0

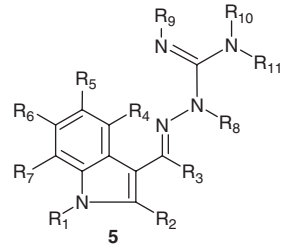
[a] Assay conditions: Ability of compounds to enhance the twitch contractions in the electrically stimulated LMMP-GPI model (n > 3).
[b] Intrinsic activity, i.a. (efficacy) relative to serotonin (1.0).

intrinsic activity (i.a.) = 0.9) with a potency (pD2 = 9.3) six times higher than that of serotonin. This compound also potently accelerated small intestinal transit in guinea-pigs and gastric emptying in rats after administration of 0.001 mg/kg i.p., but lacked adequate oral activity probably due to glucuronidation of the phenolic hydroxyl group.

Further increasing the lipophilic character of the derivatives in order to better counteract the high polarity of the hydroxyindolol carbazimidamide yielded very potent partial agonists, e.g., **5d** (R_5 = OH, R_9 = 3,4-dichlorophenetyl) (pD2 > 10, i.a. = 0.1). The very low intrinsic activity of these compounds, however, hampered *in vivo* testing. Polysubstituted derivatives were also synthesized: *N,N*-di-substitutions caused a 10-fold reduction in *in vitro* potency (e.g. **5e**). Constraining the guanidine group into a five-membered ring (e.g. R9–R10 – CH2CH2–) resulted in a 100-fold drop in *in vitro* potency and tri-substitution completely abolished activity (e.g. 5f).

However, the findings from modification of the aminoguanidine moiety, which resulted in decreased potencies, were consistent with the hypothesis of a dual-type binding mode for this head group. On the other hand, replacing one nitrogen of the guanidine with a carbon or a sulfur retained activity.

The importance of the 5-hydroxy substituent on indole as a hydrogen-bond donor is emphasized by the dramatic drop in potency and efficacy as exemplified by **5g** (R_5 = H, R_9 = *n*-pentyl) (pD2 < 6, i.a. = 0.1). Methylating the 5-OH substituent of the indole nucleus, in order to protect the metabolically vulnerable phenolic function, yielded HTF919 or tegaserod **5h** (R_5 = OMe, R_9 = *n*-pentyl), a more potent partial agonist (pD2 = 6.9, i.a. = 0.2), possibly reflecting some hydrogen-bond-accepting capability of the ether function. The compound was later shown to also bind with high affinity (pK_d 7.84 and 7.74) to calf and human caudate 5-HT_4 receptors [25,26].



5

Tegaserod potently accelerated small intestinal transit in guinea-pigs after i.p. administration of 0.001 mg/kg, and gastric emptying in rats after oral administration of 0.1 mg/kg. Moreover, the compound induced contractile activity in the intestine of fasted conscious dogs (electromyography studies) following both parenteral and oral dosing.

Increasing the size of the ether substituent (**5i**) led to a further increase in potency and efficacy *in vitro*. However, this improvement *in vitro*, which might be accounted for by secondary lipophilic interactions, did not improve efficacy *in vivo*. Substitution at the 5-position of the indole nucleus by larger polar or lipophilic substituents (**5j**) abolished activity *in vitro*.

Replacing the 5-OH group with 5-OMe or 5-OEt on the indole nucleus brought about a remarkable drop of the intrinsic activity. Finally, innovative 5-HT_4 receptor antagonists were also discovered. Compound **5k** (R_5 = OH, R_7 = Me, R_9 = *n*-pentyl) acted as a competitive antagonist vs. serotonin in the LMMP-GPI assay ($pA_2 = 8.4$). This discovery highlights the influence of subtle structural modifications on the efficacy of these ligands. A small steric hindrance or constraint might hamper the rearrangement of the receptor during the activation step, resulting in a loss of efficacy and conferring an antagonist profile on the molecules.

Finally, a variety of alternative aromatic systems were evaluated in an effort to identify surrogates for the indole. Replacement by benzothiophene, indazole or substituted phenyl groups severely reduced or abolished activity *in vitro*, emphasizing the subtle electronic and steric factors governing the aromatic binding site (compounds not shown).

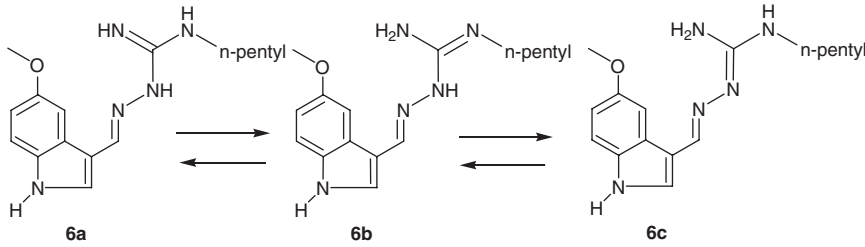
The selectivity for 5-HT_4 vs. other 5-HT receptor subtypes was examined in a set of radioligand binding assays available at the time (5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3) for some of the new compounds. While most derivatives exhibited a high selectivity for 5-HT_4 vs. 5-HT_3 receptors, structural adjustments of **5** led to profound effects on selectivity vs. other 5-HT receptors.

Introduction of the dichlorophenethyl substituent on the guanidine moiety resulted in the most selective 5-HT_4 ligand, **5d** (R_5 = OH, $R_9 = (CH_2)_2$-3,4-di-Cl-Ph), with over 4000-fold selectivity vs. all other serotonin receptors. With this substituent, we probably identified a fairly large lipophilic pocket in the 5-HT_4 recognition site, which is not present, at least at that neighborhood, in the other 5-HT receptors studied. Dialkylation of the guanidine (e.g. **5e**) led to an 8-fold decrease in selectivity for 5-HT_4 vs. 5-HT_{1A} receptors and a 40-fold increase in selectivity for 5-HT_4 vs. 5-HT_{1D} receptors.

Alkylation of the 5-OH group produced divergent effects. Methylation (e.g. **5h**) slightly decreased 5-HT_4 vs. 5-HT_{2C} selectivity, but did not alter the 25–200-fold 5-HT_4 selectivity over all other receptors studied. It should be mentioned that very recently, tegaserod was shown to antagonize 5-HT_{2B} receptors *in vitro* and *in vivo* at concentrations similar to those that activate 5-HT_4 receptors [27]. On the other hand, introduction of the dimethylallyl group (e.g. **5i**) almost completely abolished 5-HT_{1A}, 5-HT_{1D} and 5-HT_{2A} affinities. The secondary lipophilic interactions around the 5-OH binding site thus appear as a feature of the 5-HT_4 receptor, which are not shared by other members (except 5-HT_{2C}) of the serotonin receptor family.

From the indole carbazimidamides investigated, tegaserod (R_5 = OMe, R_9 = *n*-pentyl), a potent partial 5-HT_4 agonist, was selected for clinical development for the treatment of functional motility disorders based on its overall *in vitro* and *in vivo* profile.

Tautomerism. Owing to the presence of the guanidine moiety in the structure of tegaserod, the three tautomers shown below are possible. Tautomer **6a** is assigned to the solid state of tegaserod, as confirmed by X-ray structure analysis of the 5-*O*-benzyl derivative. In solution, **6b** and **6c** also exist in equilibrium depending on the experimental conditions. For instance in DMSO, the solvent used to measure NMR spectra, **6b** is preferred. Tautomer **6c** is formed in methanol after exposure to xenon light, and from this solution the tautomer can even be separated by HPLC.

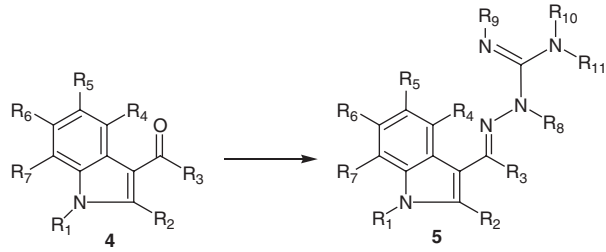


An analysis by LC-MS of the isolated tautomer as well as its HPLC-peak in the irradiated solution show mass peaks and fragmentation patterns identical to those of tegaserod demonstrating that it is not a degradation product. In the dark the tautomer HPLC-peak attributed to **6c** disappears again, thereby confirming the presence of tautomers in equilibrium in solution. We therefore assume that the biological activity of tegaserod is resulting from a combined activity of the rapidly equilibrating tautomers.

4.3 Chemical synthesis

The indole carbazimidamides **5** were obtained by condensation of appropriately substituted indole-3-carbaldehydes **4** with the respective aminoguanidine derivatives under acidic conditions [23]. Monoalkylated aminoguanidines were prepared by alkylating thiosemicarbazide with MeI, and subsequent reaction with the appropriate primary amine [24]. The sluggish reaction of *S*-methyl isothiosemicarbazide hydroiodide with secondary amines led us to prepare *N,N*-dialkylated-*N'*-aminoguanidines by a modified procedure. *t*-BuNCS was condensed with the appropriate secondary amines to yield, after cleavage of the *t*-Bu protecting group with HC1, the corresponding thioureas. Subsequent

alkylation with MeI, and final reaction with hydrazine produced *N*,*N*-dialkylated-*N*′-aminoguanidines.



Most substituted indole-3-carbaldehydes **4** were synthesized from the 2-methyl-nitrobenzene derivatives by standard procedures [24]. For example, alkylations or carbamoylations of 3-methyl-4-nitrophenol with the appropriate alkyl halides or chlorocarbonates, followed by reaction with *t*-BuOCH(NMe_2)$_2$ and hydrogenation, led to the required indole derivatives. Subsequent Vilsmeier–Haack formylation gave the corresponding aldehydes, which were condensed with aminoguanidines under acidic conditions to afford carbazimidamides. Indazoles were carboxylated at position 3 with carbon dioxide and potassium carbonate to the corresponding 3-carboxy derivatives [24]. These were reduced with lithium aluminum hydride followed by reoxidation with MnO_2 to give the required indazole-3-carbaldehydes. The aldehydes were condensed with aminoguanidines as outlined above. Azaindole carbazimidamides were obtained by similar routes [24]. For example, 6-amino-2-picoline was nitrosylated at position 3 using HNO_2 and reacted with isoamylnitrite/$CuCl_2$. The resulting 2-chloropyridine derivative was subsequently reacted with MeONa to yield 2-methyl-3-nitro-6-methoxypyridine. This was subjected to the procedures used previously for the synthesis of indolecarbazimidamide derivatives.

5. PROFILING STUDIES

5.1 Pharmacodynamic activity

Tegaserod facilitates the release of acetylcholine in electrically stimulated LMMP-GPI preparations in a partial agonist manner [28] and stimulates peristaltic activity in the guinea-pig intestine. In segments of human jejunum and rat and guinea-pig colon, tegaserod shows potent triggering of the release of calcitonin gene-related peptide (CGRP) from intrinsic sensory neurons leading to the initiation of the peristaltic reflex [29]. Use of high concentrations and long exposure times result in a desensitization of 5-HT_4 receptors, which trigger the peristaltic reflex [30,31]. Propulsion measurements using isolated segments from guinea-pig colon confirmed the promotile activity of tegaserod [18]; the compound potently and efficaciously increased the propulsion velocity of artificial fecal pellets following intraluminal administration. Concentrations higher than 100 nM lowered the propulsion velocity, indicating a bell-shaped dose–response relationship. Tegaserod also activates 5-HT_4 receptors present on isolated smooth muscle cells

of human jejunum, resulting in an increase in intracellular cAMP concentrations and a relaxant response of cholecystokinin (CCK)-contracted smooth muscle cells [32,33].

Additionally, tegaserod at low nanomolar concentrations increases intracellular cAMP concentrations in crypt cells isolated from rat distal colon and stimulates chloride and water secretion by activation of 5-HT_4 receptors [34,35]. These findings suggest a modulatory effect on intestinal electrolyte and water secretion *in vivo*.

Owing to the distribution of 5-HT_4 receptors throughout the GI tract and the selectivity of tegaserod as an agonist at these receptors, the compound acts as a promotile drug throughout the GI tract [28,29]. Stimulatory effects are observed on both normal and impaired gastric emptying in mice, rats and dogs, on small intestinal motility in dogs, on colonic transit and motility in mice and dogs [28,36–38], and on motility in healthy horses [39]. In a model of constipation in dogs using morphine, tegaserod normalized the reduced number of bowel movements and the quantity of stools. Furthermore, treatment with tegaserod significantly improved stool consistency [40]. Tegaserod does not affect gastric (acid) secretion (Figure 1).

A study using decerebrate anesthesia-free cats was performed to assess whether tegaserod had any effects on extrinsic afferents in addition to its modulating effects on intrinsic primary afferent neurons. Tegaserod dose-dependently inhibited the firing rate of rectal afferents following rectal distension [41], but did not modify the pressure–volume relationship during rectal distension (barostat system). These data suggest a role of 5-HT_4 receptors in modulating visceral sensitivity without affecting compliance of the rectal wall. Data obtained in conscious rats and mice (colorectal distension using a barostat system) [42,43]

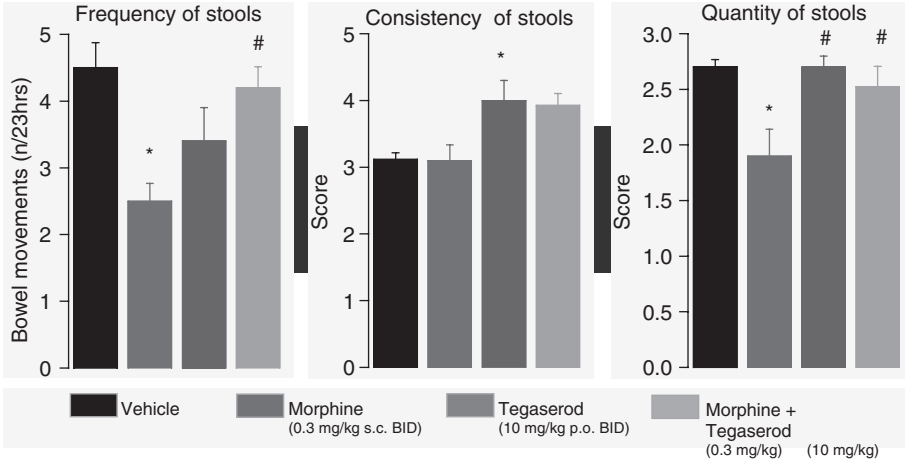


Figure 1 Effects of tegaserod in a constipation model in conscious dogs. Tegaserod normalizes stool frequency, stool quantity and softens stool consistency. *Mean ± SEM (n = 8); *p < 0.05 versus Vehicle; #p < 0.05 versus Morphine*. From Weber *et al.*, *Gastroenterology* (2003), **124**: A1806 (Please see Color Plate Section in the back of this book).

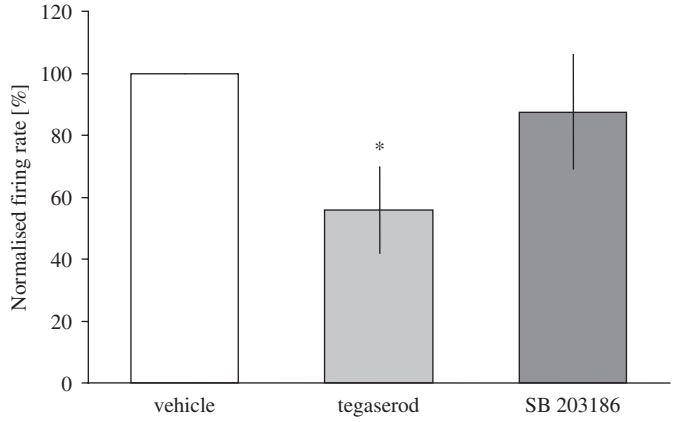


Figure 2 Inhibition by tegaserod of neuronal activity (firing rate) of rectal spinal afferents ($n = 9$) upon distention (50 mmHg) of feline colon and the antagonism by SB 203186, a competitive inhibitor at 5-HT_4 receptors. From Schikowski *et al. Neurogastroenterol. Mot.* (2002), **14**: 221–227.

confirmed the findings in decerebrate cats suggesting that tegaserod can exert antinociceptive activity during colorectal distension. In rats with sensitized colons, tegaserod potently inhibited the exaggerated visceromotor response induced by colorectal distension. Hence, tegaserod increases the pain threshold to colorectal distension in rats with visceral hypersensitivity [44,45] (Figure 2).

5.2 Pharmacokinetic properties

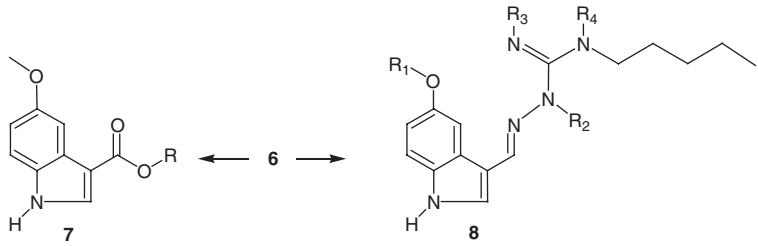
Tegaserod is rapidly absorbed following oral administration to humans; peak plasma concentrations are reached after approximately 1 h. Absolute bioavailability is about 10% under fasted conditions. Food can reduce the oral bioavailability of tegaserod by 40–65% and plasma concentrations by 20–40%. Systemic exposure to tegaserod is not significantly altered at neutral gastric pH compared to the fasted state (pH 2). Tegaserod is approximately 98% bound to plasma proteins, primarily to α-acid glycoprotein, and has a volume of distribution at steady-state of 368 ± 223 L.

The pharmacokinetics of tegaserod in patients with IBS are comparable to those in healthy individuals, and similar between men and women. No dosage adjustment is required in elderly patients or those with mild-to-moderate hepatic or renal impairment [46,47].

5.3 Biotransformation

Tegaserod is metabolized mainly via two pathways. The first is a presystemic acid-catalyzed hydrolysis in the stomach followed by oxidation and conjugation, which produces the main metabolite of tegaserod, 5-methoxyindole-3-carboxylic

acid glucuronide **7** (R = Gluc). This metabolite has negligible affinity for 5-HT receptors and is devoid of promotile activity.



The second pathway is direct glucuronidation which leads to generation of three isomeric *N*-glucuronides **8** (R_1 = Me, R_2 or R_3 or R_4 = Gluc).

In human liver microsomes, tegaserod was metabolized to *O*-desmethyl tegaserod **8** (R_1 = H, $R_{2,3,4}$ = H) at a low rate [48]. However, in human liver slices, direct *N*-mono-glucuronidation of tegaserod at the guanidine nitrogens (R_2 or R_3 or R_4 = Gluc) was found, with R_2 = Gluc being the major metabolite while *O*-desmethyl tegaserod was not detected. This discrepancy can be explained by an about 40-fold difference in the rate of glucuronidation vs. *O*-demethylation.

Interestingly, slices of human small intestine also metabolized tegaserod to the *N*-glucuronides, suggesting a contribution of the small intestine to the presystemic metabolism of the drug.

Tegaserod inhibited CYP1A2 and CYP2D6 with K_i values of 0.84 and 0.85 μM, respectively. These K_i values are approximately 140-fold greater than the maximal plasma concentrations following administration of the recommended clinical dose of tegaserod (i.e., 6 mg). The metabolite **7** (R = Gluc), the main circulating metabolite, did not demonstrate any inhibitory potential toward cytochrome P450 enzymes *in vitro*. The data indicate that cytochrome P450-mediated metabolism plays an insignificant role in the elimination of tegaserod. No clinically relevant drug–drug interactions with tegaserod have been identified.

5.4 Non-clinical safety

Tegaserod has proven safe in toxicity studies. Toxicology studies in animals showed no relevant effects on reproductive or hormonal functions, or embryo-fetal or neonatal development. Although tegaserod was detected at low levels in foetuses, radiotracer studies showed that radioactivity was about three times higher in milk than in plasma, suggesting that a suckling infant might ingest tegaserod. Tegaserod had no mutagenic or clastogenic potential, and did not induce DNA damage [46].

6. CLINICAL PHASE II/III STUDIES

The clinical efficacy of tegaserod in female patients with constipation-predominant IBS was established in two prospective well-controlled studies,

with supportive efficacy obtained in a third study. The drug dosed at 6 mg b.i.d. for 3 months showed a consistent pattern of improvement across multiple efficacy variables and study time points in all three studies. Higher response rates were observed for the tegaserod-treated group than the placebo group on a global validated measure of IBS symptom relief (Subject's Global Assessment (SGA) of Relief) [49] for each month in all three studies. In particular, tegaserod had a rapid onset of action with a therapeutic gain of 13–14% during month 1 vs. placebo. At study completion, the therapeutic gain ranged from 7 to 14%. The onset of action of tegaserod, as measured by SGA of relief, was observed as early as 1 week. Clinical efficacy persisted throughout the 12-week treatment period. Upon discontinuing tegaserod, symptoms returned within one week. Tegaserod-treated patients also showed greater improvements than placebo-treated patients in the characteristic symptoms of IBS, including abdominal discomfort and pain, bloating, and symptoms of constipation (number of bowel movements, stool consistency and straining) [50].

The efficacy of tegaserod in patients with idiopathic CC was established in two multi-center, double-blind, placebo-controlled studies with a 3-month treatment period [51,52].

The drug increased the number of spontaneous bowel movements compared to placebo. The effect was rapid (within a few hours) and sustained over 12 weeks. Patients treated with tegaserod 6 mg b.i.d. experienced a significant reduction of the straining associated with spontaneous bowel movements compared to placebo. The effect was significant during the first week of treatment and sustained over the entire 12 weeks. Moreover, patients on tegaserod experienced a clinically significant improvement in stool consistency and a greater improvement of their abdominal discomfort/pain compared to the placebo group. A significant improvement of their abdominal distension/bloating was also measured during tegaserod treatment. Clinical efficacy of tegaserod has also been shown in males suffering from chronic constipation [53].

7. CONCLUSION

The substituted indole carbazimidamides described in this paper represent a completely novel class of potent agonists at the 5-HT_4 receptor. Structural variations of **5a**, the prototype of this new class, have led to **5b** and **5c**, the most potent, full agonists known to date at this receptor species with selectivity ranging from 20- to 500-fold vs. other 5-HT receptors. Moreover, we have been able to prepare extremely potent, partial 5-HT_4 agonists exhibiting affinities similar (e.g. tegaserod) to or 100-fold higher (e.g. **5d**) than serotonin, which could be very useful in light of the propensity of this receptor to undergo densitization. The *in vitro* properties of this innovative compound class of 5-HT_4 agonists translate well into potent and therapeutically relevant effects in the gastrointestinal tract, i.e. stimulation of motility, intestinal secretion and attenuation of visceral pain. Tegaserod, a representative of this new class of compounds has shown therapeutic benefit in FGIDs exemplified by clinical efficacy and safety in patients with IBS-C and chronic idiopathic constipation.

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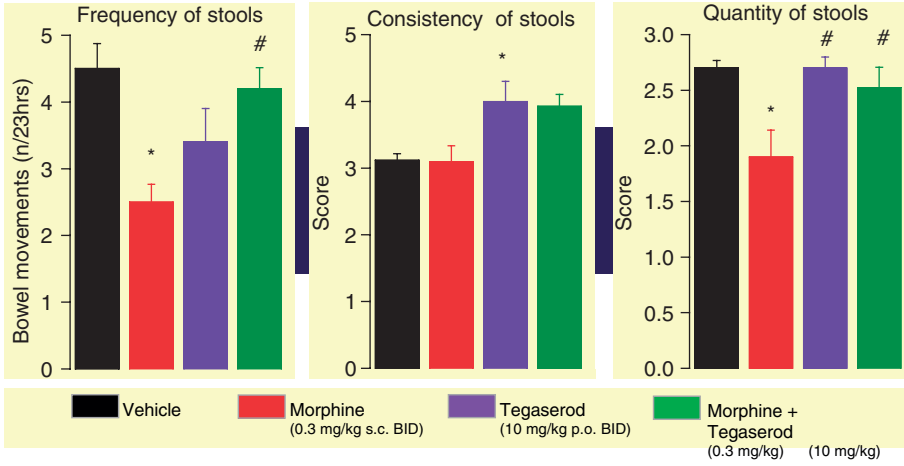

Plate 1 Effects of tegaserod in a constipation model in conscious dogs. Tegaserod normalizes stool frequency, stool quantity and softens stool consistency. *Mean* $\pm$ *SEM* ($n = 8$); *$p < 0.05$ *versus Vehicle*; #$p < 0.05$ *versus Morphine*. From Weber *et al*., *Gastroenterology* (2003), **124**: A1806 (For Black and White version, see page 204).

CHAPTER 14

Advances in the Discovery of CC Chemokine Receptor 2 Antagonists

Percy H. Carter, Robert J. Cherney and **Ian K. Mangion**

Contents

1. INTRODUCTION

CC Chemokine Receptor 2 (CCR2) is a member of the G protein-coupled receptor (GPCR) superfamily that serves as the receptor for monocyte chemoattractant proteins 1–4 (MCP-1 to -4), a group of pro-inflammatory chemotactic cytokines (chemokines). CCR2 is the primary chemokine receptor on inflammatory monocytes, and is also expressed on T-cells, dendritic cells, and endothelial cells. Upon ligand engagement, CCR2 mediates both cellular movement and activation.

Research & Development, Bristol-Myers Squibb Company, Princeton, NJ 08543-4000, USA

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The primary ligand for CCR2, MCP-1 (also called CCL2), is expressed at increased levels in many human inflammatory conditions. Moreover, both MCP-1 and CCR2 play significant roles in rodent models of human diseases as diverse as atherosclerosis and multiple sclerosis [1–3]. This strong pre-clinical validation has prompted interest in discovering viable interventions against MCP-1 and CCR2. Our review focuses on recent biological and chemical advances in this specific area (2003 to present), and thereby extends the coverage of general surveys of the field of chemokine receptor antagonism [4–6].

2. RECENT BIOLOGY

2.1 Key advances in understanding of biological mechanism

A number of recent studies investigating the mechanism of CCR2-dependent cell migration have appeared. At the cellular level, it has been shown that the MCP-1/CCR2-dependent chemotaxis event is mediated by the novel cytostolic protein FROUNT, which is co-expressed with CCR2 at the cellular [7] and tissue [8] levels, and facilitates CCR2 clustering at the leading edge of the cell after *direct* binding of FROUNT to the pro-C-terminal domain of CCR2 [7]. At a physiological level, new data have emerged to show that CCR2 is expressed on short-lived monocytes that are drawn specifically to sites of inflammation (so-termed ''inflammatory monocytes'') [9,10]. Notably, the migration of these monocytes from blood into inflamed tissue is CCR2-dependent only at early time points [11,12]. Furthermore, it appears that CCR2 plays a role in directing the emigration of these inflammatory monocytes from the bone marrow into the blood [11]. Perhaps surprisingly, MCP-3 serves as the dominant CCR2 ligand mediating this crucial function [12], an observation that adds to the growing body of literature highlighting the importance of CCR2 ligands other than MCP-1 [13,14].

2.2 Key advances in pre-clinical validation

A number of studies have demonstrated that antagonism of CCR2 and/or MCP-1 reduces disease scores in pre-clinical models of arthritis [1,3]. Recently, the first reports of the actions of small molecule antagonists in both collagen-induced arthritis (CIA) and adjuvant arthritis models have appeared [15,16], and they confirm the earlier findings: blockade of CCR2 reduced disease score. In stark contrast with the aforementioned studies, the first report of the effects of genetic deletion of CCR2 in mouse CIA showed that $CCR2^{-/-}$ mice exhibited exacerbated disease [17]. Moreover, $CCR2^{-/-}$ mice developed chronic polyarthritis after infection with *Mycobacterium avium*, whereas wild-type (WT) littermates did not [18]. Finally, an independent study documented that administration of an anti-CCR2 antibody worsened CIA when administered therapeutically (after disease initiation) but blunted disease if administered during disease initiation [19]. It is unclear how to reconcile the conflicting data at this time, but they likely point to an unappreciated complexity in CCR2 biology (e.g. a positive role in

disease resolution) that is differentially effected by the intervention employed and the exact model/species/strain studied.

Studies of pre-clinical models of other autoimmune diseases have also been reported recently. In a model of systemic lupus erythematosus, $CCR2^{-/-}$ mice exhibited prolonged survival and reduced renal disease relative to their WT counterparts [20]. Importantly, reduced macrophage and T-cell accumulation in the renal lesion sites was noted. Overall, these data are consistent with other recent studies on genetic deletion of MCP-1 [21] or administration of a peptide antagonist of CCR2 [22] in models of lupus. The recent data with experimental autoimmune encephalomyelitis (EAE, a rodent model of human multiple sclerosis) are more complex, in that different outcomes were observed depending on the system studied. Administration of a small molecule antagonist blunted EAE disease progression in C57BL/6 mice [15]. Likewise, genetic deletion of CCR2 in the C57BL/6 background also reduced EAE disease score, particularly in the early stages of disease [23]. However, $CCR2^{-/-}$ mice on a Balb/C background showed exacerbated EAE relative to their WT littermates, even though disease induction was delayed [23]. This strain dependence is reminiscent of phenomena observed in the aforementioned arthritis models [18].

Recent data have made it clear that obesity has a strong inflammatory component, and that CCR2 may play a role in this context. Strikingly, simple maintenance of a high fat diet is sufficient to increase the numbers of circulating $CCR2^{+}$ inflammatory monocytes in both WT [12] and $ApoE^{-/-}$ mice [24]. Moreover, genetic deletion of CCR2 reduces numbers of activated macrophages in adipose tissue [25], but does not affect a population of M2 adipose macrophages thought to maintain the "lean" state [26]. Consistent with this, genetic deletion of CCR2 attenuates the development of obesity in a diet-induced obesity model and improves insulin sensitivity [27]. Notably, a small molecule CCR2 antagonist also improves insulin sensitivity in this same model [27]. Likewise, in genetically predisposed mice (db/db mice) fed a high-fat diet, the development of insulin resistance (but not obesity) was blunted either via genetic deletion of MCP-1 or by gene-induced expression of a dominant negative peptide [28]. The converse was also observed: overexpression of MCP-1 in adipose tissue induced insulin resistance (both locally and systemically) and increased macrophage recruitment to the fat [29]. Conflicting data have appeared: one manuscript has shown that genetic deletion of CCR2 does not have an effect on diet-induced obesity [30], and a second has shown that genetic deletion of MCP-1 does not affect insulin resistance in the db/db mouse [31]. Thus, differences in experimental design apparently affect the relevance of MCP-1/CCR2 to the development of insulin resistance [32].

Early studies have documented that genetic deletion of either MCP-1 or CCR2 provides for reduced atherosclerotic lesion area in various murine models of atherosclerosis [1]. Recently, these findings have been extended by a study showing that bone marrow transplantation from $CCR2^{-/-}$ mice into ApoE3-Leiden mice (a transgenic mouse model with defective ApoE) dramatically inhibits early atherogenesis [33]. Likewise, population of bone marrow from WT mice into $CCR2^{-/-}$ mice was sufficient to promote atherogenesis in the presence

of a high-fat diet [34]. These data suggest that CCR2 leukocytes (as opposed to CCR2-expressing smooth muscle or endothelial cells) are involved in early atherogenesis. However, bone marrow transplant from $CCR2^{-/-}$ mice into $ApoE^{-/-}$ mice with established lesions (16 weeks of age) did not blunt the continuing course of lesion development over the subsequent 9 weeks [35]. The lack of CCR2 dependence of advanced atherosclerosis is consistent with the presence of a redundant mechanism. In this context, the recent finding that at least three different chemokine receptors (CCR2, CCR5, and CX3CR1) can facilitate the recruitment of activated ($Ly6C^{hi}$) monocytes into atherosclerotic plaques is noteworthy [24].

A role for CCR2 in neurological conditions has been highlighted recently. Relative to their WT counterparts, $CCR2^{-/-}$ mice showed altered responses to inflammatory pain, including reduced pain behavior after intraplantar formalin injection and slightly reduced mechanical allodynia after intraplantar complete Freund's adjuvant (CFA) injection [36]. Furthermore, $CCR2^{-/-}$ mice did not display significant mechanical allodynia after sciatic nerve injury. Strikingly, a small molecule CCR2 antagonist reduced mechanical allodynia to ~80% of pre-injury levels within 5 h after oral administration [37]. The small molecule was also able to block the phase 2 pain response to formalin injection. A separate line of investigation has shown that genetic deletion of CCR2 partially protects mice against ischemia/reperfusion injury [38]. Importantly, the recruitment of both monocytes and neutrophils was blunted dramatically, and blood/brain barrier integrity was partially maintained. These results are consistent with earlier studies with $MCP\text{-}1^{-/-}$ mice [39].

The role of MCP-1/CCR2 in tumor biology is complex [40]. In some instances, MCP-1 and CCR2 stimulate anti-tumor responses (as part of host defense). However, it also appears that tumors can utilize MCP-1 to promote their own growth (e.g. through angiogenesis or the recruitment of tumor-associated macrophages). Investigations of the role of CCR2 in tumor biology are ongoing [41–45].

2.3 Clinical trials of antagonists of MCP-1/CCR2

Three separate interventions against MCP-1/CCR2 have failed to provide clinical benefit in human patients suffering from rheumatoid arthritis. The diversity of modalities employed – an anti-MCP-1 antibody [46], an anti-CCR2 antibody [47], and a small molecule antagonist of CCR2 [48] – suggest that the findings have revealed a redundant role for CCR2 in this disease process. A second small molecule antagonist (INCB-3284) is currently under investigation in rheumatoid arthritis, but no data have been reported.

Other clinical trials with CCR2 antagonists are underway [1], but no reports have been forthcoming. Both MLN-1202, a humanized anti-CCR2 antibody, and MK-0812, a small molecule antagonist, were examined in rheumatoid arthritis (*vide supra*) and are also being examined in multiple sclerosis. Two additional small molecules – CCX-915 and INCB-8696 – have entered phase 1 trials with multiple sclerosis as a projected phase 2 trial. An intention to study INCB-8696 in systemic lupus erythematosus has also been declared. Finally, phase 2 clinical

trials of MLN-1202 in atherosclerosis and INCB-3284 in type 2 diabetes have been announced, but no data from these trials have been released in a scientific forum.

2.4 Potential concerns with targeting CCR2

Early studies showed that $CCR2^{-/-}$ mice exhibited an expected reduction in their ability to clear infections by pathogenic organisms [1,3]. Of particular note, one paper illustrated that $CCR2^{-/-}$ mice were unable to clear infection by intravenously-administered *Mycobacterium tuberculosis*, and showed substantially enhanced mortality [49]. Recent studies have extended this observation, and shown that both $CCR2^{-/-}$ and MCP-$1^{-/-}$ mice can clear aerosol infections of low-dose *M. tuberculosis* via a later-stage CCR2-independent immune response [50,51]. The inability of $CCR2^{-/-}$ mice to control the early stage of infections appears related to a deficiency in T-cell recruitment normally mediated by $CCR2^{+}$ monocytes and dendritic cells [52].

Two independent papers have highlighted the toxic effects of cellular products normally cleared by CCR2-dependent processes. In the first, it was shown that both $CCR2^{-/-}$ and MCP-$1^{-/-}$ mice begin to exhibit retinal degeneration after 9 months of age [53]. The authors hypothesized that impaired clearance of C5a and IgG enabled complement activation and immune complex deposition. In the second study, it was demonstrated that both $CCR2^{-/-}$ and $CCR2^{-/+}$ mice exhibit enhanced mortality in an aggressive transgenic model of Alzheimer's disease [54]. The authors hypothesized that efficient clearance of Aβ by microglial cells is a CCR2-dependent function.

Recently, some authors have raised concerns that suppressed trafficking of $CCR2^{+}$ T-regulatory cells might increase inflammation and exacerbate autoimmune responses. For example, the numbers of murine $CCR2^{+}$ $CD25^{+}$ regulatory T-cells increase during the progression phase of CIA, and the authors postulate the blockade of these cells may by responsible for the exaggerated immune response observed when an anti-CCR2 antibody was administered after disease initiation [19,55]. Of note, human regulatory T-cells ($Th^{IL\text{-}10high}$) express CCR2 and are more responsive to MCP-1 than to other chemokines [56]. Finally, MK-0812, the aforementioned small molecule CCR2 antagonist, increased C-reactive protein in rheumatoid arthritis patients and provided less clinical benefit than placebo, suggesting that the compound might exhibit pro-inflammatory activity [48].

3. RECENT MEDICINAL CHEMISTRY

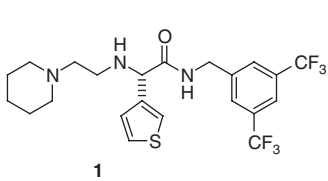
3.1 CCR2 binding pharmacology

Even though CCR2 is a GPCR, the binding of MCP-1 to CCR2 is best characterized as occurring at a protein/protein interface: the contact area between ligand (~80 amino acids) and the receptor's extracellular N-terminal domain (~40 amino acids) is large and dominated by multiple ionic interactions [4].

Accordingly, much of the early work focused on protein-based interventions (e.g. peptide antagonists, antibodies). Since 2000, however, rapid progress has been made in the discovery and development of small molecule antagonists of CCR2 [1–6]. One recent report has identified a selection of receptor residues that appear important for the binding of several different chemical series [57]. Notably, these residues all lie within the transmembrane bundles, distal to the primary binding site of MCP-1 in the receptor's extracellular N-terminus. Indeed, mutation of these residues had relatively minor effects on MCP-1 binding (generally < 3-fold) but larger effects on compound binding (> 10-fold), suggesting that the compounds studied are likely allosteric inhibitors [4]. Glutamic acid-291, a residue that is conserved at position VII:06 in ~74% of chemokine receptors (vs. ~1% of GPCRs), stands out amongst these CCR2 residues, as it was identified as a key interaction site for four of the five chemotypes examined [57,58]. The apparent importance of this glutamic acid has been observed in other chemokine receptors, and is consistent with the dominance of a general chemokine antagonist "chemotype" consisting of a central basic amine flanked by two hydrophobes [59]. As detailed below (Sections 3.2–3.5), many CCR2 antagonists fall into this general class. Exceptions do exist (Section 3.6), but the pharmacology of these latter compounds has not been disclosed in detail.

3.2 γ-Amino butyramides

A series of *N*-benzyl amides featuring substituted piperidines has been reported in the literature to display potent affinity for CCR2. For example, compound **1** exhibited a CCR2 binding (Bnd) IC_{50} of 39 nM. It was selective against other chemokine receptors, but suffered high first pass metabolism [60]. SAR studies of a screening hit arising from a neurokinin antagonist program produced spiroindenylpiperidine **2** (CCR2 Bnd $IC_{50} = 59$ nM; chemotaxis $IC_{50} = 41$ nM). While this lead had improved pharmacokinetic characteristics, it lacked selectivity against CCR5 and NK1 [61]. Cyclopropyl analog **3** features improved activity (CCR2 Bnd $IC_{50} = 14$ nM; chemotaxis $IC_{50} = 4$ nM), as well as better selectivity against chemokine receptors relative to previous examples [62]. Further exploration of the illustrated R substituent was extended into heterocycles, leading to compounds displaying potent inhibition of chemotaxis while retaining target selectivity, although with a poorer pharmacokinetic profile [63]. Such compounds also had variable murine CCR2 activity, and it was found that replacement of the spiroindenylpiperidine with phenyl piperidines was necessary to secure that activity while retaining human CCR2 binding potency.



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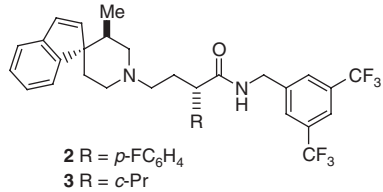
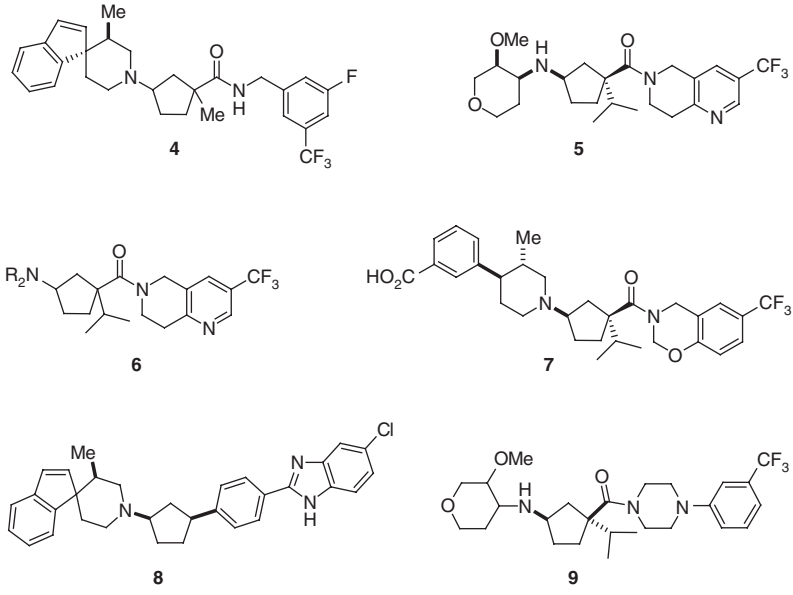


2 R = *p*-FC_6H_4
3 R = *c*-Pr

The aforementioned peer-reviewed publications concerning compounds such as **3** were expanded upon with the disclosure of a series of patent applications featuring cyclopentyl and cyclobutyl constrained analogs [64–80]. Quaternary carbon substitution of the central cyclopentane as in **4** was preferred [64], and isopropyl was the most exemplified substituent within that motif, as in **5**, **6**, and **7** [65–69]. Notable exceptions included the cyclopentyl series lacking a central amide as seen in **8** [70], and a series otherwise related to **6** based on a cyclobutyl core [71]. A diversity of tertiary and secondary amines based on the core of **6** has been extensively claimed [72–74]. The central amide has also been incorporated into a piperazine as in **9**, leaving the aryl ring as an aniline [68,69]. Notably, compound **9** was claimed as a dual antagonist of CCR2 and the highly homologous CCR5 [68], whereas close analogs with alkyl or hydrogen substituted for the methoxy group were claimed only as CCR2 antagonists [69].

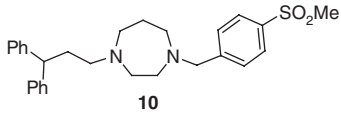


Compound **5** has recently been identified as MK-0812 [81], a potent CCR2 antagonist (CCR2 Bnd $IC_{50} = 5.0\,nM$; chemotaxis $IC_{50} = 0.2\,nM$) that has advanced into human clinical trials for the treatment of rheumatoid arthritis and multiple sclerosis (*vide supra*). It has been described in several applications and claimed as a specific salt form [65,75–78]. Benzoxazine **7** has also been specifically described as a CCR2 antagonist with possible application in combination with statins for the treatment of inflammatory conditions [67,79,80].

3.3 Glycinamide-linked antagonists

Two recent communications described the hit-to-lead optimization which ensued from the original screening hit **10** [82,83]. From this research, four new

cores, **11–14**, were discovered with substantial CCR2 activity (CCR2 Bnd IC_{50} values = 180–700 nM). The (3-trifluoromethylphenyl)glycinamide **15** was a key substituent which bestowed significant CCR2 affinity to all the cores (up to 100-fold over the methyl-substituted analog). The most potent compound was **11**, which exhibited a chemotaxis IC_{50} of 24 nM (CCR2 Bnd IC_{50} = 180 nM).



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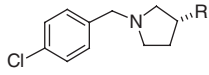

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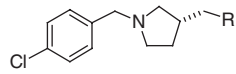

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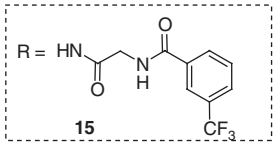

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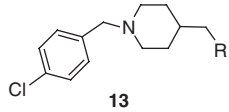

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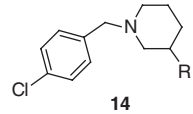
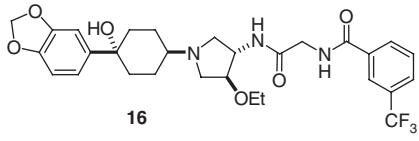


14

CCR2 antagonists with activity in murine models are rare. Compound **16** was described as an orally bioavailable murine CCR2 antagonist (mouse CCR2 binding and chemotaxis IC_{50} values = 10 nM) with good selectivity versus other murine chemokine family members, and hence it was taken into several classical *in vivo* efficacy models [15,84]. When dosed in the rat adjuvant arthritis model (100 mpk p.o. b.i.d.), compound **16** displayed 82% inhibition of joint inflammation as well as 64% inhibition of bone resorption. Compound **16** was also shown to be efficacious in several murine models: thioglycolate-induced peritonitis, delayed-type hypersensitivity reaction, diet-induced obesity, and EAE [15,27]. Recently, salt forms of a single compound, **17**, from the same genus were the subject of a separate invention [85].



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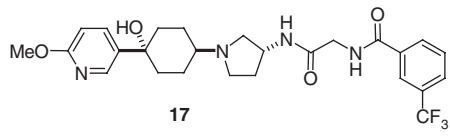
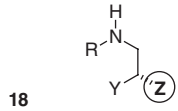


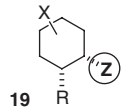
17

Additional series of putative CCR2 antagonists (CCR2 Bnd IC_{50} values <20,000 nM) containing glycinamide as a linking motif to a *meta*-trifluoromethylphenyl have been claimed in the recent patent literature. Generalized examples include acyclic **18** [86], cyclic **19** [87–89], and bicyclic **20** [90]. Malonamides [91,92] and pyrrolidinones [93,94] have also been used as linking elements. Heterocycles have been exemplified as replacements for the benzamide grouping in both the glycinamide and malonamide series.



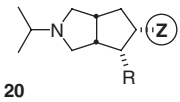
18

Y = C(O)NHR, CH(OH)alkyl
R = alkyl, arylalkyl



19

X = H, OR', $NR"_2$
R = H, substituted alkyl



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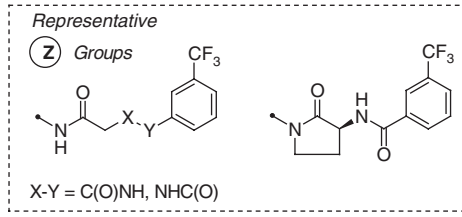
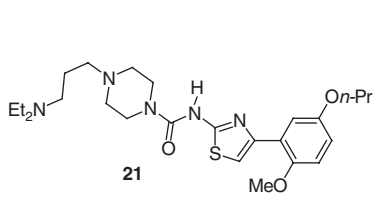
R = H, substituted alkyl



3.4 Piperazines and piperidines

Thiazoles have been used as a core element in two series of recently claimed piperidine/piperazine CCR2 antagonists. Representative compounds **21** and **22** exhibited CCR2 binding IC_{50} values of 3 and 25 nM, respectively [95,96]. A related application described the replacement of the piperazine motif in **21** with phenyl [97]. A separate series of applications has also highlighted CCR2 antagonists with unsaturated heterocyclic cores linked to piperidines. Compounds **23** (CCR2 Ca^{2+} flux $IC_{50} = 314$ nM) and thienopyrimidine **24** (CCR2 Ca^{2+} flux $IC_{50} = 379$ nM) were exemplified [98,99].



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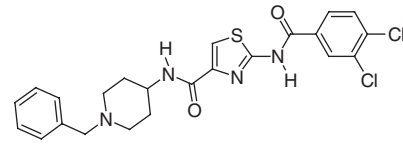

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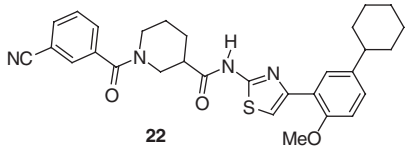

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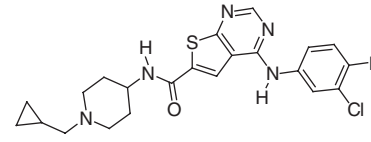
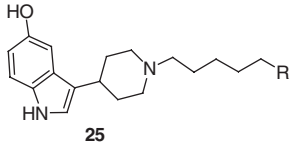


24

Indolopiperidines were described as CCR2 antagonists, and compound **25** was reported to be potent (CCR2 Bnd $K_i = 50$ nM) and selective versus CCR5. However, it also displayed unwanted activity at the 5-HT and dopaminergic receptors [100]. Important functionality included the phenol (giving a 10-fold

increase in activity) and the free indole (N-Me indole was 80-fold less active). Rigidification of these indolopiperidines gave compound **26**, which was a potent CCR2 antagonist (CCR2 Bnd $K_i = 40$ nM) with 1000-fold selectivity over 5-HT and dopaminergic receptors [101]. Recently, another set of indolopiperidines was described as CCR2 antagonists, with compounds **27** (CCR2 Bnd $IC_{50} = 1.0$ nM, chemotaxis $IC_{50} = 6$ nM) and **28** (CCR2 Bnd $IC_{50} = 0.6$ nM, chemotaxis $IC_{50} = 0.2$ nM) exemplified as potent inhibitors [16]. When dosed at 100 mpk (i.p., b.i.d.) in the mouse CIA model, compound **27** (homochiral) inhibited the clinical disease score on day 35 by 79%. Compound **27** (racemic) was also dosed (i.p., b.i.d., 100 mpk) in the rat adjuvant-induced arthritis model using three different schedules: (i) dosed from day 0–4 (resulted in insignificant swelling in the contralateral paws and 40% decrease in swelling in the injected paws); (ii) dosed day 7–4 (resulted in a 94% decrease in swelling in the contralateral paws); (iii) dosed from day 12–16 (inhibited swelling 51 and 40% in the contralateral and injected paws, respectively).



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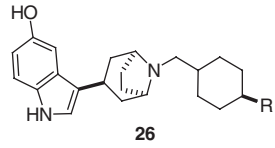

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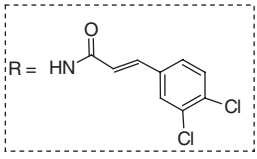
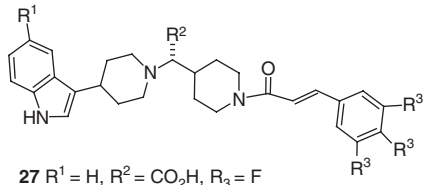
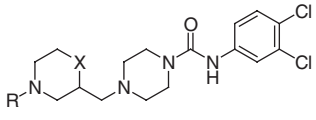


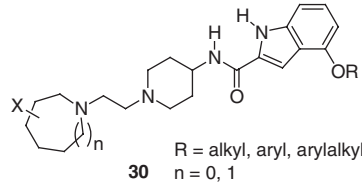


27 $R^1 = H$, $R^2 = CO_2H$, $R_3 = F$
28 (racemic) $R^1 = NHSO_2Me$, $R^2 = CH_2OH$, $R^3 = Cl$

Two distinct series of CCR2/5-dual antagonists have been claimed in recent patent applications. Series **29** included piperazine ureas linked to saturated amine heterocycles [102]. Activity ranges $<20{,}000$ nM (CCR2 Bnd IC_{50}) for all compounds were noted, with dozens of compounds also designated as having CCR5 Bnd IC_{50} values $<10{,}000$ nM (the affinity for CCR5 depended on the nature of the R substituent). Series **30** (CCR2 Bnd IC_{50} values $= 0.3$–$10{,}000$ nM; CCR5 Bnd IC_{50} values $= 4$–$10{,}000$ nM) featured 4-substituted indoles linked to saturated heterocycles via a 2-carboxamide [103].



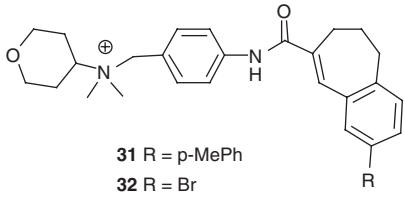
29 R = alkyl, substituted alkyl
X = O, NR′, CH_2



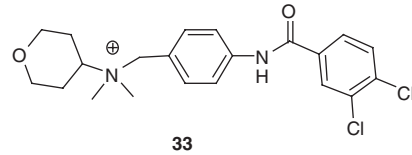
30 R = alkyl, aryl, arylalkyl
n = 0, 1
X = H, OH, NH_2, Me

3.5 Quaternary amine salts

Compound **31**, TAK-779 [104], was developed as a CCR5 antagonist and was later found to exhibit potent CCR2 activity (CCR5 Bnd $IC_{50} = 1.4$ nM; CCR2 Bnd $IC_{50} = 27$ nM). The binding of this compound to both CCR2 [57] and CCR5 [105] has been studied using a receptor mutagenesis/homology modeling approach, making it a key tool compound for understanding the binding of small molecules to these highly homologous receptors. Recently, related quaternary salts were described as CCR2 antagonists [106]. Compounds **32** (CCR2 Bnd $IC_{50} = 7$ nM) and **33** (CCR2 Bnd $IC_{50} = 8$ nM) were the most potent antagonists exemplified.



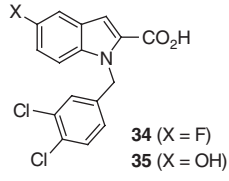
31 R = p-MePh
32 R = Br



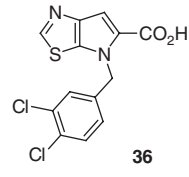
33

3.6 Unsaturated heterocycles

Although the majority of compounds described in the chemokine receptor antagonist literature contain a basic amine as a key structural element, several companies have described "non-classical" structural series as antagonists of CCR2. These compounds invariably feature an unsaturated heterocyclic core, and most feature essential acidic functionality (e.g. carboxylic acid, thiol, phenol, *N*-arylsulfonamide). The first such compound to be reported in the peer-reviewed literature was 1-(3-chlorobenzyl)-1*H*-pyrrole-2-carboxylic acid, which was disclosed as having a CCR2 Bnd IC_{50} of 11,900 nM [58]. Recently, a more potent series of 2-carboxyindoles has been described in detail [107]. After structure–activity relationship development from the initial library hit (*N*-benzyl 2-carboxyindole, CCR2 Bnd $IC_{50} = 1700$ nM) compound **34** was identified as a potent CCR2 antagonist (CCR2 Bnd $IC_{50} = 56$ nM, chemotaxis $IC_{50} = 300$ nM) that exhibited excellent selectivity against a broad panel of other GPCRs. A subsequent presentation revealed that **35** (CCR2 Bnd $IC_{50} = 29$ nM) had been taken forward into early clinical development [108]. Related compounds **36**, **37**, and other analogs have been exemplified [109,110], but are not yet described in the peer-reviewed literature.



34 (X = F)
35 (X = OH)



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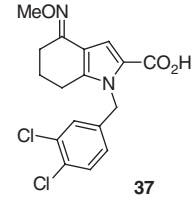
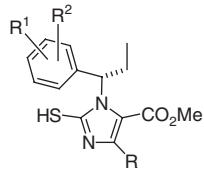
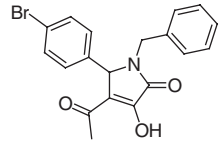


37

Mercaptoimidazoles were described as potent CCR2 antagonists [111–114]. Starting from the screening hit **38**, compounds **39** and **40** were found to be potent antagonists in the calcium flux assay ($IC_{50} = 10$ nM for both compounds). The thiol moiety was described as essential for CCR2 activity, since desulfurized analogs were inactive. The 4-ester substitution and the benzyl moiety substituted with an ethyl were found to be optimal for activity. Both **39** and **40** were active in a chemotaxis assay (human monocytes $IC_{50} < 100$ nM for both compounds) and showed good selectivity (>100-fold) over other chemokine family members.



38 (racemic) R = H, R^1 = 3-Cl, R^2 = 4-Cl
39 R = CO_2Me, R^1 = 3-Cl, R^2 = 4-Cl
40 R = CO_2Me, R^1 = 3-F, R^2 = 4-F



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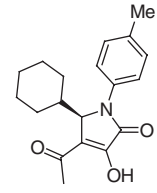
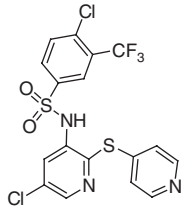
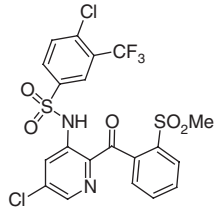


42

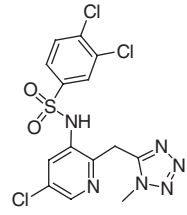
A recent paper described the discovery of 3-hydroxy-1*H*-pyrrol-2(5*H*)-ones as CCR2 antagonists [115]. Screening hit **41** exhibited a CCR2 binding IC_{50} of 8000 nM. Iterative optimization provided compound **42** (CCR2 Bnd $IC_{50} = 498$ nM, chemotaxis $IC_{50} = 603$ nM) as an orally-bioavailable lead molecule for further SAR development [115]. Additional compounds have been described in a recent patent application [116].



43



44



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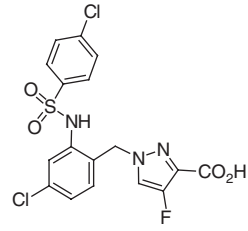
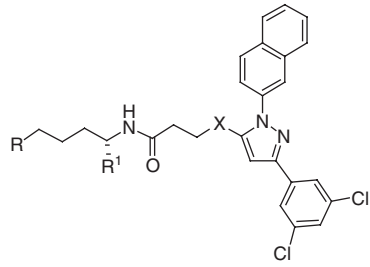


46

Several patents claiming aryl sulfonamides have recently appeared [117–121]. In the first, compound **43** was described to be effective in the rat collagen-induced arthritis model when dosed at 100 mpk (s.c., q.d., from day 9–17) [117]. This patent also reported that compound **44** significantly inhibited the number of monocytes recruited following thioglycolate injection in a rat peritonitis model. Two other patent applications describe sulfonamides **45** [120] and **46** [121] as CCR2 antagonists.



47 R = NHC(=NH)NH_2, R^1 = CO_2Me, X = CH_2
48 R = NEt_2, R^1 = $CH(Me)_2$, X = O

Diaryl substituted pyrazoles have also been described as potent CCR2 antagonists [122]. The screening hit **47** was found to have both CCR2 (Bnd IC_{50} = 221 nM) and CCR5 (Bnd IC_{50} = 63 nM) activity. The diaryl pyrazole core was quite resistant to change. Compound **48** proved to be the most CCR2 active (Bnd IC_{50} = 6 nM and chemotaxis IC_{50} = 32 nM), and also exhibited good selectivity over CCR5 (Bnd IC_{50} = 1610 nM).

4. CONCLUSION

Recent data have highlighted that CCR2 plays a more central role in immunology than had been previously anticipated, in that it governs the emigration of activated monocytes from the bone marrow in addition to directing their migration toward certain points of inflammation. New pre-clinical validation for CCR2 antagonism in rodent disease models has also been obtained, using both genetic and (for the first time) chemical approaches. Early results from phase II human clinical trials with CCR2 antagonists have been reported, and more data are expected in the near future; clinical proof-of-confidence has not yet been obtained for this mechanism. Finally, despite substantial, positive evidence for the benefits of antagonizing CCR2, recent pre-clinical data have also highlighted potential concerns with blocking this receptor.

Multiple new series of CCR2 small molecule antagonists have been described in the recent patent and peer-reviewed literature. Importantly, the diversity of structural classes recognized as CCR2 antagonists has increased. These chemical advances should allow the scientific community to test adequately the hypothesis that CCR2 plays a key role in human inflammatory disease.

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CHAPTER 15

Semicarbazide Sensitive Amine Oxidase and Vascular Adhesion Protein-1: One Protein Being Validated as a Therapeutic Target for Inflammatory Diseases

Ian A. McDonald, Jonathan Foot, Ping Yin, Eleanor Flening and **Ellen M. van Dam**

Contents

Pharmaxis Ltd, Frenchs Forest, NSW 2086, Australia

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1. INTRODUCTION

Two independent areas of research converged when the identity of two proteins under investigation were shown to be identical [1,2]. Semicarbazide-sensitive amine oxidase (SSAO), known as plasma amine oxidase and benzylamine oxidase, is identical in structure to vascular adhesion protein-1 (VAP-1). In this review, we will use SSAO/VAP-1 to refer to this protein. The SSAO/VAP-1 oxidase activity, which has been recognized for many years, was often considered more a curiosity than an activity associated with physiology or pathology [3]. The activity was best defined by Lyles as the capacity to oxidize primary amines in the presence of complete MAO-A and B inhibition, and that this residual activity could be inhibited by carbonyl reactive groups, such as semicarbazide (**1**) and hydrazines (e.g., phenylhydrazine (**2**), phenelzine (**3**) and hydralazine (**4**)) (reviewed in [3]). The discovery of VAP-1 as a protein essential to the extravasation of inflammatory cells was due to a series of very elegant studies by Jalkanen, Salmi and co-workers. VAP-1 antibodies were revealed to attenuate inflammatory processes, and together with a substantial body of evidence with *in vitro* and *in vivo* knockouts confirmed the role of this protein in inflammatory cell signaling [4–9].

SSAO/VAP-1 is an ectoenzyme containing a very short cytoplasmic tail, a single transmembrane domain, and a large, highly glycosylated extracellular domain which contains the active center. SSAO/VAP-1 is also present in a soluble form circulating in the plasma [10,11]. Using transgenic mouse models it has been shown that this form is a cleaved product of membrane-bound SSAO/VAP-1 [12], a phenomenon often seen in ectoenzymes. The genes coding SSAO/VAP-1 proteins are known as AOC genes. However, in some species a fourth gene has been cloned (AOC-4) (see Table 1 for a description of the various AOC genes) that encodes a protein which is highly homologous to plasma SSAO/VAP-1 [13]. The mouse genome only contains fragments of an AOC-4 gene and the human AOC-4 contains a single base change leading to a truncated, non-functional protein [13].

The natural ligand for the amine oxidase activity is not known for certain. While SSAO/VAP-1 will oxidize endogenous molecules such as methylamine and tyramine, the substrates associated with diapedesis are unknown. It has been speculated that leukocyte cell surface lysine residues or amino sugars, such as mannosamine residues (**5**) known to be associated with cell/cell recognition may be involved [14,15].

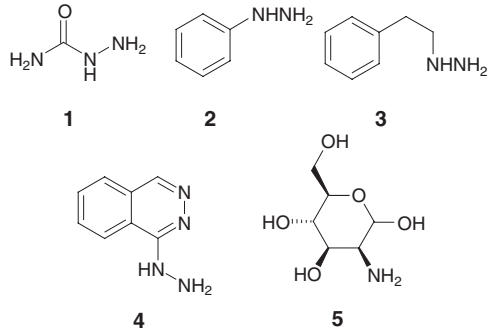


Table 1 AOC genes in *Homo sapiens*

Official symbol	Official name	Other names	Chromosome location	Genomic location	Gene size (bp)	Number of exons	Transcript length (bp)	Protein length (aa)
ABP1[#]	Amiloride binding protein 1 (amine oxidase (copper-containing))	ABP, DAO, KAO, AOC1, DAO1	7q34 – 7q36	150,180,506 – 150,189,312	8807	5	2428	751
AOC-2	Amine oxidase, copper-containing 2 (retina-specific)	DAO2, RAO	17q21	38,250,135 – 38,256,250	6116	4	2665 2584 (2 isoforms)	757 730
AOC-3	Amine oxidase, copper-containing 3 (vascular adhesion protein 1)	HPAO, SSAO, VAP1, VAP-1,	17q21	38,256,727 – 38,263, 667	6941	4	4026	763

also referred to as AOC-1

When it was proven that VAP-1 and SSAO were the same protein, Salmi, Jalkanen, and other groups then demonstrated that inhibition of the amine oxidase activity could also attenuate inflammation [16]. Antibodies and inhibitors of the amine oxidase activity of SSAO/VAP-1 have been found to interfere with leukocyte rolling, adhesion and extravasation [5,8,16–21]. They also noted that the VAP-1 antibody did not block the amine oxidase activity. This made it clear that SSAO/VAP-1 is a protein with two independent activities, both of which are important for leukocyte trafficking [16]. Mice lacking SSAO/VAP-1 (AOC-3-/-) show reduced adhesion of leukocytes to endothelial cells, reduced lymphocyte homing to the lymph nodes and a concomitant attenuated inflammatory response in a peritonitis model. AOC-3-/- mice could not be differentiated from WT mice in all other aspects. Animals were healthy, grew normally, were fertile, and examination of various organs and tissues showed the normal phenotype. SSAO/VAP-1 is now considered to be an emerging therapeutic target for inflammatory diseases. Therapeutic antibodies and small molecule amine oxidase inhibitors are being pursued as potential anti-inflammatory agents in many organizations.

For those interested in the discovery of drug candidates to attenuate SSAO/VAP-1 activity there are two properties that need to be considered. First, as mentioned above, SSAO/VAP-1 exists as a membrane bound protein and a truncated version is found in the plasma [10,11]. Second, there is tremendous species variation which is revealed in a very large range of the second order rate constant V/K, using benzylamine as substrate, [22,23], and that inhibitor potencies vary widely according to the species [24,25]. Furthermore, within a single species specific activity varies from tissue to tissue [26].

2. MOLECULAR BIOLOGY OF THE COPPER-CONTAINING AMINE OXIDASE FAMILY

The mammalian amine oxidases metabolize various mono-, di-, and poly-amines produced endogenously or absorbed from exogenous sources. They are subdivided into two main classes based on the chemical nature of the cofactors. The first group includes the monoamine oxidases (MAO) which are present in most cell types (reviewed in [27]) and use covalently bound flavin adenine dinucleotide (FAD) as the cofactor. The second group is the family of copper-containing amine oxidases with an oxidized active site tyrosine residue (TPQ) being the unusual cofactor for most of these enzymes [28]. MAO and SSAO/VAP-1 do share some substrates which includes some of the common monoamines. The systematic nomenclature for the copper-containing amine oxidases has not been completed and as a result there is often confusion in the literature. Copper-containing amine oxidases (EC1.4.3.6) is a common name for a heterogeneous group of amine oxidases which are encoded by the AOC genes. Information is obtained from NCBI Entrez Gene (build 36.2, September 2006). The human AOC genes and their products are summarized in Table 1. A fourth AOC gene has been cloned from pigs [13]; it is highly homologous to AOC-3 but codes for a protein containing a signal peptide instead of the transmembrane domain.

Interestingly, this gene is absent in rats and mice and the human AOC-4 gene encodes for a non-functional protein caused by the introduction of a stop codon [13]. AOC-4 was therefore omitted from the Table 1.

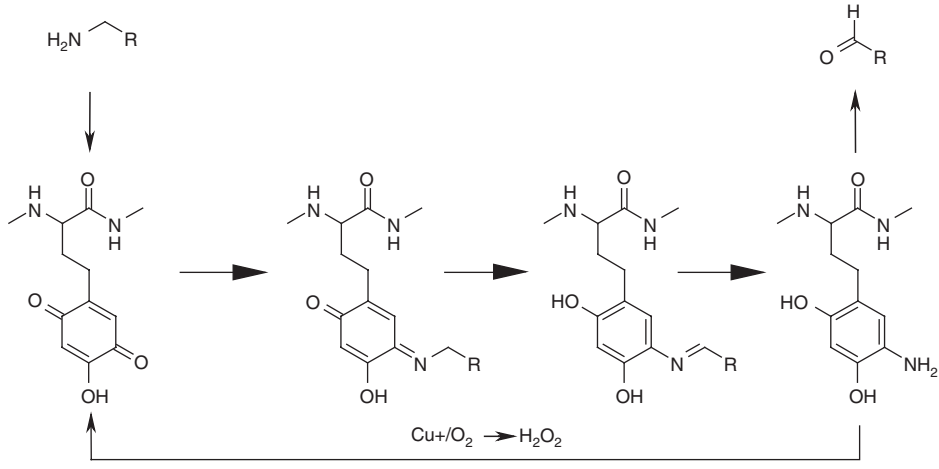
3. CO-FACTOR AND MECHANISM OF THE AMINE OXIDASE ACTIVITY

In contrast to the flavin-dependent monoamine oxidases, SSAO/VAP-1 has evolved to hydroxylate a tyrosine residue in the active site which is further oxidized to the quinone state by oxygen in the presence of copper ion releasing hydrogen peroxide [28–30]. The primary amine in the substrate ($R\text{-}NH_2$, Scheme 1) forms a Schiff-base with the quinone carbonyl group, which through a series of steps ultimately releases the aldehyde product.

The enzyme kinetics has been studied in considerable detail by the Klinman group [31–37] and others [38]. These analyses indicate that the rate-limiting step is either the proton abstraction step or the re-oxidation of reduced co-factor which depends on the species and substrate.

4. STRUCTURAL BIOLOGY

The three-dimensional protein structures of many non-human copper-containing amine oxidases have been resolved to high resolution [39]. A truncated version of the human SSAO/VAP-1 has been solved by two research groups at resolutions of 2.5 and 2.9Å [40,41]. All copper-containing amine oxidases crystallize as homodimers with a well-defined binding site containing the oxidized tyrosine cofactor (TPQ) and the copper atom. Gate keeper residues (Leu469 in human SSAO/VAP-1) block the active site, and need to rotate to accommodate substrate. A conserved aspartate residue (Asp386 in human SSAO/VAP-1) serves as an essential catalytic acid and base as the enzyme proceeds through the various proton transfer steps. The structure of the binding pocket does vary considerably



Scheme 1 SSAO/VAP-1: TPQ-catalyzed amine oxidase mechanism.

amongst species with the human form being more closely aligned to the bovine protein in comparison to the rat.

5. THERAPEUTIC OPPORTUNITIES

5.1 SSAO/VAP-1 and inflammatory diseases

Inflammation is the first response of the immune system to infection or irritation. Leukocyte migration from the circulation into tissues is essential for this process. An inappropriate inflammatory response to a normally innocuous stimulus or to a false signal can result in local inflammation of an otherwise healthy tissue which can lead to disorders such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and respiratory diseases. In order to pass through the walls of the blood vessels, the leukocytes first adhere to the endothelium via binding to adhesion molecules (reviewed in [42]). Membrane bound SSAO/VAP-1 is abundantly expressed in vascular endothelial cells such as high venule endothelial cells (HVE) of lymphatic organs and is also expressed in hepatic sinusoidal endothelial cells (HSEC), smooth muscle cells and adipocytes. Although SSAO/VAP-1 reveals no similarity to any known adhesion molecule and the leukocyte ligand for SSAO/VAP-1 has not been identified, its role in adhesion has been well established.

The expression of SSAO/VAP-1 on the cell surface of endothelial cells is tightly regulated and is increased during inflammation [43–45]. However, the signal transduction pathway regulating this mechanism is still unknown. The research into the translocation of SSAO/VAP-1 from an intracellular compartment to the cell surface during inflammation is hampered by the lack of expression of SSAO/VAP-1 in endothelial cell lines. Furthermore, primary endothelial cells in culture down-regulate the expression of SSAO/VAP-1. Expression of SSAO/VAP-1 alone is not enough to accommodate adhesion. Non-endothelial cells such as COS and CHO cells over-expressing SSAO/VAP-1 did not show adhesion to leukocytes *in vitro* [46], although this could be caused by a difference in glycosylation in these cell lines. While the mechanism is not understood, it was observed that addition of a SSAO/VAP-1 substrate (benzylamine) caused NFκB activation in HSECs and an associated up regulation of another adhesion molecule, E-selectin, and chemokine CXCL8 (IL-8) *in vitro* [21], further suggesting the important role of the amine oxidase activity of SSAO/VAP-1 in the inflammatory response.

The symptoms of many chronic diseases, such as rheumatoid arthritis, multiple sclerosis, asthma and chronic obstructive pulmonary disease (COPD) are caused in large part by an excessive and chronic inflammatory response and are therefore potential human diseases for drugs which inhibit the SSAO/VAP-1 activity. Notably, it has been recently shown that patients suffering from either atopic eczema or psoriasis, both chronic inflammatory skin disorders, demonstrate an increase in SSAO/VAP-1 positive vessels in their skin compared to skin from healthy controls [47,48].

5.2 SSAO/VAP-1 and other diseases

In addition to the presence of SSAO/VAP-1 on endothelial cells, it is highly expressed in adipocytes (1% of total membrane proteins) where it has been suggested to play a role in glucose transport independent of the presence of insulin. Addition of benzylamine enhanced the glucose uptake in adipocytes which could be inhibited by semicarbazide. It is hypothesized that the production of hydrogen peroxide causes the recruitment of glucose transporter 4 (GLUT-4) to the cell surface which accommodates the increased glucose uptake in these cells [49,50]. It was noted that levels of plasma SSAO/VAP-1 are increased in patients suffering from diabetes [10], a disease in which insulin secretion or insulin-dependent glucose uptake in cells is compromised. In addition, elevated levels of plasma SSAO/VAP-1 have been found in patients suffering from congestive heart failure and liver cirrhosis [10], even though the role of plasma SSAO/VAP-1 in physiology and/or pathophysiology remains to be resolved.

6. DRUG DISCOVERY

With the recent demonstration that SSAO and VAP-1 are the same protein and have potential as an anti-inflammatory therapeutic target, several drug discovery groups in academia and the pharmaceutical industry have launched research programs. To date, no obvious clinical candidates have emerged, although work has been published and a number of patent applications have appeared. Reviews have been published [3,51,52] that summarize early work in this area, much of which pre-dated the discovery of the anti-inflammatory potential. Recently the emergence of SSAO/VAP-1 as an anti-inflammatory therapeutic target was highlighted [53]. Many of the early inhibitors were originally designed to inhibit other therapeutic targets, notably MAO-A and B. Others were various well-known hydrazine derivatives which were tested because they possessed a carbonyl reactive group. Over the past 5 years, the same theme has continued with considerable attention directed to the design of novel hydrazine, arylalkylamine, allyl and propargylamine derivatives. Screening efforts are starting to pay dividends with the discovery of novel and competitive inhibitors.

7. SSAO/VAP-1 INHIBITORS

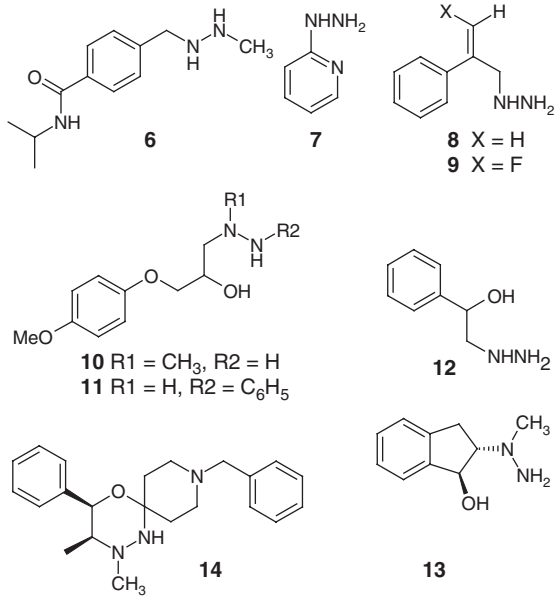
7.1 Antibodies

In an important study, Kirton *et al.* [5] engineered a mouse–human chimeric antibody and demonstrated that this was able to reduce leukocyte migration in various *in vitro* and *in vivo* mouse models. In particular, leukocyte migration to the peritoneal cavity was reduced by 40% in the thioglycollate inflammation model using mice expressing human SSAO/VAP-1 protein. The Finnish company Biotie Therapies Corporation, is in clinical development with an antibody to VAP-1 [54].

7.2 Hydrazines

Stemming from the original observation that hydrazine-containing compounds irreversibly inhibit the amine oxidase activity of SSAO/VAP-1 [3], a large number of hydrazines have been evaluated. This includes the carcinostatic agent procarbazine (**6**), the MAO inhibitor phenylhydrazine (**2**; $IC_{50} = 25\,nM$, bovine lung) and the antihypertensive hydralazine (**4**; $IC_{50} = 1.5\,\mu M$, bovine lung) [3,55]. It has been speculated that SSAO/VAP-1 inhibition may play a role in the mechanism of action of hydralazine (**4**) [56]. The exact mechanism by which 2-hydrazinopyridine (**7**) inhibits *Escherichia coli* copper-containing amine oxidase has been recently reported in some detail [40,57,58].

A group from La Jolla Pharmaceuticals has released data on their novel hydrazines in recent scientific [20,59,60] and patent literature [61,62]. A series of arylallyl hydrazines (e.g., **8** and **9**) were shown to be potent, irreversible inhibitors of rat and human SSAO/VAP-1 [59]. LJP-1207 (**8**, $IC_{50} = 2\,nM$, human) was evaluated in a series of *in vivo* inflammation models. Significant efficacy was observed in a mouse ulcerative colitis, mouse LPS-induced septic shock, and the rat carrageenan foot models [20], in a mouse model that resembles human multiple sclerosis [63], and in a transient forebrain ischemia model in estrogen-treated ovariectomized female rats [60].

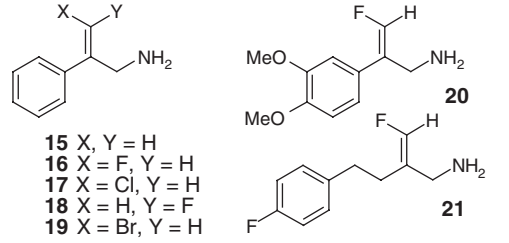


In a patent application [64], Biotie Therapies Corporation revealed that a series of hydrazines, including **10** ($IC_{50} = 350\,nM$) and **11** ($IC_{50} = 90\,nM$), were designed from an understanding of the three-dimensional environment of the binding pocket. The 2(*R*)-hydrazino alcohol (**12**) [65], was similarly also potent ($IC_{50} = 35\,nM$, human) in which the (*S*)-enantiomer is a less active and selective SSAO/VAP-1 inhibitor than the (*R*)-enantiomer. The Biotie group has also

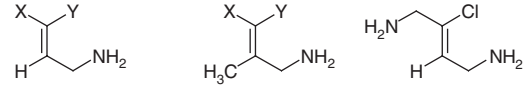
reported that the hydrazine BTT2052 (**13**) inhibited human SSAO/VAP-1 (Ki = 33 nM), and furthermore significantly attenuated inflammatory responses in various rodent models of arthritis [6]. The cyclic hydrazine **14** is a potent inhibitor against recombinant human SSAO/VAP-1 expressed in CHO cells (IC_{50} = 80 nM) with respect to both activity and selectivity over MAO [66].

7.3 Allylamines and propargylamines

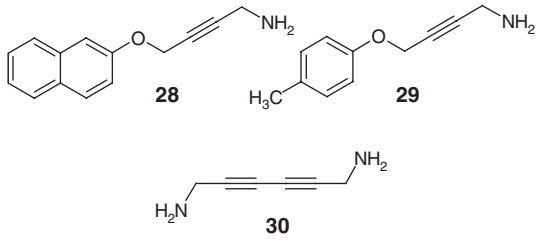
The major issue for all the hydrazine-based inhibitors is one of safety. While there are many hydrazines in clinical use, such as phenelzine (**2**) and hydralazine (**3**), this class of compounds frequently inhibit liver metabolizing enzymes which may lead to toxicity and/or drug–drug interactions [67]. Since SSAO/VAP-1 inhibitors target chronic inflammatory diseases such as rheumatoid arthritis, asthma, psoriasis, etc., it is important that very safe, potent and selective inhibitors are discovered. One family of inhibitors that offers potential are the haloallylamines, such as MDL72161A (**16**), MDL72274A (**17**), MDL72145 (**20**) and MDL72974A (Mofegiline, **21**), which have been widely studied and have provided good opportunities for lead development [68,69]. These compounds were also originally designed as MAO-B inhibitors, but were found to inhibit SSAO/VAP-1 in a time-dependent manner [68]. The parent compound (**15**), had modest potency (IC_{50} = 3 μM, rat aorta), but the chloro- and fluoro-substituted alkenes, **16–17**, were extremely potent against the rat aorta enzyme (IC_{50} = 2.5 nM and 8 nM, respectively) [68]. These compounds have been shown to be irreversible inhibitors, and in the case of **17**, highly selective for SSAO/VAP-1 over both MAO-A and MAO-B. However, both **16** and **17** are much less active against the bovine and human forms of the enzyme. MDL 72974A (**21**), however, was similarly potent against the rat, bovine aorta and human umbilical cord SSAO/VAP-1 preparations with IC_{50} values of 5 nM, 80 nM, and 20 nM respectively [70]. In a recent patent application [62] **21** was shown to be active in the mouse autoimmune encephalomyelitis model, a surrogate for human multiple sclerosis.



15 X, Y = H
16 X = F, Y = H
17 X = Cl, Y = H
18 X = H, Y = F
19 X = Br, Y = H



22 X = Cl, Y = H
23 X = Cl, Y = C_6H_5
24 X = C_6H_5, Y = Cl
25 X = Br, Y = H
26 X= H, Y = Br



Research in the Sayre laboratory has focused on analogues of 3-chloroallylamine (**22**). The inhibition profiles of the (*E*) and (*Z*) isomers of compounds **23**–**27** against six different copper-containing amine oxidases were evaluated. In a series of elegant publications, the mechanism of action of these inhibitors has been studied in detail [71–73]. The design was extended to a series of propargylamines (e.g., **28, 29**) which proved to be modest inhibitors (IC_{50} from the low to high micromolar range) of many family members of this panel of amine oxidases. Co-crystal structures of **28** and **29** with *Arthrobacter globiformis* amine oxidase, determined at 1.7Å resolution, revealed that the amino group on the reduced TPQ cofactor has alkylated the unsaturated aldehyde product [39]. The diamine **30** was the most effective of this series against the panel of enzymes.

7.4 Peptides

With an understanding of the structure of the SSAO/VAP-1 binding site, the Salmi/Jalkanen group designed a series of small peptides as inhibitors [19]. They probed the active site of the enzyme using lysine-containing peptides and found that a family of peptide inhibitors, including the small peptide GGGGKGGGG ($IC_{50} = 534\,\mu M$), behave as long lasting and highly selective inhibitors of both endothelial and recombinant SSAO/VAP-1. This compound was shown to inhibit SSAO/VAP-1-dependent lymphocyte rolling and firm adhesion to rabbit primary endothelial cells.

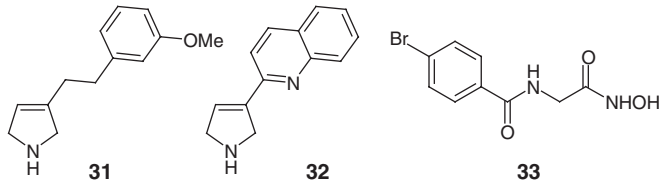
7.5 Dihydropyrroles

Two laboratories have independently disclosed an interesting series of mechanism-based inhibitors. The dihydropyrrole **31**, which appeared in a patent application [61], was reported to inhibit rat lung SSAO/VAP-1 with an $IC_{50} = 500\,nM$. Recently, the Sayre team extended earlier work [74] and showed that these inhibitors, exemplified by **32**, covalently bound to the enzyme with the cofactor in the reduced form [75]. Presumably, aromatization of the dihydropyrrole moiety accounts for the observed potencies.

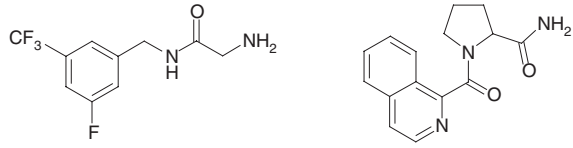
7.6 Miscellaneous structures

Novel structures are now appearing in the patent literature. A series of hydroxamic acids, such as **33** ($IC_{50} = 100\,nM$, human) were disclosed in a recent

application [76]. In another patent application [61] the LJPC group claimed a series of β-amidoamines, including **34**. This compound was a very potent inhibitor of rat lung SSAO/VAP-1 ($IC_{50} = 33$ nM, respectively). Interestingly, it was described as an irreversible inhibitor of the enzyme. A series of amide derivatives of proline were reported [75] to be very potent inhibitors of the human enzyme. For example, **35** was stated to have an IC_{50} value = 78 nM.

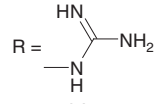


31 **32** **33**



34 **35**

Patent applications from Astellas Pharma [77,78] and Fujisawa [79] have disclosed series of guanine, imidates and aminoimidazoles built on a bis arylethane core structure. Some of these are very potent. Compound **36** inhibits the human and rat enzymes with $IC_{50} = 150$ nM and 12 nM, respectively; compounds **37**, **38** and **39** inhibit human SSAO/VAP-1 with $IC_{50} = 5.7$ nM, 5.3 nM and 2.4 nM, respectively.



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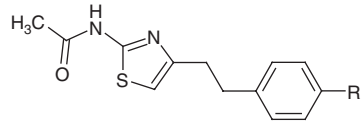

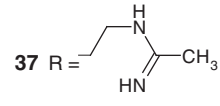

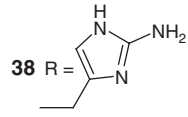

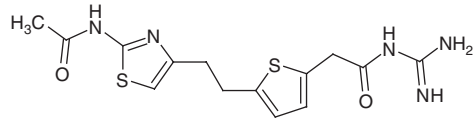

39

Other inhibitors include alginic acid hydroxamates [80], the natural products myricetin galloylglycosides [81], polyaminoglycosides [82] and a number of antidepressant drugs [83] which demonstrate various degree of SSAO/VAP-1 inhibitory property in rats, humans and cows.

8. CONCLUSION

SSAO/VAP-1 is an emerging target for drug discovery. There is considerable scientific evidence pointing to this protein as a potential target for many inflammatory diseases. The target is amenable to drug discovery, and a considerable effort to discover novel, useful drug candidates is underway. However, there is reason to question the validity of this target. Drugs such as hydralazine and phenelzine which inhibit SSAO/VAP-1 at therapeutic doses have not been reported to have anti-inflammatory properties. True validation will only come when some of the newer, very potent and selective agents are evaluated in patients suffering from these inflammatory conditions.

This is an exciting time for those seeking to better understand the precise mechanistic details by which SSAO/VAP-1 serves to facilitate inflammatory cell migration. In particular, if the natural ligand or ligands can be determined, this should elevate the profile of this inflammatory target as a drug target comparable to the selectins and integrins. This knowledge may also help with the design of more potent and selective inhibitors.

The search for drug leads is an interesting endeavor in itself. Time will tell whether a potent competitive inhibitor will have advantages over time-dependent, irreversible inhibitors, or whether the latter class will be more effective against this inducible protein. In any case, the availability of the crystal structure of a truncated human form of the protein will facilitate the search for structural cores which may lead to novel competitive inhibitors. Finally, since SSAO/VAP-1 is a protein which can facilitate cell–cell interaction and can oxidize a family of primary amines, there are in effect two targets for drug design – antagonize the adhesion binding site or inhibit the amine oxidase activity. Both would appear to be valid approaches.

Although most work to date has focused on the enzyme inhibition aspect, it is possible that the monoclonal VAP-1 antibody discussed above may well be closer to the clinic. The task of designing small molecules to interfere with cell–cell recognition is certainly feasible, but this will not be a trivial effort, more akin to the search for selective selectin antagonists which has proven to be very challenging. As confidence grows in the pharmaceutical industry that SSAO/VAP-1 is a validated target, it is inevitable that considerable resources will be directed to all avenues to block the functional action of this protein.

9. ABBREVIATIONS

ABP	amiloride-binding protein (histaminase)
AOC	amine oxidase, copper-dependent
COPD	chronic obstructive pulmonary disease

DAO	diamine oxidase
FAD	flavin adenine dinucleotide
HPAO	Hansenula polymorpha amine oxidase
HVE	high venule endothelial cells
HSEC	hepatic sinusoidal endothelial cells
KAO	kidney amine oxidase
LJPC	La Jolla Pharmaceutical Company
RAO	retina-specific amine oxidase
SSAO	semicarbazide-sensitive amine oxidase
TPQ	topa-quinone
VAP-1	vascular adhesion protein 1

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CHAPTER 16

Sphingosine 1-Phosphate Type 1 Receptor Modulators: Recent Advances and Therapeutic Potential

Nigel Cooke and **Frédéric Zécri**

Contents

1. INTRODUCTION

Sphingosine 1-phosphate (S1P) is a naturally occurring sphingolipid mediator that functions both as an intracellular messenger in many different types of cells and as an extracellular signaling molecule [1–4]. S1P plays fundamental physiological roles in a number of processes including vascular stabilization [5], heart development [6], lymphocyte homing [7], and cancer angiogenesis [8]. Extracellularly, S1P has been shown to elicit its biological effects through the activation of the endothelial differentiation gene (EDG) family of G protein-coupled receptors [9–13]. This family consists of eight highly homologous receptors; five of

Global Discovery Chemistry, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland

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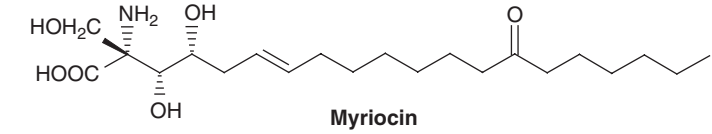
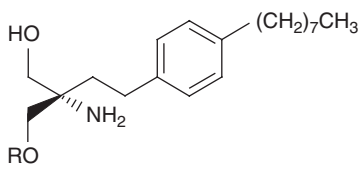
these receptors, $S1P_1$ – $S1P_5$, are specific for S1P and the other three, LPA_1–LPA_3, are specific for the related lysophospholipid mediator lysophosphatidic acid (LPA) [14]. It has recently been demonstrated in T-cells that activation of plasma membrane S1P receptors results in internalization and translocation of the receptors to nuclear envelope membranes, indicating that nuclear S1P receptors are one class of intracellular targets of S1P. Other as-yet undefined intracellular targets may also be important [15–20]. The discovery of **FTY720** and the high degree of efficacy seen with **FTY720** in a wide range of pre-clinical models of autoimmune diseases have been the catalyst for extensive research efforts into the S1P receptors [21,22]. Comprehensive surveys of the chemistry and biology of sphingolipids [23] and the role of S1P receptors in health and disease [24,25] have appeared.

1.1 Mode of action of FTY720

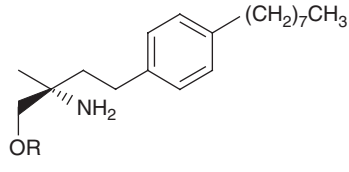
Immunosuppressants are clinically important for organ transplantation and the treatment of autoimmune diseases. Currently approved immunosuppressive drugs have a low therapeutic index and require careful drug monitoring. In particular, the use of calcineurin inhibitors such as cyclosporine A or tacrolimus (FK506), which specifically block T-cell activation, is limited by mechanism-based side effects. These adverse side effects are currently partially reduced by combining calcineurin inhibitors with antiproliferative agents such as IMPDH inhibitors (e.g. mycophenolic acid) or mTOR inhibitors, however, significant medical need remains for novel mechanisms of action in the field of immune modulation. In the early 1990s, Fujita *et al*. showed that the palmitoyltransferase inhibitor, **Myriocin**, isolated from the fermentation broth of *Isaria sinclairii*, exhibited *in vitro* and *in vivo* immunosuppressive activity with an $IC_{50} = 3\,nM$ in the mouse allogeneic mixed lymphocyte reaction (MLR). **Myriocin** prolonged survival of rat skin allografts when administered intraperitoneally at 0.3 mg/kg (Mean survival 11.2 ± 0.5 days *vs* 8 days for control). Doses of 1 mg/kg and higher resulted in death of the animals [26].



Myriocin



R = H **FTY720**
R = PO_3H **(S) -2**



R = H **(R) -1**
R = PO_3H **(R) -3**

The exploration of chemically modified derivatives of **Myriocin** led to the discovery of the structurally related compound **FTY720** which was efficacious in the rat skin allograft model at 0.1 mg/kg p.o. (Mean survival 25.3 ± 0.6 days *vs* 8.5 days for control) and inhibited lymph node swelling in a rat host-versus-graft (HvG) model ($ID_{50} = 0.2$ mg/kg p.o.). In both studies a marked reduction in the number of circulating lymphocytes was observed (50% reduction at 0.03 mg/kg p.o.; 80% reduction at 0.1 mg/kg p.o.). **FTY720** did not inhibit palmitoyltransferase, was inactive in the mouse mixed lymphocyte reaction, and only induced apoptotic cell death of lymphocytes at micromolar concentrations. The efficacy of **FTY720** in the rat HvG model is believed to result from its ability to inhibit the recruitment of specific T-cells to the antigen-draining lymph nodes, thereby reducing the swelling of these specific lymph nodes [26,27].

Several analogues having alkyl groups instead of one of the prochiral hydroxymethyl groups of **FTY720** were synthesized and evaluated for their effects on HvG inhibition and decrease in the number of T-cells in peripheral blood [28]. The (*R*)- enantiomer of 2-methyl ethanolamine analogue **1** inhibited lymph node swelling in the rat HvG ($ID_{50} = 0.09$ mg/kg) and led to a reduction in circulating lymphocytes ($ID_{50} = 0.01$ mg/kg) whereas the corresponding (*S*)- enantiomer of compound **1** had no effect on HvG or the number of circulating lymphocytes up to doses of 1 mg/kg p.o. From these results, it appears that between the two hydroxymethyl groups of **FTY720**, only the pro-(*S*) hydroxymethyl group is of critical importance for the potent *in vivo* activity [28,29].

The first insights into the mechanism by which these aminoalcohols were inducing reductions in circulating lymphocytes came from reverse pharmacology studies. Pharmacokinetic analysis of **FTY720** by liquid chromatography-mass spectrometry (LC-MS) in rats and mice revealed that **FTY720** was metabolized to the monophosphate ester **2** via sphingosine kinases [30]. Intraperitoneal or intravenous administration of **FTY720** phosphate to rats resulted in a reduction of circulating lymphocytes [31,32]. Similarly, the (*R*)- enantiomer **1** was readily phosphorylated in rat blood and led to reduction in circulating lymphocytes whereas the corresponding (*S*)-enantiomer showed only trace phosphorylation in rat blood and had no effect on lymphocyte numbers [31]. *Ex vivo o*-phthaldialdehyde derivatization and chiral HPLC analysis of blood from either rats or human subjects treated with ^{14}C labeled **FTY720** showed that only the (*S*)-enantiomer of **FTY720** phosphate **2** to be present with the (*R*)-enantiomer being below the limit of detection (<3% total **FTY720** phosphate) [33]. (*S*)- **FTY720** phosphate **2** was found to be a potent agonist on four of the five known sphingosine 1-phosphate receptors; $S1P_1$ ($EC_{50} = 0.3$ nM), $S1P_3$ ($EC_{50} = 3.1$ nM), $S1P_4$ ($EC_{50} = 0.6$ nM) and $S1P_5$ ($EC_{50} = 0.3$ nM) [30–32,34]. The phosphate (*R*)- **3** showed similar EC_{50} values for the $S1P_{1,3,4,5}$ receptors and both phosphates were inactive on the $S1P_2$ receptor [31].

Detailed mechanistic studies have shown that **FTY720** effectively inhibits the egress of T-cells [35] and B-cells [36] from lymph nodes, thereby reducing the number of activated cells that recirculate to peripheral inflammatory tissues [7,37]. Two different hypotheses have been proposed to explain these effects.

The first hypothesis suggests that **FTY720** acts as a functional $S1P_1$ receptor antagonist with agonism at the $S1P_1$ receptor inducing internalization of the receptor in lymphocytes. This results in blockade of S1P-directed migration of the lymphocytes out of the lymph nodes [7,35,38]. The second hypothesis suggests that **FTY720** acts as an $S1P_1$ receptor agonist at the lymphatic endothelium to increase barrier function and reduce lymphocyte transmigration into efferent lymph [30,39]. Recent studies with $S1P_1$ selective receptor antagonists suggest that the functional antagonism mechanism may be disfavoured and this is discussed further in Section 3 of the review.

Two $S1P_1$ independent mechanisms to explain the *in vivo* efficacy of **FTY720** have been reported. **FTY720** but not **FTY720** phosphate inhibits sphingosine 1-phosphate lyase with an IC_{50} in the micromolar range. Sphingosine 1-phosphate lyase (SPL) catalyses the degradation of S1P; therefore, inhibitors of SPL would be expected to raise S1P levels thereby perturbating immune function [40]. One group has reported that **FTY720** but not **FTY720** phosphate inhibits $cPLA_2$, a key enzyme in eicosanoid formation, and that this mechanism contributes to the inhibition of Th2-cell-mediated airway inflammation seen with **FTY720** [41]. Given the low plasma concentrations of **FTY720** and the relative weak potency of **FTY720** on these enzymes, the degree to which these additional mechanisms contribute to the *in vivo* efficacy of **FTY720** remains unclear.

1.2 Therapeutic significance

1.2.1 Efficacy of FTY720 in pre-clinical disease models

Numerous studies have demonstrated that **FTY720** synergizes with classical immunosuppressants (e.g. cyclosporine A, FK506, RAD001, steroids) in rodent and non-human primate models of solid organ and islet transplantation [24,25,37,42]. The most profound activity of **FTY720** monotherapy has been seen in experimental autoimmune encephalomyelitis (EAE) [24,31,43]. Efficacy has also been reported in models of systemic lupus erythematosus, graft versus host disease, type-1 diabetic mice, adjuvant- and collagen-induced arthritis, experimental autoimmune myocarditis, colitis, experimental autoimmune thyroiditis and uveoretinitis [24,25,44–51].

1.1.2 Clinical data of FTY720 in multiple sclerosis and transplantation

In Phase I clinical trials with stable renal transplant patients, **FTY720** led to a transient reduction in the number of circulating lymphocytes whilst being well-tolerated. Treatment was associated with a mild reduction in heart rate that was maximal 6 h after the first dose and returned to baseline with continuous treatment [52,53]. The mild effect on heart rate is believed to arise from $S1P_1$ and/or $S1P_3$-mediated activation of G-protein-gated inwardly rectifying potassium (GIRK) channels in atrial myocytes [54–56].

A phase II clinical study of **FTY720** in *de novo* renal transplant patients showed superior efficacy of **FTY720** compared to mycophenolate mofetil, if combined with cyclosporine A and steroids [57]. In a 1 year, multicentre,

randomized, Phase III study in 668 *de novo* renal transplant patients, **FTY720** given at a daily dose of 2.5 mg or 5 mg per patient was directly compared to mycophenolate mofetil for the potential to synergize with cyclosporine A to prevent rejection of the renal allografts. **FTY720** (2.5 mg) was equipotent to mycophenolate mofetil in providing rejection prophylaxis, however, a two fold higher dose of **FTY720** (5 mg) did not support a 50% reduction in cyclosporine A exposure, indicating insufficient immunosuppressive activity of **FTY720** [58,59]. A proof-of-concept/Phase II clinical trial was conducted in 281 patients with relapsing-remitting multiple sclerosis. The median total number of gadolinium-enhanced lesions on MRI was lower with **FTY720** 1.25 mg (1 lesion; $p < 0.001$) and **FTY720** 5 mg (3 lesions; $p = 0.006$) than with placebo (5 lesions). The annualized relapse rate was 0.77 with placebo, 0.35 with **FTY720** 1.25 mg ($p = 0.009$) and 0.36 with **FTY720** 5 mg ($p = 0.01$). The number of gadolinium-enhanced lesions and the relapse rate remained low in the groups that received continuous **FTY720** and both measures decreased in patients who switched from placebo to **FTY720** [60,61].

In the *de novo* renal transplantation trials, commonly reported adverse events included hypertension, anaemia, constipation and nausea, which may be expected in transplant patients. In the Phase II MS trial, the most commonly reported adverse events were nasopharyngitis, headache, nausea and diarrhoea. In all trials, **FTY720** produced a transient reduction in heart rate and an initial reduction in forced expiratory volume in one second (FEV_1), which are predictable pharmacological effects of S1P receptor modulation. In all trials, **FTY720** was also associated with elevations of liver enzymes, principally alanine aminotransferase, which were not thought to be of clinical relevance. **FTY720** was also associated with renal impairment and macular edema in the renal transplantation trials, with macular edema currently being investigated further in Phase III trials in MS. These clinical trials data have been reviewed in detail elsewhere [62–64].

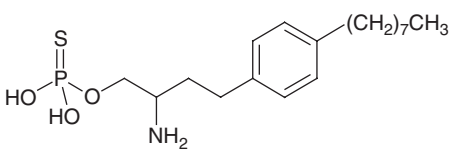
2. $S1P_1$ AGONISTS

Initial medicinal chemistry activities in the S1P receptor agonist field were focused on developing biologically stable phosphate isosteres as tool compounds that could be used to confirm that the reduction in peripheral lymphocyte levels was due to agonism of S1P receptors. A second area of interest was to develop $S1P_1$ agonists that showed a high degree of selectivity versus the $S1P_3$ receptor as it was speculated that the effects on heart rate and lung function seen in clinical trails with **FTY720** were due to agonism of $S1P_3$.

2.1 Amino alcohols and amino phosphates

Phosphothioates have been explored as analogues that had the potential to be biologically stable phosphate isosteres. Phosphothioate analog **4** (EC_{50} on $S1P_{1,3,4,5} = 49$, 600, 860 and 130 nM, respectively) showed a similar S1P receptor selectivity profile to the corresponding phosphate albeit with a ca. 10-fold loss

of potency, whereas **5** showed similar potency to the corresponding phosphate (EC_{50} on $S1P_{1,3,4,5}$ = 5.1, 120, 86 and 24 nM, respectively). Phosphothioate **5** and the corresponding phosphate ester both led to a reduction in lymphocyte count in mice when dosed at 8 mg/kg i.p. A longer duration of action was observed for the phosphothioate which was consistent with the observed slower hydrolysis of the phosphothioate *in vivo* [65,66]. Administration of the parent aminoalcohol **6** at 8 mg/kg i.p. resulted in plasma concentrations of the aminoalcohol in the range of 0.5–2.5 μM over a 24 h period but no detectable levels of phosphate ester, indicating that **6** is a poor substrate for the relevant phosphorylating enzymes (sphingosine kinases).



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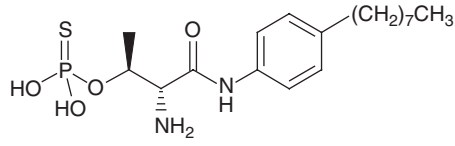

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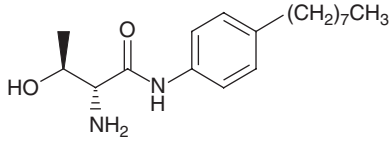

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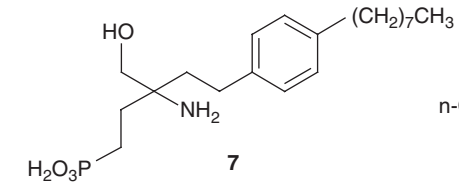
Phosphonic acids represent nonhydrolysable bioisosteres of phosphates and have been extensively explored as potential S1P receptor agonists [67]. Aminophosphonic acid **7** [30] retained moderate $S1P_1$ potency (EC_{50} on $S1P_{1,3,4,5}$ = 8.7, 510, 210 and 230 nM, respectively) and led to a ca. 90% reduction in circulating T- and B-cells in rats and mice when dosed at 5 mg/kg i.v. Modification of the phosphonic acid head group to a *N*-propylphosphonic acid [68] and optimizing the alkyl side chain resulted in the achiral phosphonate **8** (EC_{50} on $S1P_{1,3,4,5}$ = 0.16, 2.7, 8.4 and 0.73 nM, respectively) which induced 87% peripheral lymphocytes reduction in mice at a dose of 0.25 mg/kg i.v. The introduction of conformational constraint into the aminophosphonic acid head group **8** resulted in pyrrolidine **9** (*cis* isomer: EC_{50} on $S1P_{1,3,4,5}$ = 0.1, 2.8, 31 and 0.58 nM, respectively) and cyclohexane **10** (EC_{50} on $S1P_{1,3,4,5}$ = 0.33, 18, 86 and 2.2 nM, respectively) both with similar potency and S1P receptor subtype selectivity [69]. The *cis* and *trans* isomers of pyrrolidine **9** showed similar S1P receptor profiles. The two isomers of **9** and compound **10** were effective in peripheral lymphocyte lowering (89% reduction at 0.25 mg/kg i.v.). These compounds were all acutely toxic in mice when administered at higher doses which was attributed to their potency on the $S1P_3$ receptor [69]. Similar findings of acute toxicity have not been reported for other S1P receptor agonists that have a similar degree of potency on the $S1P_3$ receptor, suggesting that the acute toxicity seen with these phosphonates may be due to an additional off-target effect.



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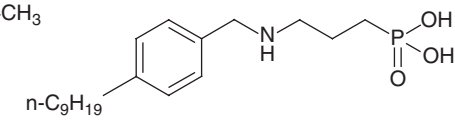

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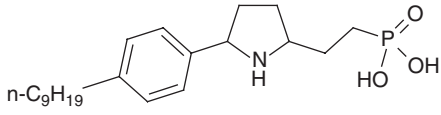

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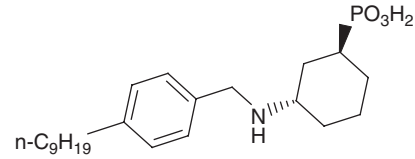

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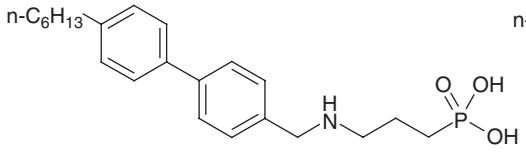

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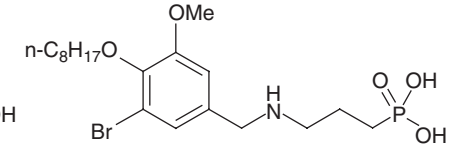


12

Steric constraints imposed by introducing substituents into the lipophilic chain of **8** led to compounds with 200–500 fold selectivity for $S1P_1$ *vs* $S1P_3$ and improved ADME properties in rats [70]. Introduction of a biphenyl moiety gave **11** (EC_{50} on $S1P_{1,3,4,5} = 0.9$, 180, 120 and 2.6 nM, respectively) which had a $Vd_{ss} = 1.8$ L/kg, $Cl_p = 4.6$ mL/min/kg and $t_{1/2} = 1.9$ h in rats. Optimal substitution of the phenyl ring afforded **12** (EC_{50} on $S1P_{1,3,4,5} = 4.1$, 2100, 80 and 10 nM, respectively) which had a $Vd_{ss} = 1.4$ L/kg, $Cl_p = 9.2$ mL/min/kg and $t_{1/2} = 2.1$ h in rats. Phosphonic acids **11** and **12** were very effective in reducing levels of peripheral lymphocytes in mice ($ED_{50} = 0.3$ and 2.1 mg/kg i.v. respectively). Bolus i.v. administration of phosphonates with different degrees of $S1P_1$ *vs* $S1P_3$ selectivity to anaesthetized rats resulted in bradycardia. The extent of the bradycardia was dose dependent with decreased $S1P_3$ affinity correlating with high doses required to elicit similar absolute heart rate lowering effects. Taken together, these data indicate that $S1P_3$ receptor agonism is not required for reduction in peripheral lymphocytes but does play a significant role in the observed heart rate lowering effects seen with nonselective S1P receptor agonists in rodents [70]. These phosphonate analogues were excellent tool compounds that helped confirm that agonism at the S1P receptor indeed resulted in the reduction of peripheral lymphocyte levels, however they all suffer from the disadvantage that they need to be administered i.v.

The application of similar strategies of rigidifying and optimizing the lipophilic tail in the aminoalcohol field has led to a number of aminoalcohol analogs

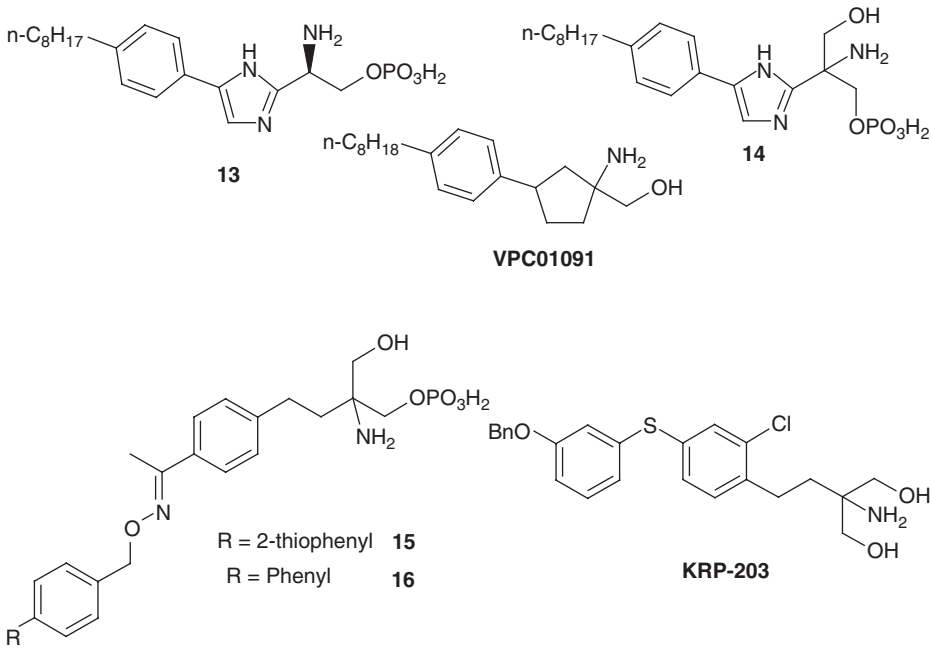
which form phosphates that are agonists at $S1P_1$ and are selective versus the $S1P_3$ receptor. These include the phenylimidazole analogs **13** (EC_{50} on $S1P_{1,3,4,5} = 4$, 330, 150 and 12 nM, respectively) and **14** (EC_{50} on $S1P_{1,3,4,5} = 7.9$, 6300, 160 and 17 nM, respectively). Interestingly, **14** was also found to be an agonist of $S1P_2$ with $EC_{50} = 18$ nM [71]. Replacing the imidazole ring by a cyclopentyl residue gave compounds such as **VPC01091**. The most active isomer of the phosphate ester of **VPC01091** is reported to be a highly selective $S1P_1$ agonist ($EC_{50} = 3.6$ nM), whilst showing no agonistic effects on $S1P_3$ but rather weak inverse agonism ($EC_{50} = 322$ nM) and no detectable effects on $S1P_4$ and $S1P_5$. **VPC01091** reduced levels of peripheral lymphocytes in mice at a dose of 3 mg/kg p.o. and at this dose showed no reduction in heart rate in conscious mice [72]. High degrees of selectivity for $S1P_1$ versus $S1P_{3,4,5}$ are also reported for a series of oximes such as **15** (EC_{50} on $S1P_1 = 0.79$ nM, and no agonism on $S1P_{2,3,4}$ up to 10 μM) and **16** (EC_{50} on $S1P_1 = 0.86$ nM, and no agonism on $S1P_{2,3,4}$ up to 10 μM). Phosphates **15** and **16** reduced levels of peripheral lymphocytes in mice with an ED_{50} in the range 0.08–0.1 mg/kg. Neither compound showed any reduction in heart rate in mice when dosed intra-peritoneally at 90 μg per mouse [73].

The aminoalcohol **KRP–203** has been reported to be highly efficacious in reducing levels of peripheral lymphocytes in rats and led to significant prolongation of survival of rat skin allografts when dosed at 1 mg/kg p.o. (Mean survival >27 days *vs* 8.8 days for control) [74]. **KRP–203** was highly efficacious in the stringent Dark Agouti (DA) to Lewis rat heterotopic heart allograft transplantation model when used in combination with suboptimal doses of cyclosporine A (combination of **KRP–203** at 1 mg/kg p.o., CsA at 3 mg/kg p.o. gave mean survival of > 30 days *vs* 9.7 and 12.5 days for the corresponding monotherapy treatments) [74]. Combination therapy of **KRP–203** and cyclosporine A was also effective in a Brown Norway to Lewis rat renal transplantation model (combination of **KRP–203** at 0.3 mg/kg p.o., CsA at 1 mg/kg p.o. gave mean survival of 25 days *vs* 10 days for CsA alone). **KRP–203** and cyclosporine A were shown to have no effect on the pharmacokinetics of each other [75,76]. **KRP–203** is phosphorylated *in vivo* and the resulting phosphate was shown to be a potent agonist on rat $S1P_1$ receptor with an EC_{50} in the low nanomolar range and equipotent to **FTY720** phosphate **2**. The phosphate of **KRP–203** was inactive on rat $S1P_3$ receptor whereas **FTY720** phosphate **2** was a potent agonist ($EC_{50} = 1.74$ nM). Intravenous administration of **FTY720** phosphate **2** at a dose of 0.01 mg/kg immediately led to acute transient bradycardia in anaesthetized rats whereas a dose of 0.1 mg/kg of **KRP–203** phosphate was needed to affect the heart rate in the same model; these findings were in line with studies in anaesthetized guinea-pigs in which doses of 0.03 mg/kg/day i.v. of **FTY720** resulted in a rapid reduction in heart rate whereas similar doses of **KRP–203** showed no effects. The observation that **KRP–203** phosphate which is inactive on the rat $S1P_3$ receptor still led to reductions in heart rate in rats supports the hypothesis that there are both $S1P_3$-dependent and $S1P_3$-independent pathways that contribute to S1P agonist mediated bradycardia and that the role of

the two pathways differs between species [75]. $S1P_1$ mRNA and protein are strongly expressed in human ventricular, septal and atrial cardiomyocytes as well as in endothelium cell layers of cardiac vessels. In contrast, $S1P_3$ receptors are found in the smooth muscle cell layer of human aorta and cardiac vessels, but only weakly expressed in cardiomyocytes from both atria and ventricles [25]. The heart effects seen with **KRP–203** along with the tissue distribution of human $S1P_1$ and $S1P_3$ receptors suggest that $S1P_1$ and $S1P_3$ receptors may play a role in the regulation of atrial myocyte function and heart rate, and that the relative contribution of the two receptors may vary depending on the species.

2.2 Homology models of S1P receptors

Homology models for human $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$ and mouse $S1P_4$ receptors have been developed that predict the critical interactions with S1P and account for the affinity of **FTY720** phosphate at the receptors [77–83]. Extensive site-directed mutagenesis studies coupled with radioligand binding, ligand-induced GTP[$\gamma-^{35}$S] binding and receptor internalization assays were used in each case to confirm the key amino acids for ligand binding and receptor function. In the case of the $S1P_1$ receptor, charged residues in the third (R3.28), fifth (K5.38) and seventh (R7.34) transmembrane (TM) domains form critical ion pair interactions with the phosphate group of S1P. Two additional interactions have been identified as important; a cation-π interaction of W4.64 with the ammonium group of S1P and an ion pair interaction between E3.29 and the ammonium group of S1P [81]. Amino acid E3.29 is conserved in the S1P receptor family whereas it is a glutamine in the lysophosphatidic acid (LPA) receptors and this difference accounts for the weak binding of S1P to LPA receptors [82]. A key difference between $S1P_1$ and $S1P_2$ models is that $S1P_2$ lacks the key basic group (R7.34) in the seventh TM domain and thus forms one fewer ion pair interaction with the phosphate group of S1P. The $S1P_4$ receptor model shows many similarities to the $S1P_1$ model with R3.28, E3.29, K5.38 and W4.64 residues and interactions being conserved. The basic residue R7.34 is also absent in the $S1P_4$ receptor. Differences in the binding pocket shape and electrostatic distributions between human $S1P_1$ and mouse $S1P_4$ result in the recognition sites for phosphate and the ammonium group being equidistant from the end of the non-polar tail in the mouse $S1P_4$ binding pocket whereas the ammonium group recognition site is 2 Å closer to the end of the non-polar tail than the phosphate recognition site in the human $S1P_1$ binding pocket [81]. According to these models, all five receptors have a conserved hydrophobic binding pocket that is localized to the TM3, TM5 and TM6 domains and consists of 18 key residues. The $S1P_5$ receptor deviates most strongly from $S1P_1$ in this pocket and $S1P_3$ shows the greatest similarity [83]. In the search for S1P subtype selective receptor modulators, exploiting small changes in the hydrophobic binding pocket as opposed to the head group recognition pocket has proven more successful [83,84].



2.3 3,5-Diaryl-1,2,4-oxadiazoles

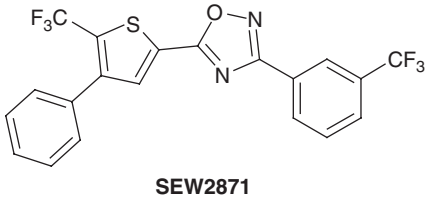
Commercially available 3,5-diaryl-1,2,4-oxadiazoles including **SEW2871** were identified as potent and selective $S1P_1$ receptor agonists by high-throughput screening [84,85]. **SEW2871** was shown to be a full agonist both on murine and human $S1P_1$ ($EC_{50} = 13$ nM on $hS1P_1$) with no agonistic activity up to 10 μM on $S1P_{2,3,4,5}$ [85]. **SEW2871** activated multiple signals triggered by S1P including guanosine 5′-3-O-(thio)triphosphate binding, calcium flux, Akt and ERK1/2 phosphorylation and stimulation of migration of $S1P_1$ but not $S1P_3$ expressing cells *in vitro* and has been shown to induce $S1P_1$ receptor internalization [79,85]. **SEW2871** reduced peripheral lymphocytes in mice ($ED_{50} = 5.5 \pm 1.04$ mg/kg p.o.) and showed a clear PK/PD relationship with plasma levels of 2 μM resulting in a 50% reduction in lymphocyte number. When **SEW2871** was tested at a dose which induced $>80\%$ reduction in number of circulating lymphocytes in rat (10 mg/kg) no bradycardia was seen in either wild type or $S1P_3^{-/-}$ mice suggesting a predominant role of the $S1P_3$ receptor for bradycardia in mice.

Studies involving docking of **SEW2871** in the $S1P_1$ homology model indicate that the affinity of this structure arises from ion–dipole interactions of the electronegative fluorine atoms of the trifluoromethyl group (phenyl group substituent) with the cationic residues R3.28 and R7.34. The less electronegative carbon of the same trifluoromethyl group is predicted to form an ion–dipole

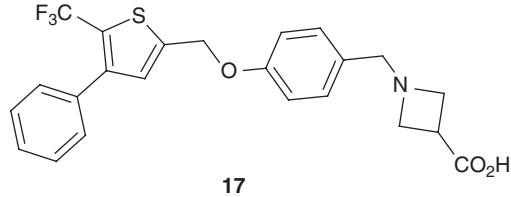
interaction with the anionic residue E3.29, whilst the aromatic rings of **SEW2871** form close contacts with multiple residues of the hydrophobic binding pocket [79,83].

2.4 Amino carboxylates

The combination of the key structural features from the phosphate bioisostere work with **SEW2871**-like lipophilic tails led to the identification of a series of amino carboxylic acid analogues as highly potent $S1P_1$ agonists [84]. Key examples of this class of agonists include the azetidine carboxylic acid **17** (EC_{50} on $S1P_{1,3,4,5} = 1.2$, 530, 1600 and 23 nM, respectively) which reduced lymphocyte counts in mice ($ED_{50} = 0.44$ mg/kg) and showed a good pharmacokinetic profile in rat, dog and monkey (e.g. $Vd_{ss} = 1.4$ L/kg, $Cl_p = 1.5$ mL/min/kg, $C_{max} = 1.8$ μM, $t_{1/2} = 12.5$ h, $\%F = 76$, dosed at 1 mg/kg p.o. in Rhesus monkeys) [84]. Extensive metabolism studies of **17** in rat and dog showed that more than 50% of the dose was excreted in bile with rat-specific metabolites including a taurine conjugate and a glucuronide conjugate arising from an azetidine lactam metabolite. The formation of a unique glutathione adduct was observed in rat bile, following a putative mechanism involving a reactive quinone methide intermediate [86]. Incorporation of a 1,2,4-oxadiazole ring led to further improvement in $S1P_1$ *vs* $S1P_3$ selectivity as shown by **18** (EC_{50} on $S1P_{1,3,4,5} = 0.6$, 12000, 70 and 1.0 nM, respectively) whilst retaining acceptable pharmacokinetic properties in rats ($Vd_{ss} = 2.8$ L/kg, $Cl_p = 4.1$mL/min/kg, $t_{1/2} = 8.5$ h). Amino acid **18** achieved maximal lymphocyte reduction in mice at 10 mg/kg p.o., rats at 0.5 mg/kg p.o. and dogs at 0.5 mg/kg p.o. Compound **18** prolonged survival of rat skin allografts when dosed at 5 mg/kg p.o. confirming that a $S1P_1$ agonist devoid of $S1P_3$ agonism retained immunosuppressive activity. Unlike $S1P_1/S1P_3$ dual agonists, **18** did not induce bronchoconstriction or elevated airway resistance in rats when administered intravenously at high doses providing further evidence that similar side effects seen in rodents with dual $S1P_1/S1P_3$ agonists result from $S1P_3$ agonism [87–89]. Replacement of the azetidine carboxylic acid by the optimally substituted 2,4-pyrrolidine carboxylic acid group led to **19** (EC_{50} on $S1P_{1,3,4,5} = 0.8$, 1100, 800 and 11.0 nM, respectively). Amino acid **19** showed similar potency to **17** leading to peripheral lymphocyte reduction in the rat with an $ED_{50} = 0.3$ mg/kg p.o. and a shorter elimination half-life in rats ($Vd_{ss} = 1.3$ L/kg, $Cl_p = 2.1$ mL/min/kg, $t_{1/2} = 5.6$ h, $\%F = 36$).



SEW2871



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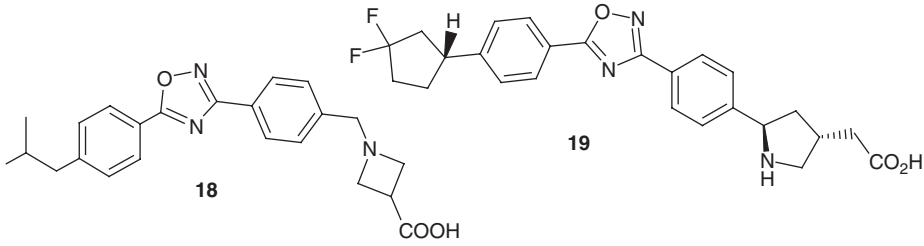
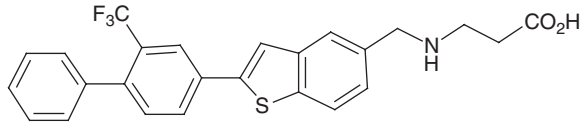


18 19



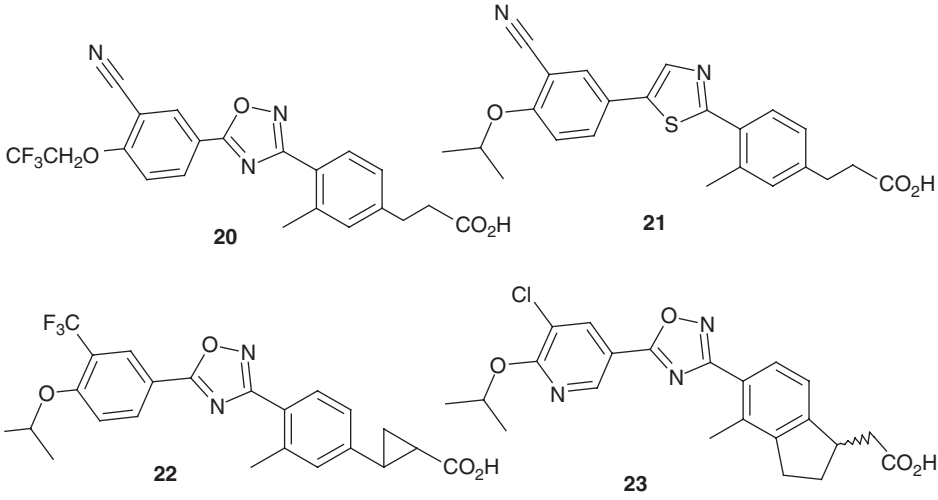
AUY954

A higher degree of subtype selectivity especially with respect to $S1P_5$ has been reported for **AUY954** (EC_{50} on $S1P_{1,2,3,4,5}$ = 1.2, >10000, 1210, >1000 and 340 nM, respectively). **AUY954** efficiently reduced lymphocyte counts in rats (ED_{50} = 0.7 mg/kg p.o.) and has good oral bioavailability and acceptable elimination half-life in rats (%F = 33, $t_{1/2}$ = 5.0 h) and monkeys (%F = 74, $t_{1/2}$ = 11.6 h) [90]. Combination therapy of **AUY954** and the mTOR inhibitor RAD001 prolonged survival of heterotopic cardiac transplant in the stringent Dark Agouti (DA) to Lewis rat transplantation model (combination of **AUY954** at 3.0 mg/kg p.o., RAD001 at 0.3 mg/kg p.o. gave median survival of >26 days *vs* 7 days for RAD001 alone). This result clearly demonstrated that $S1P_1$ agonism alone is sufficient to achieve prolongation of graft survival similar to that seen with **FTY720** [81]. In a separate study, **AUY954** and **FTY720** phosphate were shown to increase the level of ERK1/2 phosphorylation in astrocytes indicating that agonism of $S1P_1$ in the brain may contribute to the therapeutic benefits of **FTY720** in patients suffering from multiple sclerosis [91].

2.5 Miscellaneous structures

2.5.1 Carboxylic acids

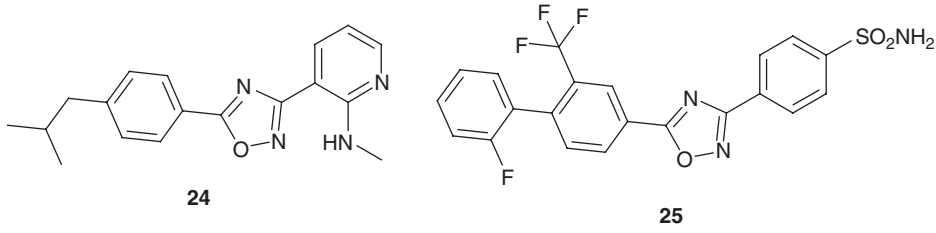
Optimization of the length between the aromatic ring and the carboxylic acid led to an interesting series of 3-arylpropionic acids, **20** (EC_{50} on $S1P_{1,3,5}$ = 0.11, 1600 and 44 nM, respectively) and **21** (EC_{50} on $S1P_{1,3,5}$ = 0.18, >1000 and 120 nM, respectively). These derivatives achieved only a modest degree of reduction in peripheral lymphocyte counts due to their short elimination half-lives and low volume of distribution in rat (for **20** %F = 59, Vd_{ss} = 0.1 L/kg , $t_{1/2}$ = 0.6 h; for **21** %F = 39, $t_{1/2}$ <1 h). Minimal efficacy in the allogenic skin graft model in rat was achieved with **20** when administered by minipump (Mean survival = 12 days when dosed at 4.5 mg/kg/day using minipump *vs* 11 days for controls) [92,93].

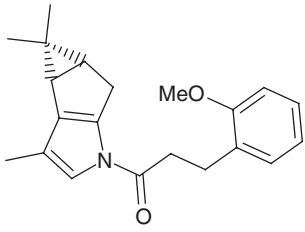


An increase in the elimination half-life in rats was achieved by blocking the benzylic position of the propionic acid chain by introduction of a cyclopropane ring **22** ($\%F = 88$, $Vd_{ss} = 1\,L/kg$, $t_{1/2} = 6.3\,h$); however, in the case of **22,** this led to a reduction in the receptor subtype selectivity (EC_{50} on $S1P_{1,3,5} = 0.21$, 123 and 5.1 nM, respectively). Similarly, blocking the benzylic position by cyclizing onto the phenyl ring to form indanylacetic acid **23** resulted in an improved elimination of half-life ($\%F = 71$, $Vd_{ss} = 0.4\,L/kg$, $t_{1/2} = 6.7\,h$). Acid **23** showed a similar pharmacokinetic profile in the dog and showed efficacy in the rat skin transplantation model when combined with sub-therapeutic doses of CsA. Compound **23** has been reported to be efficacious in the rat EAE model when dosed therapeutically or prophylactically [94–96].

2.5.2 Other structural classes

A number of other structural classes of $S1P_1$ agonists have been reported in the patent literature. These include the aminopyridine **24** which is claimed to be potent on $S1P_1$ and show a selectivity of 100-fold over $S1P_3$ [97]. A series of sulfonamides represented by **25** have been reported to be potent agonists at $S1P_1$ ($EC_{50} < 1$ nM) and to exhibit at least 20-fold selectivity versus the other isoforms [98]. The structural series represented by **26** ($EC_{50} = 31$ nM for $S1P_1$) and **27** ($EC_{50} = 1.2$ nM for $S1P_1$) has been reported to reduce peripheral lymphocytes in rats by 48% at a dose of 30 mg/kg p.o., and 56% at a dose of 10 mg/kg p.o. respectively [99–102].





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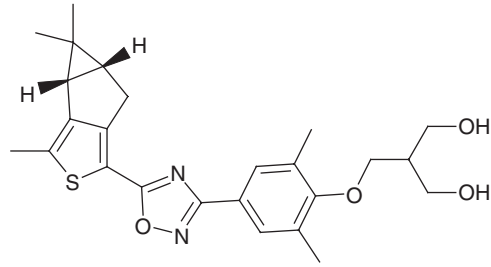
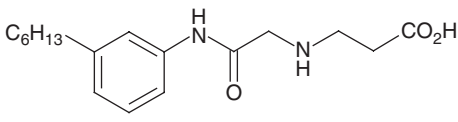


27

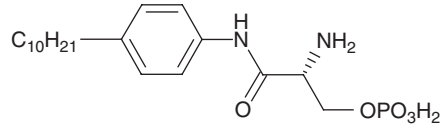
3. $S1P_1$ ANTAGONISTS

One of the two hypotheses for the mechanism of $S1P_1$ induced reduction in peripheral lymphocytes is that $S1P_1$ agonists induce internalization of the receptor [30,35,37,39]. Agonists such as **FTY720** phosphate **2** and phosphate (*R*)- **3** appear to act as supra-physiological agonists, leading to $S1P_1$ receptor internalization. Following internalization, these supra-physiological agonists stimulate polyubiquination of the receptor resulting in proteasomal degradation of the receptor. Physiological-like agonists such as S1P and **SEW2871** lead to $S1P_1$ receptor internalization and recycling of the receptor [103–105]. According to the functional antagonism mechanism, a $S1P_1$ selective antagonist should lead to similar reductions of peripheral lymphocytes.

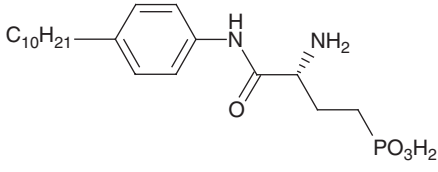
The $S1P_1$ antagonists **W123** and **VPC23019** have been used to study the effects *in vitro* of $S1P_1$ antagonists on **SEW2871** induced cell signaling [106,107]. **VPC23019** was found to be a potent dual $S1P_{1,3}$ competitive antagonist ($Ki < 20\,nM$) with agonistic activity on $S1P_{4,5}$ and no agonist or antagonist activity on $S1P_2$. **VPC23019** blocked agonist-mediated migration of T24 cells *in vitro* but insufficient stability of the phosphate moiety precluded *in vivo* experiments.



W123



VPC23019



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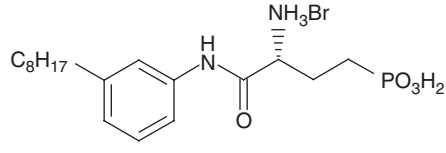

VPC44116

The phosphonic acid analogue **28** retained $S1P_1$ antagonistic activity ($K_i < 80$ nM) while showing no agonist nor antagonistic activity on $S1P_{2,3,5}$ (no data disclosed for $S1P_4$). Phosphonic acid **28** was found to completely inhibit receptor-proximal signaling events such as Gi activation induced by an agonist as well as downstream events including receptor internalization [108]. Administration of **28** at a dose of 10 mg/kg i.v. in mice led to sustained exposure (260 nM at 5 h post-injection); however, **28** did not lead to a reduction in peripheral lymphocytes at the doses and exposures tested. In a separate study, it was shown that **28** was even able to reverse the reduction in peripheral lymphocytes induced by the agonist **SEW2871**. In models of vascular endothelial growth factor (VEGF)-induced skin and lung leakage of Evans blue dye in mice, **28** led to a dose-dependent increase in dye leakage suggesting that $S1P_1$ antagonism may enhance capillary leakage and that $S1P_1$ agonism may be important for maintaining endothelial barrier function. Similar results have recently been published for the closely related antagonist **VPC44116** [109]. **VPC44116** is a potent antagonist on $S1P_1$ and $S1P_3$ ($K_i = 30$ and 300 nM, respectively), retaining agonistic effects on $S1P_4$ and $S1P_5$ ($EC_{50} = 6100$ and 33 nM, respectively) and showing no agonistic or antagonistic effects on $S1P_2$. **VPC44116** was shown to block agonist evoked reductions of peripheral lymphocytes at the doses studied.

4. CONCLUSIONS

The discovery of **FTY720** and the unique role of S1P receptors in the trafficking of lymphocytes have catalysed intensive research in this area. Two different hypotheses have been proposed to explain the mechanism by which S1P receptor agonists reduce the levels of peripheral lymphocytes. The first hypothesis suggests that agonism of $S1P_1$ receptors on lymphocytes induces internalization of the receptor, resulting in a blockade of S1P-directed migration of the lymphocytes from the lymph nodes. The second hypothesis suggests that agonism of $S1P_1$ receptors on the lymphatic endothelium leads to an increase in barrier function and a reduction in lymphocyte transmigration. Further mechanistic studies are needed to determine whether one of these mechanisms is predominant or whether both mechanisms are important. Significant progress has been achieved towards S1P subtype-selective agonists and antagonists with suitable pharmacokinetic properties for *in vivo* studies and these compounds will help to address many of these questions. The availability of agonists that show a high degree of selectivity for the $S1P_1$ versus $S1P_3$ receptor and their study in various *in vitro* and *in vivo* systems has led to the hypothesis that both $S1P_1$- and $S1P_3$-mediated heart effects occur and that there may be significant variation between species. The importance of $S1P_3$ versus $S1P_1$ effects on heart rate in man will only be answered by testing a $S1P_1$ selective agonist in man. The encouraging data from **FTY720** in the Phase II multiple sclerosis trial indicates that S1P receptor agonists have the potential to bring therapeutic benefit to these patients and may offer a breakthrough in the treatment of multiple sclerosis.

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CHAPTER 17

MAP Kinase Inhibitors in Inflammation and Autoimmune Disorders

Shripad S. Bhagwat

Contents

1. INTRODUCTION

Cells have evolved to orchestrate gene transcription and other cellular functions by signaling through a variety of intracellular protein mediators such as kinases, phosphatases, ligases and transcription factors. Mitogen-activated protein kinases (MAPKs) are a family of serine, threonine phosphorelay enzymes activated by cytokines, growth factors, stress, immune receptors and G-protein coupled receptors (GPCRs). The phosphorelay system consists of three tiers of kinases, MAPKs, MAPK kinases (MAPKKs or MAP2Ks or MKKs or MEKs) and MAPKK kinases (MAPKKKs or MAP3Ks or MEKKs or MKKKs). The MKKKs transduce cellular signals by phosphorylating and activating MAPKKs, which in turn, phosphorylate and activate MAPKs. Once activated, MAPKs are often translocated to the nucleus and phosphorylate their respective substrates such as transcription factors and/or other effector proteins. The nature of stimuli, cell types and substrates involved in signal transduction dictate the observed pharmacologic effect (Figure 1) [1–4].

Ambit Biosciences, 4215 Sorrento Valley Boulevard, San Diego, CA 92121, USA

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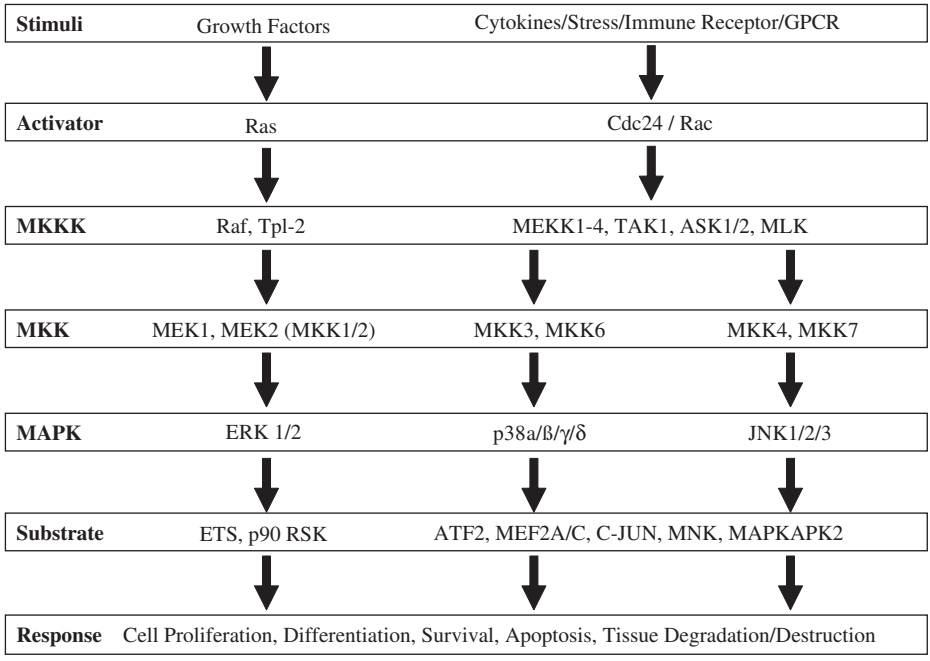


Figure 1 MAP kinase phosphorelay signaling pathways in cells.

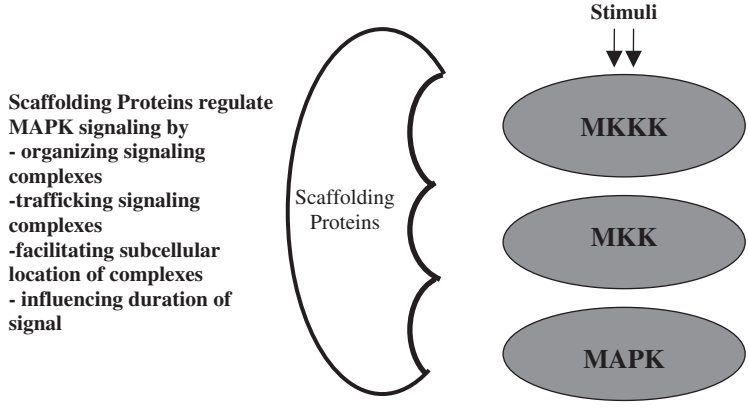


Figure 2 Scaffolding proteins facilitate MAP kinase signaling in cells.

Scaffolding proteins, whose primary role in cells is binding and organizing multiple signaling proteins in a complex and trafficking the complex to appropriate sub-cellular locations, often facilitate the MAPK phosphorelay signal transduction. The type and location of the scaffolding proteins regulates the outcome of the signaling pathway (Figure 2) [5,6].

The three major MAPK families, whose regulation and function have been conserved during evolution in eukaryotic cells, are extracellular

signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38. In inflammatory cells such as macrophages, stimulation of selected Toll family receptors like TLR4 by microbial pathogens or other immunogenic factors leads to the activation of the ERK pathway [4]. Stimulation of TLR4 activates Tpl-2, a MKKK, which phosphorylates and activates MEK1/2 (MKK1/2) leading to the activation of ERK1/2. Activated ERK1/2 regulates expression of tumor necrosis factor-α (TNFα) and other cytokines, which are the key mediators of inflammation and immune responses. The ERK pathway can also be activated in other cell types, by binding of a growth factor such as epidermal growth factor (EGF) to its receptor resulting in the activation of Ras which then activates Raf serine/threonine kinase (MKKK). Raf kinase phosphorylates MEK1/2 which activates ERK1/2 by phosphorylating its serine/threonine and tyrosine residues leading to mitogenic responses. Activated ERK phosphorylates the transcription factor Elk-1, ribosomal S6 kinase RSK and others. The scaffolding proteins, KSR, MP1 and β-arrestin-1, regulate the signal transduction in the ERK pathway.

Signal transduction in the JNK pathway is also largely dependent on scaffolding proteins, sub-cellular location and cell type [4,6]. JNK1, 2 is expressed ubiquitously, while JNK3 is expressed primarily in brain, heart and testes. The scaffolding proteins, JNK inhibitory protein (JIP) and CrkII, facilitate JNK1, 2 mediated signal transduction. The scaffolding proteins bind JNK1, 2, MKK7 or 4 and members of mixed lineage kinase family (MLK) or MEKK1. In neuronal cells, β-arrestin-2 and JIP-1b are the scaffolding proteins that facilitate signaling through ASK1, MKK7 and JNK3. Once activated, JNK is translocated to the nucleus where it phosphorylates transcription factors (AP-1, ATF-2, Elk-1, NFAT and p53) that regulate gene expression of matrix metalloproteases (MMPs) and pro-apoptotic proteins. Activation of JNK1, 2 leads to inflammatory signals, while activation of JNK3 in the brain produces neurodegenerative responses.

The stimulus- and location-selective activation of the p38 MAPK pathway is also controlled by the scaffolding proteins, TAB1 and OSM. TAB1 recruits TAK1, MKK4 or 6 and p38, while OSM recruits MEKK3, MKK3 and p38. Activation of p38 leads to phosphorylation and activation of MAPKAPK2 (MK2) and transcription factors such as ATF-2 [3,4]. Activation of p38 pathway primarily leads to inflammatory and autoimmune responses, although mitogenic effects have also been observed.

The effect of MAPK activation on cellular processes that affect cell function and the resulting pharmacology has been delineated using modern techniques such as knock-out cells and animals [1,3,6]. Activation of MAPK in inflammatory cells such as T-cells, B-cells, macrophages and eosinophils leads to expression and/or activation of pro-inflammatory genes and mediators such as interleukin-1β (IL-1β), TNFα, IL-6, chemokines [e.g., IL-8, macrophage inflammatory factor-1α,β (MIP-1α,β)], MMPs and toxic molecules such as free radicals and nitric oxide [1,3]. These pro-inflammatory mediators induce cellular proliferation, differentiation, survival, apoptosis and tissue degradation/destruction and help induce chronic inflammation. Inhibition of any one or more of the MAPK family

of enzymes is therefore anticipated to produce beneficial pharmacologic and therapeutic effects [7,8].

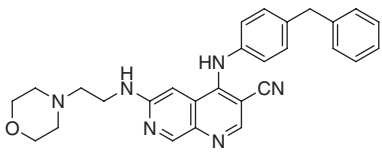
2. INHIBITORS OF ERK PATHWAY

Inhibitors of Tpl-2 (MKKK), MEK1/2 (MKK1/2) and ERK1/2 have been reported. Macrophages from Tpl-2 knockout mice lack ERK1/2 activation leading to loss of TNFα expression and are insensitive to LPS-induced endotoxin shock [9]. Inhibition of Tpl-2 and MEK1/2 produces potent anti-inflammatory effects in cellular and animal models.

A series of naphthyridine- and quinoline-3-carbonitriles, compounds **1**, **2** have been reported to be potent Tpl-2 inhibitors with IC_{50} values = 2 and 19 nM, respectively [10,11].

Compound **1** was selective against a series of six kinases known to regulate TNFα production including the downstream Tpl-2 substrate MEK1 (IC_{50} = 630 nM). However, due to its poor activity in whole blood (IC_{50} = 13.6 μM) [10], its solubility, cell permeability and protein binding needed significant improvement. Compound **2** on the other hand, inhibited Tpl-2 with an IC_{50} = 19 nM and showed improved selectivity and improved physical properties leading to increased whole blood potency (IC_{50} = 3.3 μM). When administered intraperitoneally to mice at 25 mg/kg dose, **2** inhibited LPS-induced TNFα production by 70% [11].

A number of MEK1/2 inhibitors have been reported to display anti-inflammatory properties. PD98059, an ATP non-competitive, flavone derivative, inhibits the inactive form of MEK1/2 with IC_{50} = 2–10 μM by blocking the phosphorylation required for its activation [12,13]. PD98059 is a fairly specific inhibitor of MEK1/2 because it does not inhibit phosphorylation mediated by c-Raf, JNK, p38, PKA, PKC, ν-Src, the active form of MEK1/2 and several other serine/threonine and protein tyrosine kinases. Efficacy with PD98059 has been demonstrated in animal models of pain, arthritis and asthma [14–16].



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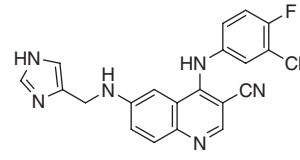

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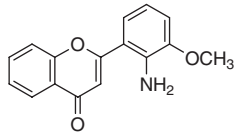
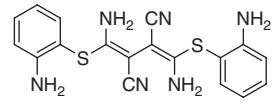
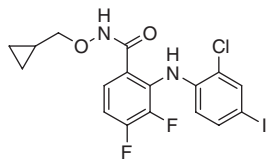


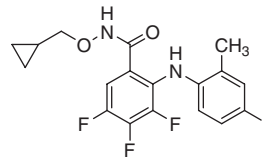
PD98059



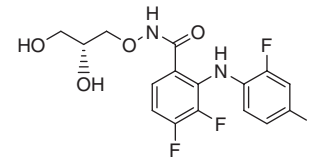
U0126



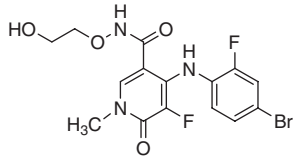
CI-1040 (PD-184352)



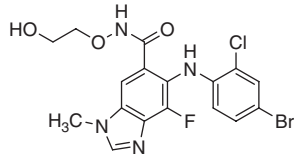
PD-198306



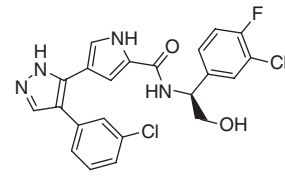
PD-325901



ARRY-438162



ARRY-142886



3

U0126 represents another ATP non-competitive and selective inhibitor of MEK1/2 with no activity toward Raf, MEKK, ERK, MKK-3, -4 and -6 [17]. Activity of U0126 in cells is also limited to those with ERK pathway activation. For example, U0126 inhibited ras-transformed cell growth without affecting normal cells. In an adjuvant-induced arthritis model in rats, U0126 inhibited hyperalgesia to heat and mechanical sources [18].

CI-1040 (PD-184352) is a potent and selective ATP non-competitive inhibitor of MEK1 and MEK2 with $IC_{50} = 17$ nM for purified MEK1 [19]. The compound inhibits phospho-ERK (pERK) and proliferation of a number of relevant cells with $IC_{50} = 100–200$ nM and inhibits tumor growth in xenograft models. As a result, this compound did progress through Phase I clinical trials for the treatment of cancer however was later dropped from further investigation due to less than expected efficacy in Phase II [20]. The exquisite MEK1/2 selectivity of this compound is explained by the observation that in the X-ray structure of analogs of CI-1040 in MEK1 and MEK2, the inhibitors bind in a hydrophobic pocket adjacent to but distinct from the ATP-binding site which may not be available in other kinases [21]. It is likely that the structurally similar and ATP non-competitive MEK1/2 inhibitors discussed below have a similar binding mode in the active site.

The second generation compound PD-198306 is a structurally close analog of CI-1040 that inhibits MEK1/2 with $IC_{50} = 8$ nM and MEK activity in synovial fibroblasts at concentrations of 30–100 nM, depending on the species [22]. The paper mentions (without any data) that this selective MEK inhibitor has good oral bioavailability (F = 62%) and is efficacious in a number of arthritis models in rats including streptococcal cell wall and adjuvant-induced arthritis (ED_{50} values = 11.2 and 6.6 mg/kg, respectively). The authors demonstrate efficacy of PD-198306 in a rabbit model of osteoarthritis when administered at 30 mg/kg orally. At this dose, there was nearly 50% reduction in the area of cartilage macroscopic lesions, in synovial inflammation and in pERK levels in the cartilage.

PD-325901 is another analog of CI-1040 that is also a potent, selective and ATP non-competitive inhibitor of MEK1/2 [23]. The compound inhibited MEK1 with $K_i = 1.1$ nM, MEK2 with $K_i = 0.79$ nM and pERK in C26 cells with $IC_{50} = 0.43$ nM. PD-325901 is efficacious in a number of xenograft models and is currently in Phase I/II trials in cancer patients.

ARRY-438162 is a recently disclosed potent and selective ATP non-competitive MEK1/2 inhibitor that is in Phase Ib clinical trials as an anti-arthritic agent [24]. ARRY-438162 inhibited the MEK1/2 enzyme with an $IC_{50} = 12$ nM and pERK in cells with an $IC_{50} = 11$ nM. ATP non-competitive inhibition may be responsible for equipotent inhibition of MEK1/2 *in vitro* and pERK in cells. The compound was selective against a panel of 220 other kinases.

ARRY-438162 was found to be efficacious in a number of animal models of inflammation. For example, in a collagen-induced arthritis model in rats, oral administration of ARRY-438162 at 10 mg/kg q.d. significantly inhibited paw swelling which was accompanied by inhibition of cartilage damage in the joint and $>80\%$ inhibition of pERK in the tissue from the foot with induced arthritis.

In a Phase Ia study in healthy volunteers, ARRY-438162 was well tolerated up to 20 mg/kg q.d. oral dose. There was a dose proportional increase in plasma concentration and decrease in the production of IL-1β, TNFα, IL-6 and pERK in the *ex-vivo*-stimulated whole blood from drug treated volunteers. Array has initiated a Phase Ib study of ARRY-438162 in combination with methotrexate in rheumatoid arthritis patients with the goals of assessing safety, tolerability, pharmacokinetics (PK), biomarkers and initial signs of efficacy.

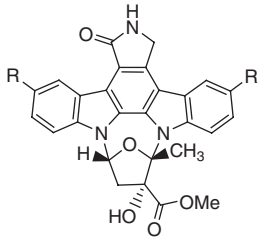
ARRY-142886 (AZD6244) is another potent, selective and ATP non-competitive MEK1/2 inhibitor with $IC_{50} = 12$ nM. This compound inhibited cellular pERK with an $IC_{50} < 10$ nM and tumor growth in a number of xenograft models and is reported to be in Phase II clinical trials in cancer patients [25].

To date the only ERK1/2 inhibitor reported is compound **3** with $IC_{50} = 2$ nM for the inhibition of ERK2 [26]. This compound inhibited proliferation of the Colo205 cell line with $IC_{50} = 540$ nM and was selective against 12 other kinases. During the optimization of *in vitro* activity of this series of compounds, it was discovered that the binding modes of an analog of **3** to ERK2 and JNK3 are significantly different.

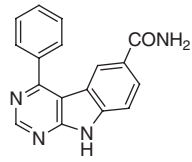
3. INHIBITORS OF JNK PATHWAY

Inhibitors of MLK (MKKK) [27], MKK4, 7 and JNK [6,28,29] have been disclosed to date. CEP-1347, a semi-synthetic analog of the natural product K252a, inhibits MLKs in the JNK pathway with $K_i = 17$ nM [30–32]. This compound has shown neuroprotective effects in cellular and animal models [33]. CEP-1347, an orally available compound that was well tolerated in the clinic, was advanced to Phase II/III trials for assessing efficacy in Parkinson's disease. However, the clinical trial was stopped due to a lack of significant efficacy [34].

Compounds **4** and **5** and their analogs have been disclosed as MKK4 and 7 inhibitors [35,36]. The compounds in these patent applications are claimed to inhibit the two enzymes with IC_{50} <1 μM; no information on kinase specificity or other biological properties was disclosed. There are numerous other compounds that are disclosed in the patent literature to inhibit many kinases including MKK4 and 7; such compounds are not listed here.



K252a, R=H
CEP-1347, R=$CH_2SCH_2CH_3$



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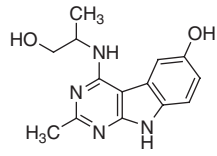

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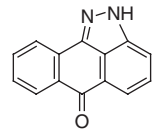
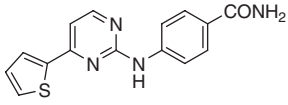


SP-600125

A number of distinct chemotypes have been reported as JNK inhibitors. In addition, a significant number of p38 inhibitors have also been found to have JNK inhibitory activity. This review will primarily focus on specific JNK inhibitors which have little or no p38 activity.

SP-600125 was one of the first JNK inhibitors to be reported with potent JNK 1, 2 and 3 inhibitory activity (IC_{50} = 40, 40 and 90 nM, respectively) [37]. This tool compound has been studied extensively in a variety of cellular and animal models of inflammation and neuroprotection, among others. The profile of SP-600125 has been discussed in a number of reviews [6,28,29] and will not be discussed here.

CC-401, whose structure has not yet been reported, is a potent and selective inhibitor of JNK1, 2 and 3 (pan-JNK inhibitor), that has been advanced to Phase II clinical trials [38]. Celgene has disclosed analogs of compounds **6** and **7** as JNK inhibitors [39,40]. However, a recent patent application claims the crystal forms and other properties of **8**, suggesting that it is likely to be a clinical candidate [41].



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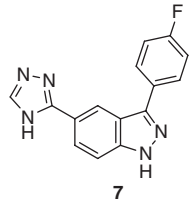

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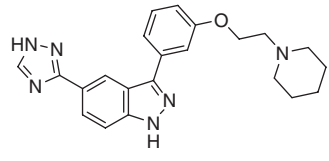
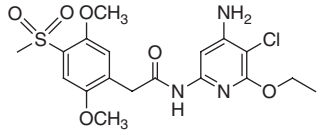


8

Two series of aminopyridine carboxamide derivatives have been reported to be potent, selective and ATP-competitive pan-JNK inhibitors [42–45]. Compound **9** containing a 6-aminophenylacetamide group inhibits JNK1 and JNK2 with IC_{50}

values of 36 and 70 nM, respectively and inhibits p-c-jun in HepG2 cells with an $IC_{50} = 1.7\,\mu M$. The compound inhibited JNK3 with similar potency [42]. The X-ray structure of an analog of **9** complexed to the JNK1 enzyme indicated that while hydrophobic interactions contribute to significant binding interactions, the NH- of the 4-amino group and the oxygen of the 6-aminocarboxamide group form weak H-bonding interactions with the backbone residues of Glu109 and Met111, respectively. The authors hypothesize that these weak backbone interactions could be the reason for the >100-fold selectivity of this series of compounds against a panel of 74 kinases. Compound **9** showed a desirable PK profile in Sprague–Dawley (SD) rats at a dose of 5 mg/kg p.o. with AUC of 1.5 μg h/mL and good oral bioavailability (F = 49%). An analog of **9** containing a cyano group in place of the chloro- showed a similar overall profile. The efficacy profile of **9** and analogs has not been reported to date.



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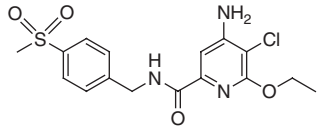

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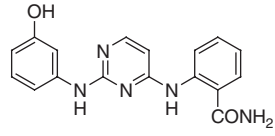


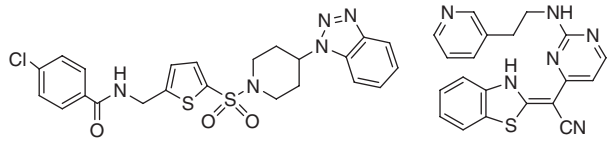
11

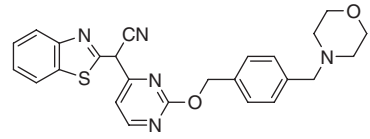
A "reverse amide" analog, **10**, also has been reported to be a potent, selective and ATP-competitive pan JNK inhibitor [43]. Compound **10** inhibited JNK1 and 2 with IC_{50} values = 24 and 74 nM, respectively and inhibited p-c-jun in HepG2 cells with $IC_{50} = 480$ nM. At a 5 mg/kg oral dose in SD rats, the PK profile of **10** appeared to be superior to **9** with an AUC = 15.4 μg h/mL and %F > 100. Compound **11** has been reported to be a potent JNK1 inhibitor ($IC_{50} = 9$ nM) with selectivity against a panel of 12 kinases [46]. The IC_{50} for inhibition of phosphorylation of c-jun in HEPG2 cells was found to be 1.2 μM.

AS600292, AS601245 and their analogs have been reported to be pan JNK inhibitors [47,48]. AS600292 inhibits JNK2 and 3 with IC_{50} values = 520 and 150 nM, respectively, and has significant selectivity against a panel of 80 other kinases [47]. In a cell assay assessing JNK3 activity, AS600292 inhibited anti-NGF antibody-induced neuronal apoptosis of SCG cells with an $IC_{50} = 1.7\,\mu M$. The shape of neurons appeared to be normal indicating no compound-related cytotoxicity. AS600292 and analogs were found to have poor water solubility (1 μg/mL in PBS) and a poor PK profile (i.v., i.p. and p.o.) in rats.

AS601245 has been reported to be an ATP-competitive inhibitor of JNK1, 2 and 3 with IC_{50} values = 150, 220 and 70 nM, respectively, with minimal to no activity in a panel of 25 other kinases [48]. In Jurkat T-cells, AS601245 at 10 μM inhibited IL-2 production induced by phorbol-12-myristate-13-acetate (PMA) by 90%. The weaker cell activity could be due to poor cell permeability. The oral bioavailability of AS601245 in rats was 38%. In mice, AS601245, when dosed at 60 mg/kg p.o. in a developed arthritis model induced by collagen, showed

moderate beneficial effects as measured by paw swelling and histopathological analysis of the joint.





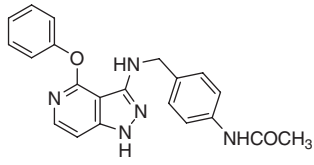
AS602801 has been reported to inhibit JNK1 and 2 with $IC_{50} = 80$ and 90 nM, respectively, and JNK3 with $IC_{50} = 230$ nM [49]. The compound was selective against a panel of 100 other kinases. Anti-inflammatory effects of AS602801 have been demonstrated by measuring inhibition of TNFα release upon LPS-stimulation and by demonstrating protective effects in animal models of multiplesclerosis (MS), lung inflammation and fibrosis. AS602801 showed good bioavailability in rats (F = 50%) with 98% brain penetration. In a Phase I study in normal volunteers, 80–570 mg b.i.d. oral doses of AS602801 were well tolerated and the drug showed a desirable PK profile in humans. The efficacy of AS602801 is being evaluated in patients with MS.

The indazole derivatives **12**, **13** have been reported to inhibit JNK1 and JNK3, respectively [50,51]. Compound **12** is reported to inhibit JNK1 with a $pIC_{50} = 6.8$ and to have good oral bioavailability in rats (F = 50%) [50]. Compound **13** inhibits JNK3, JNK1 and p38α with IC_{50} values of 3, 101 and 903 nM, respectively [51]. The binding modes of compounds **12** and **13** complexed to JNK1 and JNK3, respectively, have been determined using X-ray crystallography, however, additional biological data have not been disclosed. Compound **14** has also been reported to inhibit JNK3, JNK1 and p38α with IC_{50} values of 7, 384 and 180 nM, respectively. This compound was found to have oral bioavailability of 16% in rats. Additional biological data have not been reported [52].

The enantiomers of **15**, with structural similarity to the imidazole-based p38 inhibitors, have been reported to be potent inhibitors of JNK3 (IC_{50}~3 nM) and p38 (IC_{50}~30 nM) with significant neuroprotective effects in cells [53].

Recent patent literature disclosures include a number of JNK inhibitors with little or no biological data reported. This review will focus on some of the patent applications that have appeared after the publication of reviews that covered the patent literature through 2004 [6,28,29]. Compound **16** and analogs are claimed as sub-micromolar inhibitors of JNK1 [54]. The pyrrolo-triazine **17** and analogs are claimed as equipotent, sub-micromolar inhibitors of JNK2 and p38α [55]. Aminopurines similar to **18** have been claimed as pan-JNK inhibitors with IC_{50} values = 0.001–10 μM [56]. Imidazo-pyrazine derivatives like **19**, are claimed in

a patent application as pan-JNK inhibitors. Compound **19** inhibits the JNK isoforms with IC_{50} values <200 nM [57].



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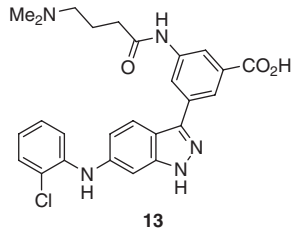

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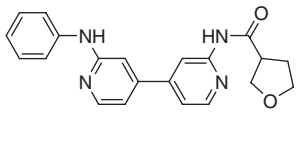

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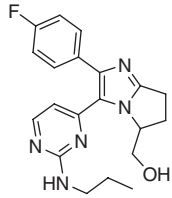

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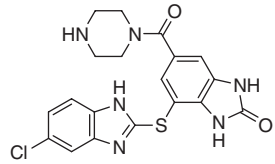

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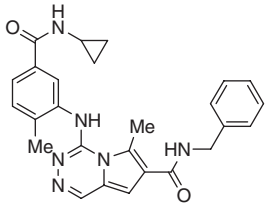

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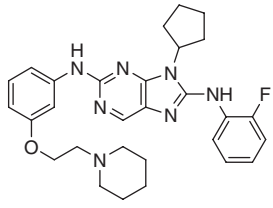

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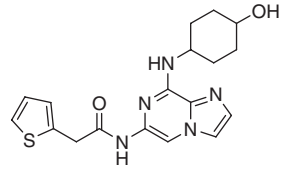

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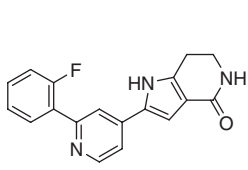
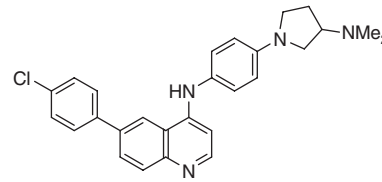
4. INHIBITORS OF p38 PATHWAY

A large number of p38 inhibitors have been discovered in the past 15 years. Many excellent reviews cover this topic well [58–60] and hence will not be discussed here. Compounds that specifically inhibit MKK3 and/or 6 or upstream kinases at the MKKK level in the p38 pathway have not been reported to date, although a number of patents on kinase inhibitors disclose compounds that inhibit MKK3, 6 along with a list of other kinases. PH-089 is a potent, selective and ATP-competitive MK2 (MAPKAPK2) inhibitor with $IC_{50} = 126$ nM [61]. The compound inhibited TNFα production in human peripheral blood monocytes (PBMC) with $IC_{50} = 1.08$ μM. PH-089 at an oral dose of 60 mg/kg, b.i.d., inhibited paw swelling by 55% in a streptococcal cell wall-induced arthritis model in rats. This result is in line with the data from the MK2 knock-out mice which were resistant to collagen-induced arthritis [62]. Compound **20** has also been claimed in a patent application as a MK2 inhibitor with no activity information [63]. Compound **21** has been reported to be a potent inhibitor of MK2 ($IC_{50} = 130$ nM) with the ability to inhibit PMA-induced TNFα production in U937 cells ($IC_{50} = 130$ nM) [64].

At a dose of 20 mg/kg i.p., **21** inhibited LPS-induced TNF production in Lewis rats by 68%. Compound **22** and analogs are reported to inhibit MK2 with $IC_{50} < 2\,\mu M$ [65].



PH-089



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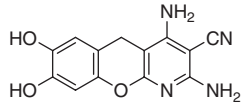

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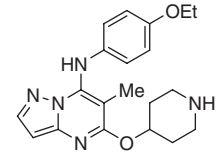

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5. CONCLUSION

The search for potent and selective p38 inhibitors has been ongoing for more than 15 years. More than a dozen p38 inhibitors have been advanced to clinical trials however none has been approved for human use yet. It is hoped that the newest generation of p38 inhibitors will be suitable for treating inflammation and autoimmune disorders. Discovery of drugs inhibiting other targets in the MAP kinase pathway has received increased attention in the past decade. A number of MEK and JNK inhibitors are currently being evaluated for safety and efficacy in a variety of indications including inflammation and cancer. It remains to be seen if these drugs will make it to the market. It is anticipated that novel drugs inhibiting additional targets in the MAP kinase pathways will be discovered, advanced to clinical trials and found useful for treating human diseases in the coming years.

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CHAPTER 18

Recent Progress on Novel HCV Inhibitors

Christoph W. Zapf, Jonathan D. Bloom and **Jeremy I. Levin**

Contents

1. INTRODUCTION

Hepatitis C (HCV) is a blood-borne pathogen belonging to the *Flaviviridae* [1] family of viruses, which also includes the West Nile, Yellow Fever, and Dengue viruses. It has been referred to as the ''silent killer'' because many infected patients will be unaware of their disease for two decades before symptoms first appear. Although acute liver disease arising from HCV is uncommon [2], as many as 85% of infected patients will progress to a chronic infection [3]. Over the course of 20 years, 15–20% of these patients will develop cirrhosis and hepatic carcinoma [4]. HCV infection is the leading cause of liver transplants in the United States and Europe [5]. The disease affects 4 million people in the US and 170 million worldwide [6], making it about 5 times as prevalent as AIDS [7].

Despite a proliferation of pharmaceutical and academic research in the past decade, improved therapies for HCV remain an enormous unmet medical need.

Wyeth Research, Pearl River, New York 10965

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In contrast to AIDS, there are no specific antiviral agents available for the treatment of HCV. Rather, interferon (IFN) [8], or more recently, pegylated IFN (PEG-IFN) [9], which act by modification of immune function, typically in combination with ribavirin, a viral mutagen [10,11] that acts synergistically with IFN, are the only approved treatments for HCV infection. Furthermore, the sustained viral response is 50–80%, depending on the genotype [12], and the regimen is poorly tolerated, making patient adherence suboptimal [13]. Thus, the need for HCV-specific small molecule inhibitors is acute. As a result, there are over 50 documented potential therapies, small molecules and immune modulating proteins, in various stages of development [14].

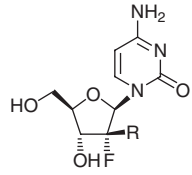
By analogy to AIDS, most small molecule inhibitor approaches to HCV have been focused on inhibition of essential viral targets, particularly the NS3-4A protease and the NS5B RNA-dependent RNA polymerase although other targets are being pursued [15,16]. Recent reviews have examined the HCV genome [17] and efforts to discover small molecule HCV inhibitors [13,16,18–21]. The following report outlines some of the major advances in this area over the last few years. Inhibitors are, in general, evaluated *in vitro* in both enzyme assays and cell-based replicon assays. Care must be taken when comparing replicon data from different research groups, because a variety of replicon constructs have been used. Preliminary *in vivo* data is less readily available due to the lack of a simple and inexpensive animal model to test inhibitors of HCV.

2. NOVEL COMPOUNDS IN THE DISCOVERY/PRECLINICAL STAGE

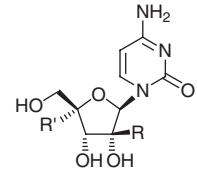
2.1 Nucleoside inhibitors of HCV RNA-dependent RNA polymerase (RdRp)

Inhibitors of viral polymerases have provided successful therapeutics for the treatment of hepatitis B, herpes simplex, and HIV viruses. A variety of nucleoside competitive inhibitors of HCV NS5B, the RdRp encoded at the 3′-terminal portion of the HCV genome and required for viral replication, have also been thoroughly studied and advanced to clinical trials. These inhibitors function as chain terminators and are usually modified in the 2-′ or 3′-position of the sugar moiety. For example, 2′-deoxy-2′-fluorocytidine (FdC), **1**, wherein a fluorine has replaced the 2′-hydroxyl of cytidine, has an EC_{90} of 5 μM in an HCV replicon assay [22]. However, FdC inhibits cellular polymerases other than the NS5B enzyme, as well as a variety of viral targets in addition to HCV, limiting its utility. In order to increase the selectivity of FdC the analogous 2′-deoxy-2′-fluoro-2′-*C*-methylcytidine, **2**, was prepared and found to have an EC_{90} equivalent to FdC in the replicon assay, but unlike FdC, **2** does not induce cytostasis at this concentration [23]. The activity of **2** is not retained on replacement of the cytidine moiety with either adenine or guanine [24]. Another cytidine-bearing inhibitor of HCV replication is 4′-azidocytidine (R1479), **3** [25] which was identified through targeted screening in combination with rational drug design. R1626, a pro-drug

of **3**, has advanced into Phase 2 clinical trials [26]. Nucleoside derivative **3** is a competitive inhibitor of RNA synthesis with an IC_{50} of 1.28 μM in a replicon assay, and no effect on cell viability at concentrations up to 2 mM. This is equivalent to that of 2′-*C*-methylcytidine, **4** [27]. The 3′-*O*-valinyl ester prodrug of **4**, valopicitabine (NM283) was in Phase 2 clinical trials for HCV, but has been discontinued [28–31]. The triphosphate of **3** is a substrate for incorporation into nascent RNA, but blocks subsequent elongation, presumably due to the 4′-azide moiety. In addition, upon extended incubation of **4** with replicon cells at its IC_{90}, it is able to clear completely HCV replicon RNA without selecting for resistance, and it is not affected by mutations that confer resistance to 2′-*C*-methyl-nucleosides.



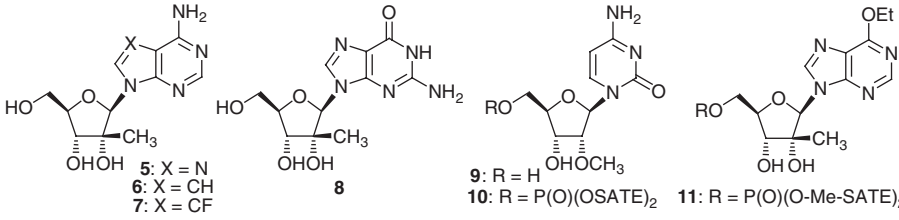
1: R = H
2: R = CH_3



3: R = H, R' = N_3
4: R = CH_3, R' = H

The triphosphate of 2′-*C*-methyl derivative **5** is a 1.9 μM inhibitor of NS5B-catalyzed RNA synthesis, with excellent activity ($EC_{50} = 0.3$ μM) in a replicon assay [32]. Inverting the 2′-methyl or alcohol groups of **5** destroys activity, as does methylation of the 2′-hydroxyl. The 3′-*C*-methyl analog corresponding to **5** is also inactive. The analogous guanine derivative **8** is 10-fold more active against NS5B enzyme, but has an EC_{50} of only 3.5 μM in the replicon assay. In hepatoma cells, uptake and metabolism of **8** to its triphosphate are less efficient than for adenine analog **5**. In an effort to correct enzymatic liabilities associated with **5** that resulted in poor bioavailability, modifications of the heterobase were explored resulting in the identification of the 7*H*-pyrrolo[2,3-*d*]pyrimidine scaffold as a replacement for adenine [33]. Compound **6** (MK-0608) [34], the 7-deaza analog of **5**, is 17-fold more potent than **5** in a polymerase assay ($IC_{50} = 0.108$ μM) and has an EC_{50} of 0.3 μM in the replicon assay [35,36]. It also has sub-micromolar EC_{50}s against BVDV and rhinovirus type 2 polymerases. Incorporation of a fluorine into **6** provides **7** which is a potent inhibitor in the HCV replicon assay ($EC_{50} = 0.07$ μM) perhaps in part due to a direct interaction between the fluorine and a lysine residue of the protein. Neither **6** nor **7** is cytotoxic at 100 μM in a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, and both have greatly improved pharmacokinetic properties (rat F = 51%) relative to **5**. Replacement of the fluorine of **7** with a nitrile or amide group results in the 2′-β-*C*-methyl analogs of the naturally occurring nucleoside antibiotics toyocamycin and sangivamycin, respectively, for which efficient synthetic routes have been described [37]. Variations at C4 and C5 of toyocamycin have been described with the C4 methoxy and

thiomethyl analogs providing the best activity with EC_{50}s of 0.4 μM in a replicon assay [38].

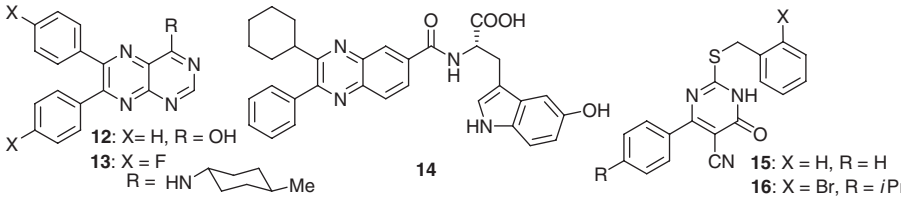


A prodrug approach utilizing a bis(*t*BuSATE) monophosphate [39] (SATE: *S*-acyl-2-thioethyl) has been applied to nucleoside HCV inhibitors in order to improve cell activity of compounds in which cell uptake or initial monophosphorylation is problematic [40]. Thus, in a replicon assay 2′-*O*-Me-cytidine, **9**, has an EC_{50} of 21 μM, though its triphosphate has an IC_{50} of 3.8 μM in the polymerase assay [41]. Conversion of **9** into the corresponding neutral 5′-SATE-monophosphate ester, **10**, improves its activity in the replicon assay 7-fold ($EC_{50} = 3$ μM). This increased cellular potency may be due in part to protection of this analog from hydrolytic deamination by cytidine deaminase [40]. Application of the SATE-prodrug approach after an extensive survey of the effect of substitution at the 6-position of 9-(2-β-*C*-methyl-β-D-ribofuranosyl)-purines provided **11**, with an EC_{50} of 0.08 μM in an HCV replicon assay [42]. The 6-substituents that afford the greatest activity in the replicon assay are an aminomethyl moiety ($EC_{50} = 1$ μM) and a hydroxylamine ($EC_{50} = 1.8$ μM). A similar approach extended to SATE prodrugs of cyclic and acyclic monophosphates of purine 6-sulfonohydrazides yielded low nanomolar replicon EC_{50}s [43,44]. Additional *C*-6 heteroaryl substituted purine analogs have also been prepared in a ribose series. Although some of these display activity in the replicon assay, they have significant toxicity [45]. A small series of 6-substituted L-ribonucleosides also failed to provide inhibitors with EC_{50}s better than 40 μM [46]. Similarly, a group of 5-alkynyl and alkenyl substituted 2′-*O*-methyluridine derivatives display no replicon activity at concentrations up to 300 μM [47].

Two approaches that have been validated for HIV inhibitors, nucleoside 5′-phosphonates [48] and 1,3-dioxolane analogs [49] have proven unsuccessful when applied to HCV inhibitors. Phosphonodiphosphates have been synthesized and are incorporated by NS5B RdRp, but V_{max}/K_m for these chain terminators is 10–100-fold less than for ATP, and potency must be greatly improved for analogs of this type to have utility [50]. A small series of 1,3-dioxolanes also failed to afford active inhibitors of HCV, or HIV, despite the addition of a 5-methyl substituent to impose the desired conformational preference [51]. Ring expanded nucleobases [52,53] and AICAR analogs have also been synthesized as HCV inhibitors which provide only weak replicon activity [54].

2.2 Non-nucleoside inhibitors of HCV RNA-dependent RNA polymerase

Pteridine **12** was found to display weak inhibitory activity against HCV NS5B in a high-throughput enzymatic assay ($IC_{50} = 50\,\mu M$). The pteridine nitrogens are required for activity and *para*-fluoro substituents are optimal on the phenyl rings. Compounds bearing a hydrophobic amine have improved cellular activity, with cyclohexyl-derivative **13** being the most active analog in this series ($IC_{50} = 1.3\,\mu M$, $EC_{50} = 18\,\mu M$) and display no cytotoxicity up to $250\,\mu M$ [55].



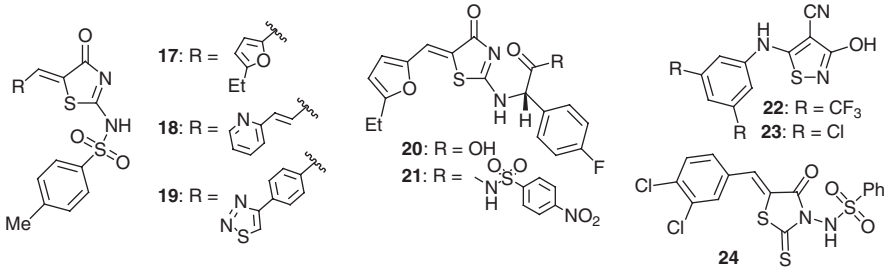
Structurally related quinoxalines have been identified as sub-micromolar HCV polymerase inhibitors in a high throughput screen (HTS). Derivatives that are unsymmetrically substituted with hydrophobic groups on the pyrazine moiety, and with a pendant hydroxy-tryptophan side-chain that has been reported previously (*vide infra*), provide the greatest activity (**14**, $IC_{50} = 0.6\,\mu M$) [56].

Uracil derivatives have also shown activity as NS5B inhibitors. Thus, the activity of **15,** with an IC_{50} of $27\,\mu M$, is increased 7-fold for analog **16** ($IC_{50} = 3.8\,\mu M$), though **16** is only slightly active in a cell-based replicon assay ($EC_{50} = 32\,\mu M$). The free NH on the uracil core is required for activity [57].

Thiazolone lead **17** ($IC_{50} = 1.4\,\mu M$, $EC_{50} = 25\,\mu M$), represents another class of compounds that has received substantial attention. Thiophenes are less active than the analogous furans, and an ethyl substituent in the 5-position of the furan is optimal. Analysis of an X-ray crystal structure revealed a shallow, hydrophobic binding pocket that accommodates this moiety. A sub-micromolar enzyme inhibitor is obtained when the furan is replaced by a 2-pyridyl ring attached to the thiazolone scaffold via a vinyl linker (**18**, $IC_{50} = 0.6\,\mu M$). This compound however is slightly less active in the cell-based assay ($EC_{50} = 35\,\mu M$) compared to the ethyl-furan analog. In this series the greatest cellular activity and least cytotoxicity is obtained by replacing the ethyl-furan moiety with *para*-substituted phenyl rings (**19**, $EC_{50} = 9\,\mu m$) [58].

Further optimization of this scaffold using structure-based design was directed at exploiting interactions with Lys533 in the binding pocket of the thiazolones. Phenylglycine analog **20** was selected by computational methods and X-ray crystallography confirmed that this compound indeed establishes an additional hydrogen bond between Lys533 and the carboxylate group. Unfortunately, this additional interaction does not result in improved polymerase activity [59]. Variation of the α-amino acid of **20**, or replacement of the carboxylic acid with a tetrazole also does not increase potency [60]. X-ray crystallographic analyses of these compounds revealed an opportunistic binding pocket formed

by His475 and Lys533. Acylsulfonamide **21** contains an acidic hydrogen while retaining an aromatic moiety for potential π–π stacking interaction with His475. When evaluated against a BK strain of NS5B polymerase enzyme, **21** is active in the low micromolar range ($IC_{50} = 7\,\mu M$) and X-ray analysis confirmed the expected binding mode [61].

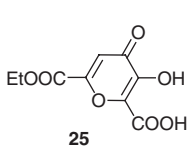


A series of isothiazoles exemplified by **22** was reported to bind in the active site of NS5B. An X-ray analysis revealed that these compounds bind in the RNA-binding site and react with Cys366, leading to cleavage of the isothiazole ring and the formation of a disulfide bond. This finding explains the observed structure-activity relationship (SAR), as the more electron deficient analogs have an increased propensity for ring-opening and therefore more readily form covalent bonds with the enzyme. Also, replacement of the isothiazole core with an isoxazole almost abolishes activity. When evaluated in the cell-based replicon assay, isothiazole **23** ($EC_{50} = 0.1\,\mu M$) was reported to be the most active member of this series with an IC_{50} of $0.2\,\mu M$ and a CC_{50} of $52\,\mu M$ [62].

Rhodanine derivatives have also been identified as non-nucleoside allosteric inhibitors of NS5B [63]. Compound **24**, was shown by X-ray crystallography to bind covalently to the polymerase via Michael addition of Cys366 to the olefin. This binding event was shown to be reversible ($IC_{50} = 0.2\,\mu M$). Since the hydrogenated analog also shows activity, it was concluded that both covalent bond formation and other specific interactions with the enzyme are important contributors to activity. This compound displays low clearance, moderate half-life and high exposure following oral administration in the rat.

Related to a class of α,γ-diketoacids that has previously been shown to bind to NS5B [64], is the mono-ethyl ester of meconic acid **25**. This compound was identified as a selective inhibitor of NS5B HCV polymerase ($IC_{50} = 2.3\,\mu M$) and is competitive with the diketoacids. SAR studies have demonstrated the requirement for the carboxylic acid. A variety of different permutations of esters, acids, amides, and decarboxylated compounds were prepared without any improvement in binding affinity or in the cell-based replicon assay [65]. The 4,5-dihydroxypyrimidine-6-carboxylic acids, a hybrid of the α,γ-diketoacids and meconic acid, envisioned as chelators of the essential Mg^{2+} ions in the active site of NS5B, are also active in the polymerase assay (**26**, $IC_{50} = 5.8\,\mu M$). While alkylation of the phenol of the hybrid is tolerated, methylation of the heterocyclic hydroxyl groups or the carboxylic acid, as well as decarboxylation, leads to

complete loss of activity [66]. The phenol can be replaced by a thiophene ($IC_{50} = 2.6\,\mu M$) but the pyrimidine cannot be altered without substantial loss in activity in the polymerase assay [67]. None of the compounds in this series show activity in the replicon assay. The SAR of the thiophene at the 2-position of the pyrimidine was explored to increase cellular activity. Thiophene analogs with urea and carbamate linkers to a chlorinated phenyl ring show sub-micromolar activity in the enzyme assay and low micromolar potency in the replicon assay, with urea **27** being the most potent derivative ($IC_{50} = 0.15\,\mu M$, $EC_{50} = 9.3\,\mu M$) [68].



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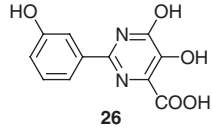

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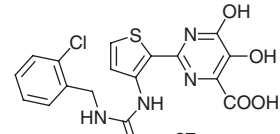
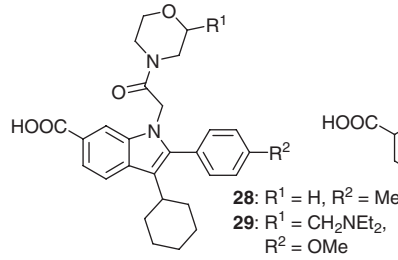


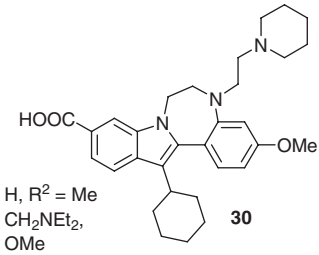
27

A series of indole allosteric inhibitors of NS5B with excellent enzyme ($IC_{50} < 6\,\mu M$) and cell potency ($EC_{50} = 500\,nM$), exemplified by **28**, has been disclosed, related to a similar series of benzimidazole derivatives [69]. Decorating the core with an aryl group at C-2, cycloalkyl moiety at C-3, and a carboxylic acid at C-6 provides compounds with the greatest potency. A polar, neutral acetamide side chain on the indole nitrogen is optimal, and incorporating lipophilic substituents at the *para*-position of the phenyl ring increases potency further. Compound **28** has an IC_{50} of 11 nM, an EC_{50} of 0.3 μM and a $CC_{50} > 50\,\mu M$ [70], but protein binding is a potential liability for this series [71].

An X-ray crystal structure of **28** bound in the thumb-region of the NS5B polymerase showed little interaction of the acetamide moiety with the protein. Alterations at this position were explored in order to improve the physical properties of the compound. Incorporation of basic amines as part of this side-chain, leading to zwitterionic compounds, reduces plasma binding and has a beneficial effect on cell activity and pharmacokinetic profiles. In the cell-based replicon assay, racemic **29** has an EC_{50} of 152 nM in the presence of 10% fetal calf serum and 376 nM in the presence of 50% normal human serum [71].



28: R^1 = H, R^2 = Me
29: R^1 = CH_2NEt_2, R^2 = OMe

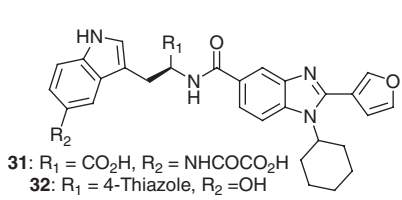


30

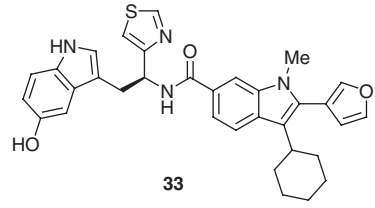
Additional work around the benzimidazole scaffold on compounds related to **29** [72,73] suggested that the dihedral angle between the heterocycle and the phenyl ring is a crucial determinant of binding affinity, leading to the design of tetracyclic compounds with the aromatic moieties linked [74]. Thus, indole

derivative **30** is very potent in the enzyme assay ($IC_{50} = 9$ nM) and replicon assay ($EC_{50} = 35$ nM), with little change in the presence of human serum albumin ($EC_{50} = 84$ nM). This compound is only weakly cytotoxic ($CC_{50} > 20$ μM) and has good permeability in Caco-2 cells.

The 2-position of the benzimidazole-carboxylic acids is also amendable to structural alterations, with small heterocycles preferred [75]. Furthermore, coupling of the 5-carboxylic acid to α-amino acids provided substantial improvements in enzyme activity, exemplified by L-tryptophan derivative **31** ($IC_{50} = 8$ nM), but without any significant cellular activity [76]. By converting the tryptophan carboxylate to the more lipophilic thiazole, and incorporating a free hydroxyl on the tryptophan indole, moderate cellular activity can be achieved (**32**, $EC_{50} = 1.7$ μM) [77]. The less polar *N*-methyl indole analog **33** shows a 22-fold improvement in cell-based activity ($IC_{50} = 100$ nM, $EC_{50} = 50$ nM) despite very poor aqueous solubility [78].



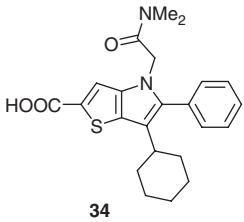
31: $R_1 = CO_2H$, $R_2 = NHCOCO_2H$
32: R_1 = 4-Thiazole, R_2 =OH



33

Numerous replacements for the indole core have been investigated, including imidazoles, thienoimidazoles, pyrroloimidazoles, quinolines, indolizines and several aza-indoles. Attachment of an *N*,*N*-dimethylacetamide side-chain to the thienopyrrole scaffold gives compound **34** with nanomolar enzyme activity ($IC_{50} = 58$ nM) and enhanced cell activity ($EC_{50} = 2.9$ μM) compared to the unalkylated scaffold [79].

Acylated *Z*-dehydroalanine derivatives substituted with aromatic moieties at the β-carbon are allosteric polymerase inhibitors with enzyme activity in the micromolar range. These inhibitors bind in a hydrophobic pocket near Cys366 of NS5B polymerase, in a site that overlaps with the binding region of rhodanines **24**. Carboxylic acid analogs, exemplified by **35**, with biaryl ether substituents provide submicromolar enzyme activity, but only moderate potency in cells, presumably due to low permeability and significant plasma binding [80]. The analogous amides, such as **36**, display similar enzyme ($IC_{50} = 2.7$ μM) and cellular activities ($EC_{50} = 8$ μM). Amide **36** has 76% oral bioavailability and a half-life of 1 hour at 5 mg/kg po in rats [81].



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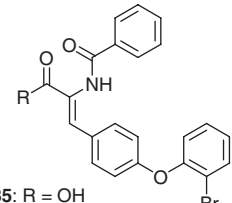
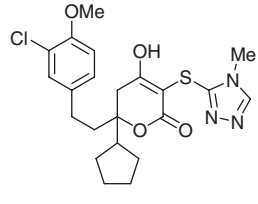


35: R = OH
36: R = $NHCH_2CH_2OH$



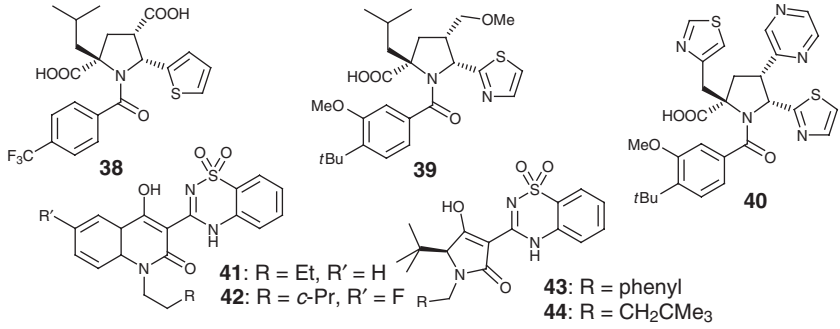
37

A series of non-GTP competitive, reversible, allosteric inhibitors with a dihydropyrone core has been reported. Analysis of an X-ray crystal structure defined the binding site of the pyrones as a region close to the junction of the thumb and finger domains and identified three proximal binding pockets that accommodate substituents of the lead structure. SAR studies on two of the three binding moieties provided analog **37** containing a substituted phenyl ring, an unoptimized cyclopentyl substituent, and an *N*-methyl triazole side-chain, with an enzyme IC_{50} of 38 nM, but no activity in the replicon assay [82].

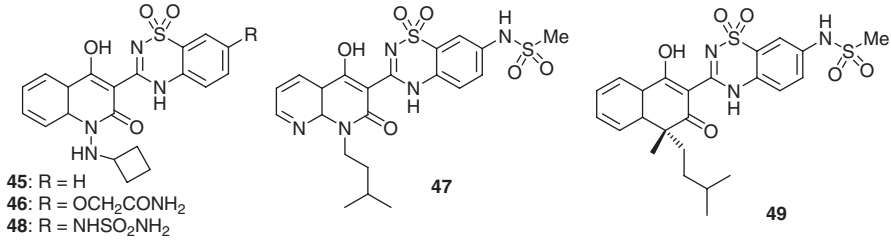
Reversible, non-competitive inhibition of polymerase is also afforded by a series of *N*-benzoyl pyrrolidines. Substitution on the benzoyl moiety with a *para*-trifluoromethyl group is optimal in this series. Bulky, hydrophobic groups at the 2-position of the pyrrolidine ring increase activity, and the 5-position tolerates a wide range of substituents, indicative of a solvent exposed portion of the inhibitor. Compound (+)-**38**, containing a 2-thienyl moiety at the 5-position, has an IC_{50} of 190 nM in the enzyme assay while its enantiomer is almost 100-fold less active [83].

Further optimization of this compound [84] led to analog **39**, GSK-3082 [85]. When screened against individual subtypes of HCV, it was shown that **39** is active against subtype 1b with an IC_{50} of 16 nM, but has an IC_{50} of only 2.3 μM against 1a. A similar disparity was seen in the cellular assay (1b: 160 nM, 1a: 20 μM). The three mutations that are critical for influencing the binding affinities of the acyl-pyrrolidines are N316C, Y415F and Q446E. Further optimization of the substituents at the pyrrolidine 2-, 4- and 5-positions led to **40**, with enzyme IC_{50}s of 6 nM and 79 nM for subtypes 1b and 1a, respectively, and EC_{50}s of 6 nM and 410 nM in cells. Despite this substantial discrepancy in activity against subtypes 1a and 1b, compound **40** has a significantly improved profile when compared to **39** [86].

Hydroxyquinolinone **41** was identified as a non-competitive inhibitor of NS5B in a high-throughput scintillation proximity screen as well as in a cell-based assay ($IC_{50} = 32$ nM, $EC_{50} = 417$ nM). SAR studies revealed that structural changes are tolerated at the *N*-alkyl substituent and the 6-position. The best compound in this series, **42**, has an IC_{50} of 10 nM and EC_{50} of 38 nM and is potent against subtypes 1a, 1b, and 2a, while its potency against subtype 3 is greatly diminished. X-ray crystallographic analysis showed that these compounds bind to the palm/thumb interface and that the quinolinone and thiadiazine moieties are distorted from planarity when bound to the enzyme. Compound **42** has moderate half-life (97–182 min), low clearance, and $>35\%$ bioavailability, resulting in good exposure after oral dosing in rats, dogs and monkeys. The potential liabilities of low volume of distribution and high plasma binding may be offset by good distribution into the liver. A 4-day toxicology study in rats at doses up to 300 mg/kg/day showed no adverse effects [87]. Mimicking the quinolinone core of **42** with substituted tetramic acids led to **43** and **44** with excellent enzyme IC_{50}s of 22 nM and 1.7 nM, respectively. However, analog **44** is only weakly active in the replicon assay ($EC_{50} = 13$ μM) while no data was provided for compound **43** [88].

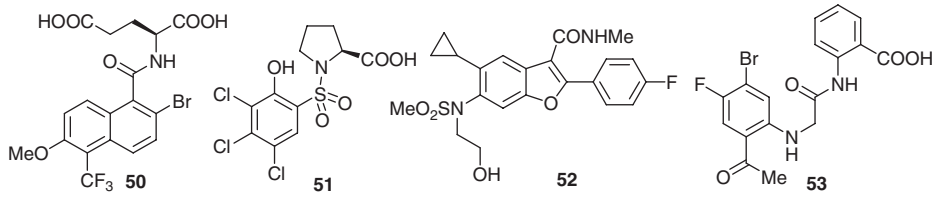


Hydroxyquinolinones bearing a heteroatom at the *N*-1-position as well as 1,8-naphthyridone analogs have also been explored as HCV inhibitors. While the oxygen-linked analogs show poor enzyme activity, many of the nitrogen derivatives display sub-micromolar binding affinities in the enzyme (1a and 1b genotype) and cell-based assays. Cyclobutyl analog **45** is equipotent in the 1b enzyme ($IC_{50} = 108$ nM) and replicon assay ($EC_{50} = 103$ nM) [89]. Further investigation of the benzothiadiazine core in conjunction with the 1,8-naphthyridine moiety revealed that substituents in position 7 enhance binding affinity [90]. An analog of **45**, acetamide **46** (A-782759) in combination with BILN-2061 led to a 7 log reduction in cellular RNA levels after 16 days and was deemed unlikely to develop resistance mechanisms [91,92]. Sulfonamide **47** was reported to inhibit the polymerase with IC_{50}s of 6 and 2 nM for genotype 1b and 1a, respectively. The EC_{50} of this compound is 3 nM in the replicon assay, however it is more than 99% protein bound and in the presence of 40% human serum its activity drops to 1.31 μM. Substitution of the alkoxy group of **46** by a primary sulfamide group provided **48**, which has slightly improved binding affinity to genotypes 1a and 1b ($IC_{50} = 5.2$ nM and 0.4 nM, respectively). More importantly, the cell-based activity of this compound is substantially improved in the presence of 5% fetal calf serum and 40% human serum ($EC_{50} = 3$ nM and 81 nM, respectively) [93]. Compounds with the related naphthalene scaffold, such as **49** (A-837093), are potent in a polymerase assay ($IC_{50} = 19$ nM and 32 nM for genotype 1a and 1b, respectively) as well as in the replicon assay against genotype 1a ($EC_{50} = 134$ nM) [94].



A series of pyranoindole polymerase inhibitors has been disclosed [95,96], and this research was expanded to include benzothienopyrans and

pyranobenzofurans [97] as well as tetrahydrocarbazoles and cyclopentaindoles [98]. Another series of NS5B inhibitors is the naphthalene-1-carboxamides [99]. The bis-carboxylates, such as **50**, bind to the polymerase with moderate affinity ($IC_{50} = 120$ nM), but the low cellular permeability of these compounds prevents significant efficacy in the replicon assay. In a similar manner, a proline sulfonamide lead was optimized by varying substituents around the aromatic ring yielding phenol **51** as the most active compound in this series with an IC_{50} of 80 nM. These compounds display low permeability in a parallel artificial membrane permeability assay (PAMPA) and are only weakly active in the replicon assay [100]. Benzofuran-based analog **52**, HCV-796, has been reported to inhibit NS5B with an IC_{50} of 20–50 nM (genotype 1b and 1a) [101]. Its EC_{50} in the replicon assay ranges from 4 to 16 nM. HCV-796 is currently in Phase 2 trials.



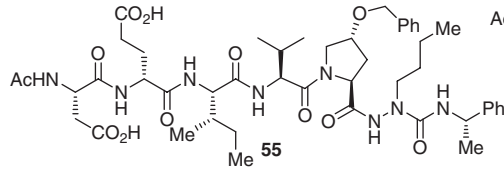
Most recently, anthranilic acid derivatives were found to bind to an allosteric binding site between the palm and thumb region of NS5B. The lead structure was identified in an HTS assay ($IC_{50} = 1.6\,\mu M$) and confirmed by X-ray crystallography. Optimization of the screening hit gave anthranilate **53** that has substantially improved enzyme activity ($IC_{50} = 10$ nM) and an EC_{50} of 1.95 μM in the cell-based replicon assay while lacking toxicity (IC_{50} MTS $> 122\,\mu M$). It was further demonstrated that these analogs showed significant selectivity over a variety of related enzymes [102].

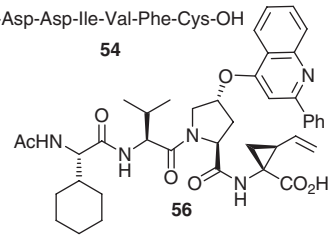
2.3 Peptide mimetics as inhibitors of NS3 protease

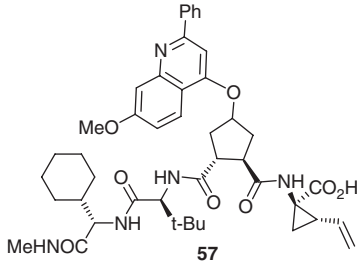
By analogy to AIDS, where HIV protease plays a crucial role in processing mature virions, HCV uses the NS3-4A (aka NS3) protease in a similar manner making it a very attractive inhibition target. The key difference between the two proteases is that the HIV protease active site forms a well-defined active site while the NS3 protease has a shallow cleft with fewer opportunities to bind to small molecules. As a result, NS3 inhibitors have been generally more complex, more peptide-like and larger than those for HIV. Nonetheless, there are a substantial number of different chemotypes being investigated for this target.

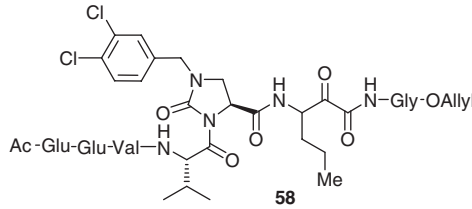
The first protease inhibitor studied in human clinical trials was ciluprevir (BILN-2061), whose discovery was predicated on earlier studies that identified **54**, a 6 amino acid N-terminal cleavage product of an NS5A/5B substrate, as a competitive inhibitor of NS3 [103,104]. Although the development of ciluprevir was halted due to cardiotoxicity in animals, it demonstrated that a potent

inhibitor of HCV ($K_i < 1$ nM vs. enzyme, 4 nM vs. 1b replicon) translated into an impressive 2-3-log reduction of HCV in humans after only two days of treatment [105].



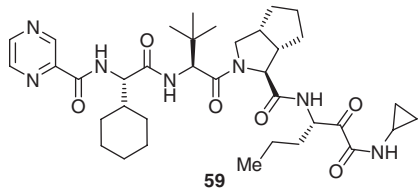






This medicinal chemistry strategy has been further exploited to prepare acyclic derivatives of **54**, including azapeptide **55** ($IC_{50} = 99$ nM vs. NS3, > 400-fold selectivity vs. HLE) [106] and a vinyl-substituted cyclopropyl tetrapeptide **56** ($IC_{50} = 19$ nM) [107]. Substituting a novel cyclopentane-1,2-dicarboxylic acid scaffold for the proline ring of **56** gives a highly active series of inhibitors, of which **57** ($K_i = 1.3$ nM) is the most potent [108]. Isosteric replacement of carbon by nitrogen in the proline moiety gives a series of novel oxoimidazolidine-4-carboxylic acids, with compound **58** ($K_i = 310$ nM) being the only sub-micromolar inhibitor reported [109].

Other potent peptide mimetic NS3 protease inhibitors have been reported that incorporate a serine trap on the C-terminal end of the peptide. Thus, the inhibitory activity of telaprevir (VX-950, **59**), (7 nM vs. NS3, ~300 nM vs. the 1a replicon) is based on truncation of the polypeptide substrate, maximizing binding by alteration of amino acids at the scissile site, and capping both N- and C-terminal ends, the latter with a known dicarbonyl serine trap. This compound has exhibited impressive antiviral activity in animals, and showed a 4.4 log drop in viral load in genotype 1-infected patients in a Phase 1b clinical trial [110]. Telaprevir is expected to enter Phase 3 clinical trials in 2007. Additional bicycloproline-based P2 tetrapeptides, represented by analog **60** ($K_i = 22$ nM), have been explored. Although the compounds are selective inhibitors of NS3, little or no cell-based replicon activity was reported, presumably due to poor cellular permeability [111–114]. A diastereomer of telaprevir, has been reported to inhibit the replicon with an EC_{50} of 0.55 μM [115].



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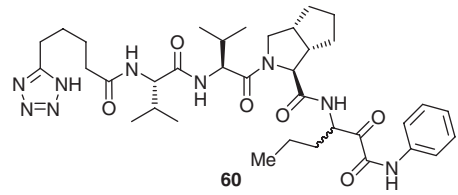
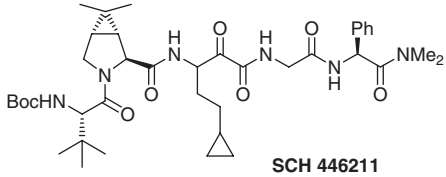
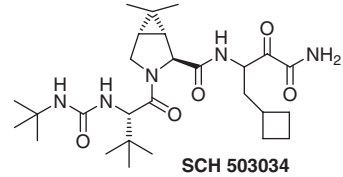


60

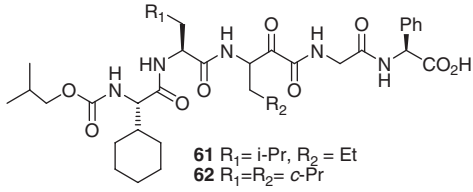
Following the identification of the undecapeptide as a potent ($K_i = 1.9$ nM) inhibitor of NS3 spanning the P6′- to P5′ region of the NS5A/5B cleavage site, a series of smaller peptide mimics was pursued [116]. Guided by analyses of X-ray crystal structures, truncation and optimization led to SCH 446211, which retains excellent potency ($K_i = 3.8$ nM vs. NS3, Replicon $IC_{90} = 100$ nM), but is not orally active [117,118]. Further modification led to the lower molecular weight, orally active SCH 503034 ($K_i = 14$ nM, Replicon $IC_{90} = 350$ nM), currently in Phase 2 trials [116]. Similarly, other potent NS3 inhibitors, including **61** ($K_i = 66$ nM) and **62** ($K_i = 15$ nM) [119], have been reported in which the ketoamide serine trap is retained and the P2-P1 bond has been optimized [120].



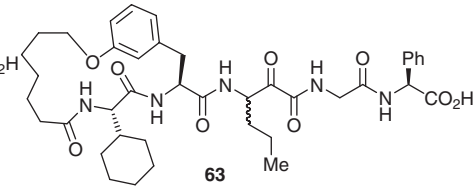
SCH 446211



SCH 503034



61 R_1= i-Pr, R_2 = Et
62 R_1=R_2= *c*-Pr



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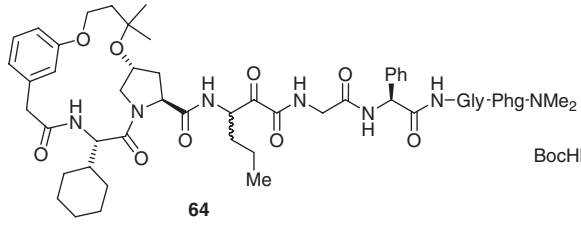

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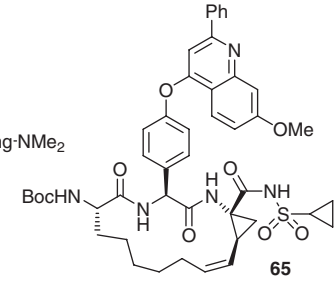
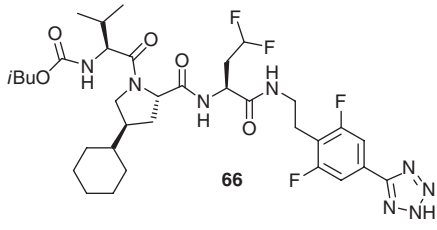


65

In order to minimize peptide-like properties, macrocycles have been incorporated into these protease inhibitors. For example, connecting the P2 aryl side and P3 capping group gives **63**, a 160 nM inhibitor (K_i) [121]. The optimal 17-membered macrocycle reportedly enhanced binding with NS3 by forming an

additional interaction between the new ring and Ala156, resulting in about 50-fold greater potency than the corresponding acyclic analog [121,122]. Different ring sizes were also examined [123]. Other macrocycles have been prepared, as exemplified by **64**, involving cyclization of the proline subunit of SCH 503034 with the N-terminal amine [124,125]. Replacement of the proline group of ciluprevir with an arylated tyrosine derivative led to a series of very potent phenylglycine-based macrocyclic inhibitors, exemplified by **65** ($K_i = 76$ nM, replicon $EC_{50} = 4.6\,\mu$M) [126], although this series displays a different SAR pattern from the standard proline-based inhibitors, suggesting a different binding mode.

A series of phenethylamide-based NS3 inhibitors was disclosed [127] and subsequently elaborated to yield more potent analogs, such as **66** ($K_i = 100$ nM) [128]. Similarly, a second series of phenethylamide [129] and sulfonamide-containing serine traps, exemplified by **67** were prepared and exhibit potent (20–60 nM) protease inhibition, although no cell-based data is given [130].



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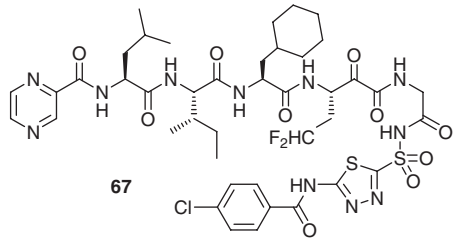

67

3. CONCLUSION

Since the discovery of Hepatitis C 20 years ago, the molecular machinery of this virus has become well understood and significant progress has been made in identifying inhibitors of essential viral targets. At the time of this review, despite enormous efforts put forth by the pharmaceutical industry for over a decade, no effective single agent therapy has yet been discovered. However, novel and highly sophisticated drug candidates continue to progress through clinical trials. It appears likely that a combination therapy for the disease, probably incorporating polymerase and protease inhibitors will enter the market in the not too distant future, providing a much needed effective and better tolerated treatment for this important infection.

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CHAPTER 19

Small Molecule CCR5 and CXCR4-Based Viral Entry Inhibitors for Anti-HIV Therapy Currently in Development

Wieslaw M. Kazmierski*, **Kristjan S. Gudmundsson*** and **Stephen C. Piscitelli****

Contents

1. INTRODUCTION

There are more than 40 million people living with HIV worldwide (http://www.unaids.org/en/HIV_data/), the etiologic cause of the acquired immune

* GlaxoSmithKline, Infectious Diseases Center for Excellence in Drug Discovery, 5 Moore Drive, Research Triangle Park, NC 27709, USA
** GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, NC 27709, Clinical Pharmacology & Discovery Medicine-Infectious Diseases

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deficiency syndrome (AIDS) and there were about 5 million of AIDS-related deaths worldwide in 2004. The number of people infected with HIV is projected to significantly climb in the next several years and estimates indicate that about 100 million people could die from AIDS in the next 25 years.

While the discovery and use of HIV protease inhibitors and of second-generation non-nucleoside reverse transcriptase inhibitors resulted in a significant decrease in the number of AIDS-related deaths in the US starting in mid-late 1990s, there is a continuing need for new and improved drugs, due to limitations of the existing therapies. Thus, the highly active antiretroviral therapy (HAART), drug cocktails of several antiretroviral drugs, can be associated with significant side effects. Loss of efficacy of HAART due to the development of viral resistance requires close patient monitoring and continued adjustment of drug regimens in HAART. Simplified treatment regimens involving fewer pills and less-frequent administration are needed to improve patient compliance, which in turn could slow down the development of viral resistance. Along these lines, it is believed that new, potent and highly bioavailable antiretrovirals that target cellular proteins utilized by the HIV in its replication cycle may hold particular promise in addressing issues of current therapies.

The goal of this report is to present the current state of this field by summarizing results of molecules currently in the clinic as well as by providing a snapshot of the diverse preclinical CCR5 or CXCR4 binders that were recently disclosed in patent applications and the peer-reviewed literature. Additional background and earlier developments can be found in earlier reviews [1–6].

2. CCR5 INHIBITORS

2.1 Rationale for CCR5-based entry inhibitors as anti-HIV agents

More than 19 currently known chemokine receptors belonging to the 7TM class of receptors are involved in pathology of many diverse diseases such as sepsis, COPD, RA, transplant rejection, asthma, IBD, cancer, HIV and others [7–10]. There are more than 45 known endogeneous ligands of chemokine receptors, reflecting the significant redundancy and complexity of the chemokine signaling network. For example, while endogenous chemokines CCL3 (MIP-1α, Macrophage Inflammatory Protein type 1α), CCL4 (MIP-1β), and CCL5 (RANTES, Regulated on Activation, Normal T Expressed and Secreted) bind CCR5, CCL5 also binds two other chemokine receptors, namely CCR1 and CCR3.

Several chemokine receptors, such as CCR2, CCR3, CCR8 and CX3CR1 have been reported to support HIV infection, but CCR5 and CXCR4 are thought to be the primary, physiologically relevant chemokine receptors that mediate entry of the non-syncitium-inducing HIV-1 isolates (R5 virus), and of the syncitium-inducing HIV-1 isolates (X4 virus), respectively. A switch in co-receptor use from viruses utilizing only CCR5 to viruses using both CCR5 and CXCR4 eventually occurs in approximately 50% of HIV-infected individuals [11]. Whether this change is a cause of disease progression or an effect of a declining immune system remains a controversial research topic.

Epidemiology data suggested strongly that individuals homozygous for the δ32 CCR5 allele (leading to non-expression of CCR5) are resistant to HIV-1 infection. In addition, these individuals were otherwise healthy suggesting, as a possible manifestation of the chemokine redundancy, that the blockade of this target may not necessarily lead to side effects [12].

In addition, individuals heterozygous for δ32 CCR5 (one δ32 and one normal CCR5 gene) show retention of CCR5 in the endoplasmic reticulum and thus have lower cell surface levels of CCR5. These individuals have slower progression to AIDS and longer survival periods [13,14]. Along these lines, individuals with high levels of CCL4 were found to have lower rates of HIV progression and other CCR5 ligands like CCL5, AOP-RANTES, CCL3, Met-RANTES and LD78β were found to protect *in vitro* against R5 virus infection [15–19]. More recently, a number of short- and long-term clinical studies conducted with small-molecule R5-antagonists, such as maraviroc (MVC, scheduled to reach the market in 2007), vicriviroc (VCV), aplaviroc (APL) and INCB9471 confirmed that antagonizing CCR5 blocks HIV infection (further reviewed in Section 2.3).

2.2 Preclinical data

The discovery of Maraviroc (MVC, UK-427,857) has been recently described [20–24]. Accordingly, ligand **1** emerged from the high throughput screen of the compound collection in CCR5- expressing HEK-293 against MIP-1β (Figure 1). Subsequent chemistry optimization and removal of the pyridyl imidazole nitrogen, thought to be involved in a potent inhibition of 2D6 P450 cytochrome by **1**, yielded analogue **2**. The high lipophilicity of **2** was then in turn addressed by introducing the more polar amide moiety contained in **3** [24]. Additional optimization in the acyl region followed by homochiral synthesis of both enantiomers resulted in the identification of lead compound **4**.

Further explorations around **4** led to identification of molecules such as tropane-based **5** and **6** (Figure 2), which were practically equipotent in both the MIP-1β inhibition of binding and in the PM-1/BaL antiviral assay [23]. However, **6** also proved to be a potent inhibitor of hERG ion channel (99% inhibition at 1 μM concentration [23] and 80% inhibition at the rate of 300 nM [22]).

Subsequent efforts focused on reducing hERG inhibition. This goal was successfully accomplished using the triazole moiety instead of benzimidazole in

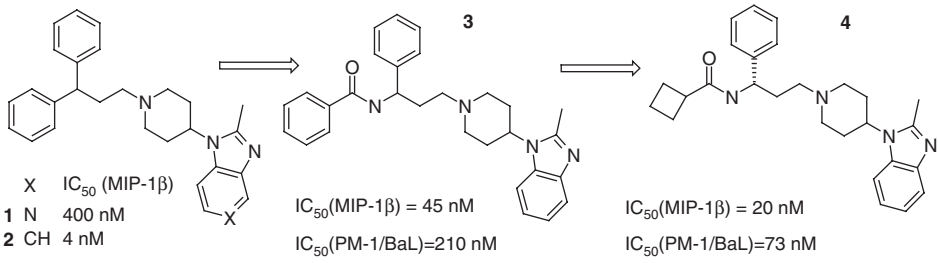


Figure 1 Early SAR in the development of Maraviroc.

7 and the difluorocyclohexane moiety instead of cyclobutyl in **8** (MVC), which was additionally characterized by a relatively low protein binding of 51%, 63.7% and 75.5% in rat, dog and human plasma [25], respectively. MVC, **8**, was subsequently progressed to the clinic.

In rat and dog i.v. PK study, MVC $t_{1/2}$ were 0.9 and 2.3 h, reflecting its relatively high clearance of 74 [mL/min/kg] and 21 [mL/min/kg] in both species, respectively. Oral administration of MVC in rats indicated bioavailability $F = 6\%$ at 10 mg/kg dose (dose-normalized DNAUC = 12.4 ng · h/mL), and $F = 42\%$ at 1 mg/kg in dogs (AUC = 335 ng · h/mL). Following oral administration, the $t_{1/2}$ in dog was 2.3 h at 1 mg/kg, and 8.9 and 10.6 h at respectively 30 mg (0.43 mg/kg assuming 70 kg individual) and 300 mg (4.3 mg/kg) in humans. Human DNAUC, DNC_{max} and elimination half-life $t_{1/2}$ were 272 ng · h/mL, 36 ng/mL, 8.9 h and 537 ng · h/mL, 144 ng/mL, 10.6 h at doses 0.43 and 4.3 mg/kg, respectively [25]. These results suggest a significant interspecies PK difference for MVC.

Piperidino-piperazine-based Vicriviroc (SCH 417690, VCV) **9**, and piperidino-piperidine-based Sch-C **10**, were discovered through high throughput screening leads **11** and **12** (Figure 3). Both leads exhibited a significant binding to the muscarinic M2 receptor, and thus in addition to improving the PK and CCR5 binding, lead optimization focused on tuning out the muscarinic activity. Sch-C

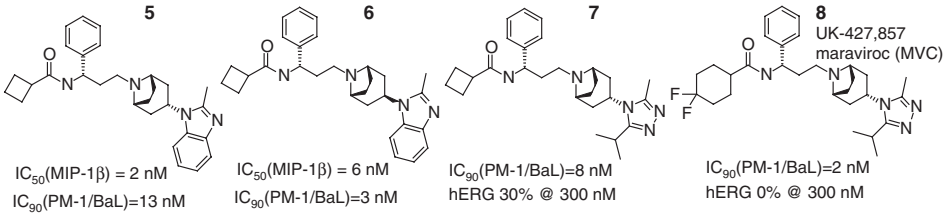


Figure 2 Late SAR and the structure of Maraviroc.

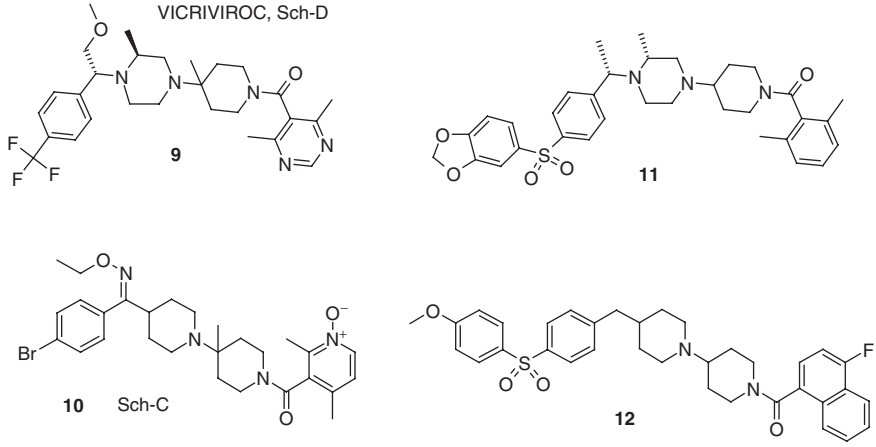


Figure 3 Sch-C and Sch-D (Vicriviroc).

was later found to cause acute CV (prolongation of cardiac QTc intervals associated with peak plasma concentration c_{max} at the highest dose) and CNS side effects, but VCV progressed to the clinic and is further discussed in Section 2.3. In the preclinical studies VCV demonstrated high bioavailability in rat, $F = 100\%$ at 10 mg/kg dose and in monkey, $F = 89\%$ at 2 mg/kg dose, respectively. At these oral doses, the $t_{1/2}$ was 7.9 and 3.4 h, respectively. VCV is moderately protein bound (16% free fraction) and inhibits hERG potassium ion channel to lesser degree than SchC (5.8, vs. 1.1 μM), which could be consistent with no QTc findings at plasma concentrations up to 3.5 μM in monkeys and 6 μM in dogs for VCV [26,27].

TAK-652 (Figure 4) is yet another small-molecule CCR5 inhibitor currently in the clinic. Published data suggest that TAK-652 potently inhibits the macrophage-tropic HIV-1 clinical isolates in peripheral blood mononuclear cells with the mean $IC_{90} = 0.25$ nM [28].

The *in vivo* $t_{1/2}$ half-lives of TAK-652 in rat, dog and monkey plasma administered i.v. at 1 mg/kg dose were 4.85, 5.04 and 6.09 h, respectively. Evaluation of TAK-652 PK in rat, dog and monkey at 3 mg/kg p.o. dose yielded AUC $_{0\rightarrow 24h} = 2320\,ng \cdot h/mL$ (corresponding to bioavailability $F = 10.2\%$), $6010\,ng \cdot h/mL$ ($F = 88.5\%$) and $670\,ng \cdot h/mL$ ($F = 15.6\%$), respectively. TAK-652 also demonstrated a favorable drug–drug interaction profile with other antiretrovirals, such as AZT, lamivudine, indinavir, efavirenz and T-20 *in vitro* [29]. Oral administration of TAK-652 as solution to 24 human subjects as single doses of 25, 50 and 100 mg, was not associated with ECG or QTc prolongation issues and the compound was generally well tolerated [30]. At these doses, the concentration of TAK-652 at 24 h was 7.2 ng/mL (9.1 nM), 14.4 and 42.3 ng/mL and the corresponding AUC values were 416, 1040 and $2760\,ng \cdot h/mL$, respectively. The human $T_{1/2}$ ranged from 8.39 to 12.2 h.

Earlier CCR5 clinical compounds included an injectable quarternary salt TAK-779 [31], which was discontinued due to irritation around the injection site, and TAK-220 [32–34], Figure 5 (clinical status unknown).

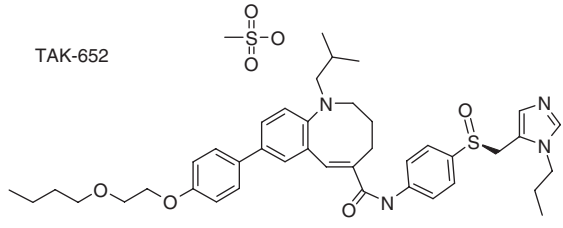


Figure 4 TAK-652.

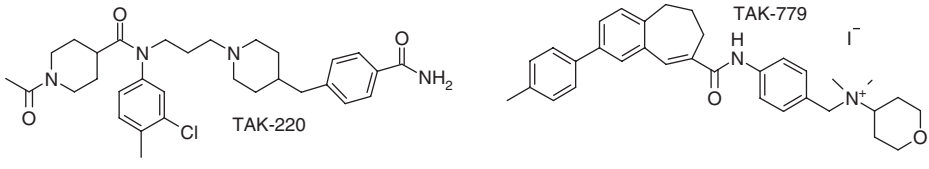


Figure 5 TAK-220 and TAK-779.

INCB9471 is the latest CCR5 ligand to enter the clinic (phase I completed, see Section 2.3). Relatively little preclinical data are available at this time for INCB9471 and the structure has not yet been disclosed. Public reports claim that INCB9471 anti-HIV IC_{50} potency in PBMC is in the range 0.5–2.6 nM against many R5 strains and that INCB9471 is a non-competitive, reversible CCR5 antagonist with slow off-rate, akin to other known ligands. The compound is reported to be active on PI, NRTI, NNRTI-resistant strains, and to have excellent ADME properties, such as no CYP isozyme inhibition, albeit it is also a CYP3A4 substrate and undergoes metabolic transformation mediated by that isozyme. Preclinical PK evaluation suggests low clearance and good PK in rat, dog and monkeys.

2.3 Clinical data for small molecule CCR5 ligands

The CCR5 antagonists are an attractive target for HIV drug development. The allosteric binding of these drugs to host cells differentiates this mechanism from all other antiretroviral classes which target viral enzymes. As noted above, CCR5 is one of two receptors for HIV and the vast majority of HIV-infected patients harbor R5-tropic virus [11]. Persons lacking CCR5 through a genetic deletion are apparently healthy and these individuals demonstrate significant protection against HIV infection. Further support for this target is that CCR5 is a member of the 7TM GPCR protein family which are historically tractable targets for drug development. There are currently several compounds in clinical development, one of which (MVC) has been issued an approvable letter by FDA at the time of this writing.

2.3.1 Efficacy

The antiviral activity of CCR5 antagonists has been demonstrated in short-term monotherapy studies in HIV-infected patients with R5-tropic virus. Four separate compounds have produced a $>$ 1.5 log drop in HIV RNA after 10–14 days of treatment [35–37]. CCR5 antagonists have an extended antiviral effect as demonstrated by the nadir viral load value occurring 2–7 days after treatment has been discontinued. It is hypothesized that this effect is due to prolonged binding of these compounds to CCR5 as pharmacokinetic properties alone would not explain the activity.

Longer-term data with CCR5 antagonists as part of combination therapy have begun to emerge [38,39]. Phase 2/3 data with MVC in treatment-experienced patients having R5-tropic virus demonstrated potent antiviral effects. Over 1000 patients in two identical trials were randomized 2:2:1 to MVC 150 mg QD, MVC 150 mg BID, or placebo in combination with an optimized background regimen based on resistance testing. Both MVC treatment arms were significantly better than the placebo. The mean change in viral load from baseline was -1.82 log for MVC QD, -1.95 log for MVC BID and -1.03 log for placebo in the first study with similar results reported in the second. CD4 cell increases were also higher in the MVC-treated patients. Approximately 60% of patients in the BID arms and 55% of patients in the QD arms achieved a viral load of <400 copies/mL

compared to 23–31% of patients on placebo. Results have not been as positive for treatment-experienced patients with dual or mixed tropic virus (D/M). In a study of 191 patients using a similar design and MVC doses as above, no difference in viral load change from baseline was observed between MVC-treated patients and those receiving placebo after 24 weeks of therapy. Mean viral load decreases were −0.91 for MVC QD, −1.20 for MVC BID and −0.97 for placebo. However, MVC-treated subjects did have CD4 cell increases of approximately 60 cells/mm^3 compared with 36 cells/mm^3 for placebo. Studies with MVC in treatment-naïve individuals with R5-tropic virus have not yet been reported. These studies are evaluating MVC (300 mg QD or BID) plus two reverse transcriptase inhibitors (NRTIs) compared to efavirenz plus two NRTIs. The QD arm was terminated during the trial for inferior efficacy compared with the efavirenz arm. The BID arm continued and results should be available later in 2007.

Mixed results have been shown for VCV in Phase 2 studies [40]. Treatment experienced patients with R5-tropic virus receiving an optimized background regimen including a RTV-boosted protease inhibitor were randomized to three doses of VCV (5, 10 or 15 mg) or placebo. The 5 mg dose was terminated early for inferior efficacy. Potent antiviral responses were seen with the 10 mg dose (−1.86 log) and the 15 mg dose (−1.68 log) compared to placebo (−0.29 log). CD4 cell increases were approximately 140 cells/mm^3 while no change was seen in the placebo group. However, this durable antiviral activity has not been demonstrated in treatment-naive patients. Three doses of VCV (25, 50 or 75 mg QD) were compared to the standard of care efavirenz with all arms receiving two NRTIs. All VCV treatment arms were terminated early due to inferior efficacy. The reasons for the virological failures are unclear since the pharmacokinetics of VCV support QD dosing. New Phase 2 studies have been initiated in treatment-naïve subjects using higher VCV doses to improve efficacy.

2.3.2 Safety

The long-term safety of CCR5 antagonists continues to be a subject of intense debate. There are a variety of theoretical concerns that mechanism-related effects could lead to toxicity and immunologic adverse events. The development of aplaviroc was discontinued due to idiosyncratic hepatotoxicity observed in Phases 2 and 3 [41]. These findings fueled questions as to whether the hepatic effects were drug-related or class-related. Additionally, patients receiving VCV in a Phase 2 trial had a significantly higher incidence of malignancies compared with patients on placebo which led to further concerns about the safety of this mechanism [40]. While concerns exist around immune dysfunction and hepatotoxicity with long-term therapy, MVC continues to progress forward. Phase 3 studies did not show significant safety issues suggesting that the adverse events seen with APL and VCV were compound specific. Other studies with CCR5 antagonists include long-term follow-up for safety and further data will be forthcoming.

2.4 Challenges and promises of CCR5 antagonists for HIV treatment

CCR5 antagonists have the potential to significantly alter the landscape of HIV treatment by providing a new class of drug that targets a host cellular receptor rather than a viral enzyme. These compounds, along with integrase inhibitors and next generation NNRTIs, will provide potent options for patients with drug-resistant HIV strains. CCR5 antagonists could also provide alternative treatment paradigms such as HIV prevention and initiation of early treatment.

The major challenges with this class of compounds revolve around tropism testing, safety and their place in the HIV armamentarium. The available data in treatment-experienced patients demonstrate that R5-tropic patients have a significantly better benefit than D/M-tropic patients. This will lead to a scenario of all patients requiring a tropism test before initiation of therapy. However, treatment-naïve subjects with D/M-tropic virus have not been evaluated and this population may have a different outcome with a CCR5 antagonist-containing regimen. The tropism issue is related to the question of how best to use these drugs. Since 80–90% of treatment-naïve subjects have only R5-tropic virus detectable, it would be rational to use these drugs as early therapy in this population. However, due to safety concerns and the available potent and convenient first-line drugs, CCR5 antagonists will initially be used in later lines of therapy where about half of subjects have R5-tropic virus and the other half have D/M-tropic. Additionally, the first indication for MVC will be in the treatment-experienced population. In these patients, CCR5 antagonist will be combined with a variety of drugs based on resistance testing including RTV-boosted HIV-protease inhibitors, integrase inhibitors and enfurvitide. As safety and clinician experience increases, CCR5 antagonists will move into earlier lines of therapy. Although there is concern that treatment with a CCR5 antagonist could promote a change in tropism from R5-tropic to D/M-tropic, there are currently no data demonstrating that such a treatment effect is clinically detrimental or irreversible. Safety and tolerability will be the major drivers for the use of these drugs in treatment-naïve subjects.

2.5 Other small molecule CCR5 ligands

Many other families of CCR5 antagonists have been described in the last several years. Selected structures are provided along with references.

Aplaviroc (AK-602, GW873140, Figure 6), discontinued due to idiosyncratic liver toxicity findings in phase II [41], was discovered in the lead optimization effort, which originated from CCR5 hits in the synthetic library of spirodiketopiperazines [42–44].

Several diverse classes of potent CCR5 antagonists, Figure 7 have recently been reported [2]. Scaffolds reported to support high potency against CCR5 in antiviral cellular assays included the aminocyclohexyl (compound **13**) [45], 4,4-disubstituted piperidine (compound **14**, GSK163929) [46,47], tetrahydro-2*H*-1,3-oxazine (compound **15**) [48], pyrrolidine (compound **16**) [49], cyclopropyl (compound **17**) [50] and 2,3-dihydro-1*H*-indene (compound **18**) [51].

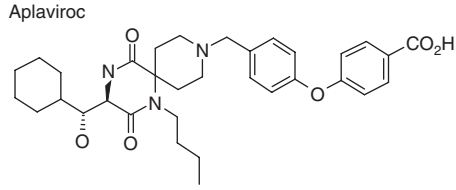


Figure 6 Aplaviroc GW873140.

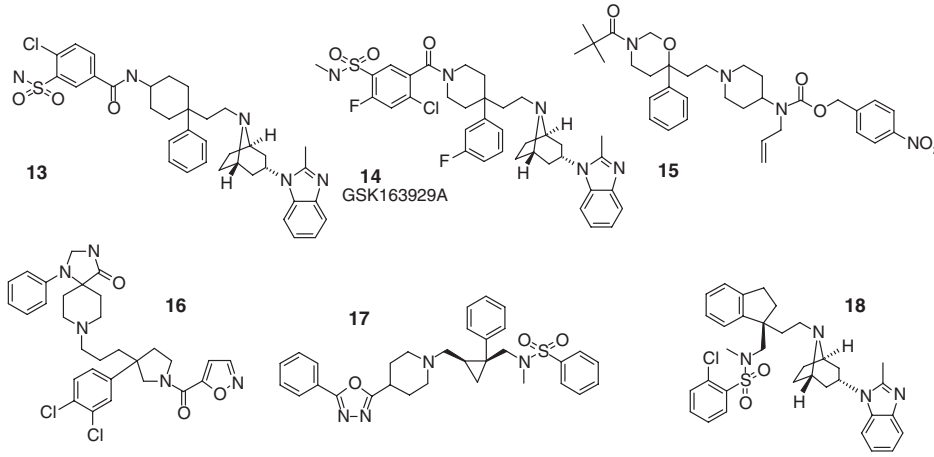


Figure 7 GSK CCR5 inhibitors.

GSK163929 is a potent CCR5 antagonist that inhibits HIV-1 replication at low nanomolar level. GSK163929 has successfully completed preclinical studies [47].

Kilogram scale synthesis of the key GSK163929 precursor has been described [52,53].

CCR5 ligands **19–31** [54–66], have recently been reported either in the patent or the primary literature. Their clinical status has not been disclosed. When published, antiviral potency of a compound is included, otherwise randomly selected patent examples are shown in Figure 8.

3. CXCR4 INHIBITORS

3.1 CXCR4 pharmacology

CXCR4 is a 7-transmembrane spanning receptor comprised of 352 amino acid residues that displays 33% homology to other CXC and CC members of the chemokine receptor family [67]. Unlike most of the other receptors within the chemokine family, which tend to have a number of distinct ligands, CXCR4 has only one known natural ligand, stromal cell-derived factor (SDF-1). SDF-1 is a highly basic protein with about 20% of its 68 amino acids (for SDF-1α) being arginine, lysine or histidine, while CXCR4 is rather unusual among chemokine

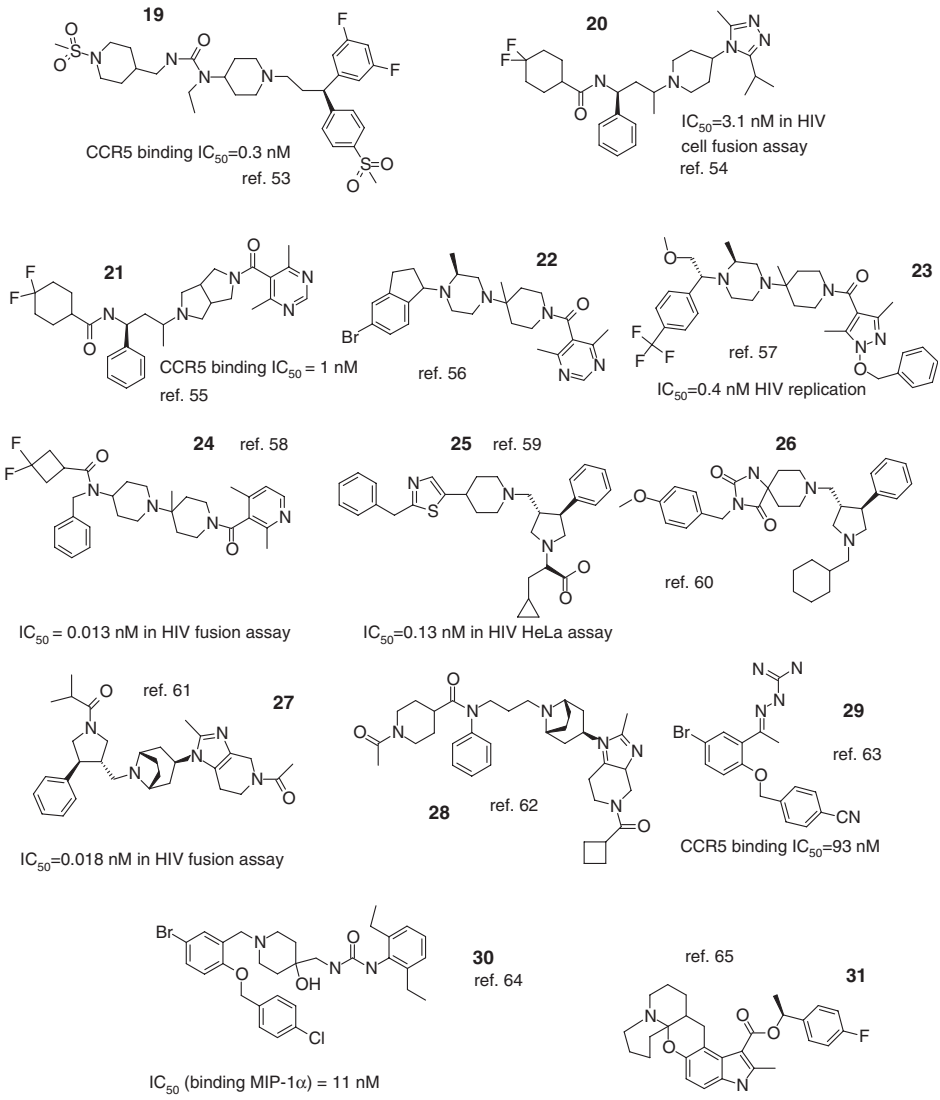


Figure 8 Other selected CCR5 inhibitors from patent and peer-reviewed literature.

receptors in being strongly negatively charged. The lack of redundancy of the SDF-1/CXCR4 system and its unique importance not only to the function of the immune system but also for embryonic development have been demonstrated in mice knockout studies, where knockout of either SDF-1 or CXCR4 is embryonically lethal [68,69]. However, recent short-term clinical trials with CXCR4 antagonists have shown that these are safe, suggesting that a functioning SDF-1/CXCR4 may only be vital during embryonic development (reviewed in Section 3.3). Ongoing studies on CXCR4 antagonists should provide further information about their safety.

CXCR4 antagonists have multiple potential therapeutic uses. CXCR4 is known to be a co-receptor for a number of specific strains of HIV-1 [70]. However, it is the CCR5 (R5) utilizing HIV-1 strains that are generally associated with the initial infection phase of the virus. This apparent selectivity may be related to the higher expression level of CCR5 on target cells within or near the genital mucosa [71,72]. In addition, these tissues also express relatively high levels of SDF-1 that may bind to the CXCR4 receptor blocking the interaction with CXCR4 virus [73]. However, as the disease progresses towards AIDS, variant forms of the virus emerge that has the ability to either utilize both CCR5 and CXCR4 chemokine receptors (R5X4 dual tropic or mixed tropic viruses) or solely the CXCR4 chemokine receptor (X4 viruses) to gain entry and infect the host cells [74]. Independent of which co-receptor is utilized, all viruses require co-expression of CD4 on the host cell to allow the formation of the tertiary complex formation of co-receptor/CD4/gp120 that is essential for viral entry. R5 strains primarily infect both macrophages and primary T cells, while X4 strains infect T cells and with much lower efficiency macrophages [75].

CXCR4, and its interaction with SDF-1, has also been shown to play a role in a number of physiological processes. CXCR4 plays a role in the homing of immune cells such as T cells to sites of inflammation [76] and the interaction of CXCR4 and SDF-1 has been shown to have a role in maintaining the cellular micro-environment of the bone marrow [77]. A CXCR4 antagonist could thus be used to mobilize hematopoietic stem cells from the bone marrow [78]. Also by blocking the homing of inflammatory cells to inflamed joints CXCR4 antagonists may be of value in the treatment of rheumatoid arthritis [79,80].

Additionally, the CXCR4/SDF-1 interaction has been shown to play a role in metastatic spread and in directly regulating the growth and/or survival of several types of cancer, including prostate cancer [81,82], neuroblastoma [83], non-Hodgkins lymphoma [84], lung cancer [85], several hematopoietic malignancies and breast cancer [86–88]. Hence, there may be a role for CXCR4 antagonists in the treatment of certain forms of cancer [9,89].

SDF-1 has been shown to inhibit infection of CD4+ cells by X4 HIV strains [90]. The antiviral activity (IC_{50}) of SDF-1 (SDF-1α) is reported to be 79 nM [91]. The exact mechanism by which SDF-1 interfers with virus entry is unknown, but could include competitive binding of gp120 and SDF-1 with CXCR4, downregulation of the CXCR4 receptor and receptor dimerization. The concentration of SDF-1 required for HIV inhibition is significantly higher than that required for receptor signaling or binding. N-terminal truncations or changes in amino acid composition of the N-terminus of SDF-1 have demonstrated the importance of this portion of SDF-1 for signaling and for anti-HIV activity [91]. While antiviral and signaling properties of SDF-1 are partially retained by peptides corresponding to its N-terminus and several SDF-1 like peptides have been synthesized and studied, smaller peptides have received more interest as starting points for discovery of 'drug-like' CXCR4 antagonists.

3.2 Peptides targeting CXCR4

N-α-acetyl-nona-D-arginine amide (ALX-40-4C; Allelix-40-4C] was initially designed to mimic the arginine-rich sequence in the TAT domain of HIV and inhibit TAT from binding to its target, TAR [92]. Subsequently, this highly cationic peptide was shown to prevent viral entry into cells via blockage of the CXCR4 receptor [93]. ALX-40-4C was initially tested in HIV patients before its mechanism of action had been elucidated, making it the first co-receptor inhibitor to be tested in humans [92]. No reduction in viral load was seen, and this compound is no longer in development, but importantly these initial trials, while not definitive, argue that CXCR4 antagonists can be used safely [94].

Several small peptides (17–19 amino acids) containing KGVSLSYR sequences, similar to the first 8–9 amino acids of the N-terminus of SDF-1 are moving forward through development [95]. The CXCR4 antagonist, CTCE-9908, has recently completed phase 1 study in healthy adults (single i.v. administration of 0.5, 2 and 5 mg/kg doses) and did not show any significant toxicity. It is being progressed into cancer patients and has received an orphan drug designation from the FDA for treatment of osteogenic sarcoma [96,97].

Polyphemusins are self-defense proteins isolated from horseshoe crab. These had been known for some time as inhibitors of HIV-1 replication and virus-cell fusion, before they were identified as CXCR4 antagonists [98]. T22 is a synthetic polyphemusin (18 amino acid residues, 9 positive charges (arg/lys)). Further SAR studies of T22 subsequently led to development of downsized analogs such as T140 (14 amino acid residues, 7 positive charges) (Figure 9) [99]. Discovery and development of these have been recently reviewed [100]. In addition to anti-HIV activity, T140 has been shown to have anticancer-metastatic acitivity and anti-rheumatoid arthritis activity. Subsequent studies showed that Arg^2, L-3-(2-naphthyl)alanine $(Nal)^3$, Tyr^5 and Arg^{14} constitute the critical pharmacophore of T140. Screening of pentapeptide libraries containing these critical residues resulted in identification of the small cyclic pentapeptide FC131 (cyclo(-Nal-Gly-D-Tyr-Arg-Arg-) that has similar HIV activity as

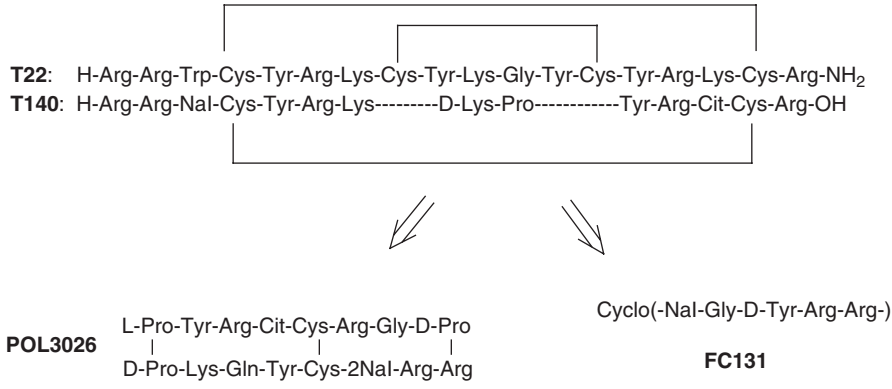


Figure 9 Peptide CXCR4 antagonists.

T140 [101]. Further optimization has led to identification of additional potent small cyclic peptide analogs [102].

A group of Swiss researchers has also used the beta-hairpin motif in the polyphemusin and T22 as well as beta-hairpin protein epitope mimetics (PEM) in the form of template-bound macrocyclic peptides to design CXCR4 inhibitors. Optimization of these resulted in identification of POL2438 and POL3026. POL3026 showed excellent stability to proteolytic degradation in human plasma and good pharmacokinetics when dosed subcutaneously in dogs. POL3026 is a very potent CXCR4 antagonist, with the potential to be used against HIV, as anti-cancer agent or for mobilization of stem cells [103].

3.3 Small molecules that target CXCR4

A series of CXCR4 antagonists based on an arginine scaffold have been described. Among these, KRH1636 is a potent CXCR4 antagonist, Figure 10 [104]. KRH1636 has also been shown to block HIV replication *in vivo* in a SCID mouse model. KRH1636 and methanesulfonated KRH1636 were absorbed into blood after intraduodenal administration to rats in a cyclodextrin formulation, but a more conventional oral pharmacokinetic study in rats has not been reported for KRH1636. More recently, alkyl amine analogs of KRH1636 (KRH2731 5HCl) that are reported to be orally bioavailable (37% in rats) and have potent activity against HIV have been described [105]. While the structure of KRH2731 has not been disclosed, several recently published patent applications claim compounds related to KRH1636 where the arginine has been replaced by a dialkylated ornithine (e.g. compound **32**) [106]. Recently, applications have been published where these molecules are further simplified to non-amino acid-based derivatives that were reported to have potent activity against HIV (e.g. compounds **33** and **34**) (Figure 10) [107].

The study of small molecules that block CXCR4 has until recently been dominated by the bicyclam class of molecules being developed. The discovery of

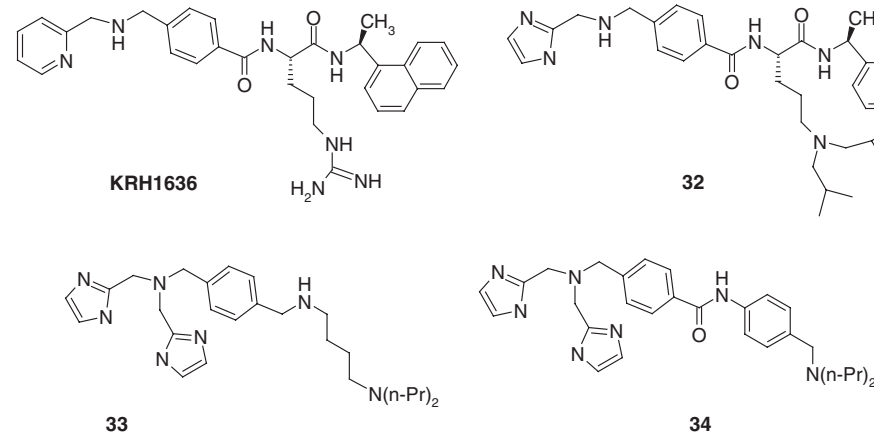


Figure 10 Arginine and non-amino acid-based CXCR4 small molecules.

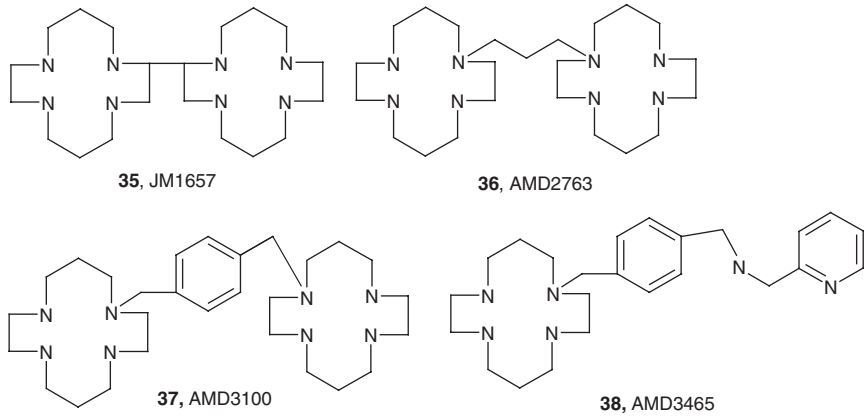


Figure 11 Bicyclams.

the cyclams (JM1657, **35**) as an impurity in a commercial cyclam preparation, and the research that resulted in the discovery of AMD3100 (**37**) has been reviewed [108]. The bicyclams contain two macrocyclic polyamine cyclam (1,4,8,11-tetraazacyclotetradecane) rings connected by an aliphatic linker (e.g. AMD2763, **36**) or an aromatic linker (e.g. AMD3100, **37**) (Figure 11).

AMD3100 shows potent activity against several strains of HIV-1, in the 1–10 ng/mL range [109], but suffers from the disadvantage of requiring intravenous or subcutaneous administration [110]. In an open label dose-escalating phase I/II trial in HIV patients, one patient with pure X4 virus exhibited a clear viral load reduction that could be interpreted as evidence for antiviral efficacy [111]. AMD3100 did induce cardiovascular adverse effects and was discontinued as an anti-HIV agent, but development of AMD3100 for stem cell mobilization is being continued (Plerixafor; Mozobil) [112]. A US NDA is expected in the 2nd half of 2007, with Canadian and European filings in 2008 [113]. Later generations of these cyclams include AMD3465 (compound 38) and related analogs, where one of the cyclam units of AMD3100 has been replaced with a heterocyclic moiety, without adversely affecting HIV activity [114,115].

More recently antiviral and pharmacokinetic data on a new CXCR4 antagonist, AMD070 (compound **41**, Figure 12) was disclosed [116]. AMD070 has a protein-adjusted EC_{90} of 125 nM against HIV-1 in MT-4 cells and bioavailability of over 20% in rats and about 80% in dog. Phase 1 studies in healthy volunteers have showed acceptable human PK for progression [117]. Transient increases in white blood cells were observed in these studies demonstrating expected pharmacological effects of CXCR4 inhibition. AnorMED has recently reported preliminary results from two phase IIa trials of AMD070 in HIV-infected patients. The preliminary endpoint in these trials was a reduction in relative light units (RLUs) from baseline which is the output from the cellular tropism assay and demonstrates an indirect measure of X4-tropic anti-HIV activity. Patients with X4-tropic or dual/mixed tropic virus were treated with 200 mg AMD070 twice daily for 10 days in both trials. Of 16 total patients treated in these studies, 7 demonstrated a $\geqslant$ 1 log reduction in RLU. HIV viral load and CD4 count were

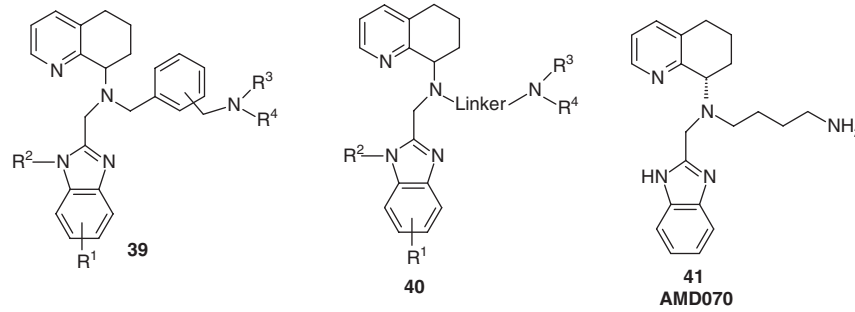


Figure 12 Tetrahydroquinoline-based CXCR4 antagonists.

not altered by treatment with AMD070 [118,119]. Adverse effects were reported as mild-to-moderate and included diarrhea, dizziness, flatulence and headache. These studies suggest AMD070 may have activity against X4-tropic HIV. Unfortunately, clinical trials AMD070 have been placed on hold due to hepatotoxicity in preclinical studies.

Several patent applications have been filed claiming CXCR4 antagonists, where the cyclam structure has been replaced with a *N*-(1H-benzimidazol-2-ylmethyl)-5,6,7,8-tetrahydro-8-quinolineamine pharmacophore, like compounds **39** and **40** (Figure 12) [120–124]. Salt forms suitable for development have been described for **41** (AMD070) in a recent patent application [125]. These filings describe a novel tetrahydroquinolineamine pharmacophore.

Molecules in Figures 10 and 12 highlight exciting progress toward development of orally bioavailable small molecules as CXCR4 antagonists. In general both the peptides as well as these small molecules contain multiple protonable sites giving them a cationic character. Although only one small molecule inhibitor has progressed through phase 1 clinical trials there are several additional highly basic pharmacophores that have recently been reported in the literature. These include isothiourea derivatives [126], substituted indole derivatives [127], substituted benzodiazepine derivatives [128], substituted benzylamine derivatives [129,130] and dipicolylamine derivatives [131].

4. CONCLUSIONS

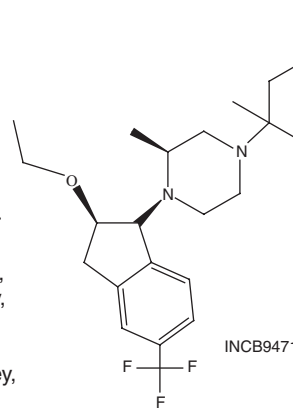
The discovery of co-receptors and their relationship to HIV entry sparked an exciting flurry of research and drug development. CCR5 and CXCR4 antagonists represent the first potential antiretrovirals that act upon a host target and not a viral enzyme. CCR5 antagonists have demonstrated proof of concept as antivirals and long-term efficacy and safety data are now becoming available. The first approved member of this class is expected in 2007. CXCR4 antagonists are at a much earlier stage and the development of these compounds will be more complex. Long-term safety will be the biggest issue for both classes, especially the CXCR4 compounds. Tropism testing and their place in therapy are additional questions which will be clarified as better assays are developed and clinicians

gain experience with these drugs. The development of entry inhibitors will provide a potent option for HIV-infected individuals at all stages of disease.

NOTE

The structure of INCB9471 has recently been disclosed.

TUAB106 - Potent antiretroviral activity of the once-daily CCR5 antagonist INCB009471 over 14 days of monotherapy
C. Cohenl, E. DeJesus, A. Mills, G. Pierone, Jr., P. Kumar, P. Ruane, R. Elion, G. Fusco, R. Levy, K. Solomon, S. Erickson-Viitanen
4th IAS Conference on HIV Pathogenesis, Treatment and Prevenation, July 22–25, Sydney, Australia



INCB9471

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CHAPTER 20

Small-Molecule Inhibitors of Glutathione *S*-Transferase P1-1 as Anticancer Therapeutic Agents

Guillermo A. Morales* and **Edgardo Laborde****

Contents

1. INTRODUCTION

The Glutathione *S*-Transferase family of enzymes (EC 2.5.1.18), also referred to as glutathione transferases or GSTs, comprises a group of isoenzymes present in most aerobic eukaryote organisms. Discovered in 1961, GSTs were initially thought to act as carrier proteins and were originally called ligandins [1,2]. GSTs are found in mammalian tissues (e.g., muscle, liver, brain, testis, kidney, spleen, skin, placenta) and constitute approximately 1–4% of the total cellular protein content [3–5]. GSTs play a role in the synthesis of prostaglandins [6], steroids [7,8] and leukotrienes [9,10], as well as in the intracellular transport of molecules including metabolites [11], hormones [12–18], neurotransmitters [12], bilirubin [14,19,20], hemin [21,22], heme [22], thyroid [23], bile acids [24–26], and steroids [16,17,27,28].

One of the most important functions of GSTs is the detoxification of endogenous as well as exogenous substances via conjugation with glutathione

* Semafore Pharmaceuticals Inc., 8496 Georgetown Road, Indianapolis, IN 46268, USA
** Telik Inc., 3165 Porter Drive, Palo Alto, CA 94394, USA

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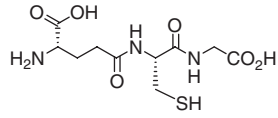
(γ-L-glutamyl-L-cysteinylglycine, GSH, **1**). GSH is a thiol-containing tripeptide found in 0.5–10 mM concentration in the cytosol of a vast number of cell types [29] and in micromolar concentration in plasma [30].



1

GSTs contain a site that accommodates GSH (“GSH binding domain”) [31], where the proton of the GSH’s thiol group is abstracted, promoting the nucleophilic conjugation of the thiolate to electrophilic substrates. The resulting adducts become more water-soluble and are then eliminated by a phase II detoxification mechanism.

GSTs are of therapeutic interest because their overexpression has been associated with diseases such as chronic renal failure [32], neurodegeneration [33–37], multiple sclerosis [33,38,39], asthma [33,40–44], and particularly prostate [45], colon [46], and ovarian cancers [47].

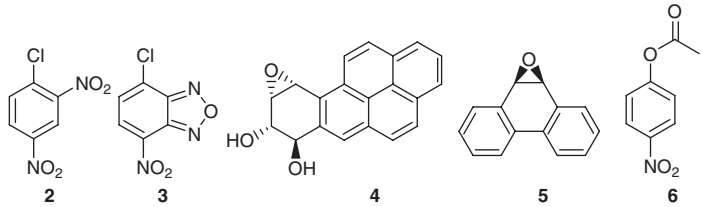
To date, 16 GST isozymes have been found in humans [48]. Studies of several cancer tissues have revealed the overexpression of different GST isozymes, with GST P1-1 (GST Pi, GST π) being the most predominant. For this reason, GST P1-1 is regarded as a potential tumor marker [5,49–53]. The high expression levels of GST P1-1 (up to 2.7% of the total cytosolic protein [52]), combined with its detoxification role against xenobiotics, make GST P1-1 a major player responsible for drug resistance in patients undergoing anticancer chemotherapy [49].

This report will cover inhibitors of GST P1-1 based on their mode of action.

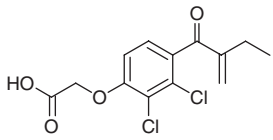
2. TYPES OF INHIBITORS

2.1 Suicide inhibitors

Suicide or irreversible inhibitors of GST P1-1 include agents that bind covalently to glutathione, thereby forming thioether adducts that are stabilized at the active site of the enzyme. These agents include activated aromatic systems (**2**, **3**), epoxides (**4**, **5**), esters (**6**), and Michael acceptors such as ethacrynic acid (**7**), cycloalkenones (**8**, **9**), and haloenol lactones (**10**–**13**), among others [3,48,54–57].



2 **3** **4** **5** **6**



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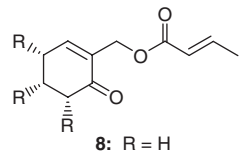
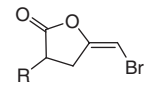
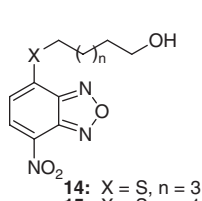


8: R = H
9: R = OH

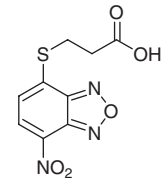


10: R = *trans*-PhCH=CHCH$_2$
11: R = Ph-CH$_2$-CH$_2$-CH$_2$
12: R = Cy-HexCH$_2$
13: R = 2-naphthyl-CH$_2$

Recently, 7-nitro-2,1,3-benzoxadiazole thioethers (**14**, **15**, **17**, **18**) have been reported as a new class of suicide GST P1-1 inhibitors. GST P1-1 catalyzes the nucleophilic addition of GSH to C-4 of the benzoxadiazole ring forming a sigma-complex intermediate that is stabilized at the active site of the enzyme. This results in the dissociation of the complex between JNK and GST P1-1, inducing apoptosis in K562, HepG2, and GLC4 cancer cell lines. The requirement of the thioether group for activity became clear as amino analog **16** did not inhibit GST P1-1 at concentrations as high as 100 μM. The observed SAR indicates that thioether hexanol **14** inhibits GST P1-1 at submicromolar concentrations ($IC_{50} = 0.8\,\mu M$), while **15**, **17**, and **18** inhibit GST P1-1 in the single-digit micromolar range ($IC_{50} = 2$, 5.7, and 6.3 μM, respectively) [53,58].



14: X = S, n = 3
15: X = S, n = 1
16: X = N, n = 3



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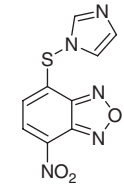
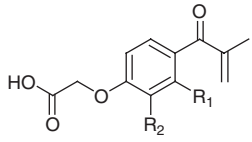
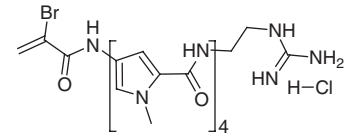


18

Several Michael acceptors have been studied as irreversible GST P1-1 inhibitors. A recent SAR study of analogs of **7** in HL-60 cells revealed that substitution at the R_1 position is essential for GST P1-1 inhibition (**7**, **19**, **20**: 89–94% inhibition at 40 μmol/L), while substitution at the R_2 position did not contribute significantly towards activity (**21**–**23**: <19% inhibition at 40 μmol/L) [59].



19: R_1 = Br, R_2 = H
20: $R_1 = R_2 = CH_3$
21: R_1 = H, R_2 = Cl
22: R_1 = H, R_2 = F
23: R_1 = H, $R_2 = CH_3$



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The DNA minor groove binder brostallicin (PNU-166196, **24**), currently in Phase 2 clinical trials for the treatment of soft-tissue sarcoma, exhibited potent

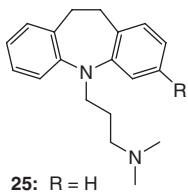
activity against GST P1-1 ($IC_{50} = 103$ nM) in GST-transfected MCF-7 cells where the intracellular GST P1-1 activity is high (78 nmol/min/mg) [30,60].

2.2 Competitive inhibitors

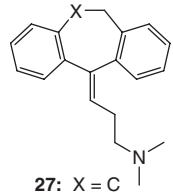
Competitive inhibitors of GST P1-1 fall under two categories: non-glutathione- and glutathione-based compounds. The former group covers a broad range of chemical structures such as tricyclic-based dibenzazepines, polyphenolic natural products, alkaloids, pyrimethamine, and dyes. The latter group, as its name indicates, covers compounds whose main structure or backbone is that of GSH.

2.2.1 Non-glutathione-based small molecules

Tricyclic antidepressants such as imipramine (**25**), clomipramine (**26**), amitriptyline (**27**), and doxepine (**28**) were found to be weak inhibitors of GST P1-1 *in vitro*. Inhibition of GST P1-1 was enhanced with the introduction of a chloro group on the dibenzazepine ring (**25**: 40% inhibition at 15 mM; **26**: 70% inhibition at 10 mM). The same result was observed with the substitution of an oxygen for a carbon in the heptadiene ring (**27**: 18% inhibition at 10 mM; **28**: 48% inhibition at 15 mM) [35].

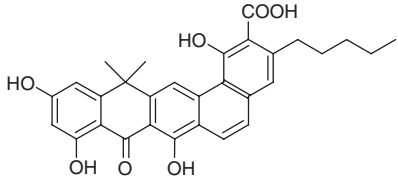


25: R = H
26: R = Cl

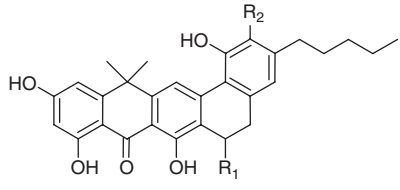


27: X = C
28: X = O

Natural products such as benastatin A (**29**), benastatin B (**30**), bequinostatin A (**31**), and bequinostatin B (**32**), all isolated from the culture broth of *Streptomyces* sp. MI384-DF12, were tested for inhibition of GST P1-1. Compounds **29** and **30** exhibited similar binding affinity towards murine-derived GST ($K_i = 3.5 \times 10^{-6}$ M and 4.2×10^{-6} M, respectively). Studies with compounds **31** and **32** indicate that the carboxylic acid group is required for inhibition of human-derived GST P1-1 (**31**: $IC_{50} = 0.6$ μg/mL; **32**: $IC_{50} = 100$ μg/mL). Compounds **29–32** exhibited low toxicity when tested in mice at 100 mg/kg i.p. [61,62].

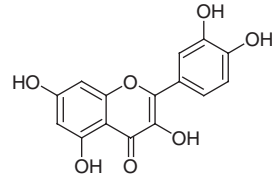


29



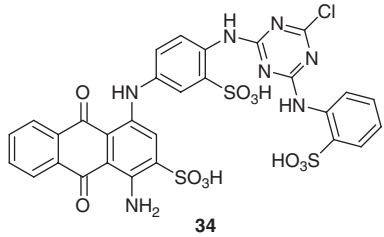
30: R_1 = H, R_2 = CO_2H
31: R_1 = OH, R_2 = CO_2H
32: R_1 = OH, R_2 = H

Phase 1 clinical trials of the natural polyphenol quercetin (**33**) against several types of cancer have shown that this compound is well tolerated when administered at a dose of 70 mg/kg by i.v. bolus [63,64]. Further studies revealed that **33** inhibits GST P1-1 completely after 2 h at a concentration of 25 μM; however, addition of GSH partially restores activity. HPLC and LC-MS studies indicate that **33** inhibits GST P1-1 through the formation of a covalent yet reversible bond with GST P1-1 cysteine residue at position 47.

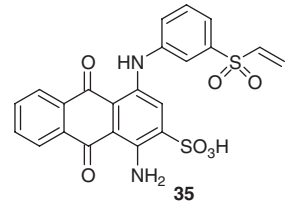


33

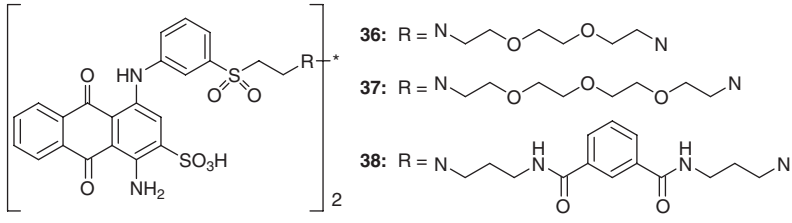
The anthraquinone analog cibacron blue (**34**) has been co-crystallized with GST P1-1 (PDB entry: 20GS). Using the vinyl sulfone Uniblue A (**35**) as the binding moiety, bivalent-based inhibitors **36–38** were synthesized incorporating different linkers. It was observed that analogs **36** and **37**, both containing flexible PEG-based linkers, exhibited higher affinity towards GST P1-1 ($IC_{50} = 44$ and 72 nM, respectively) than the more rigid isophthalamide-linked analog **38** ($IC_{50} = 440$ nM). It is worth noting that compounds **36–38** were highly selective GST P1-1 inhibitors, as none of them inhibited GST A1-1 at concentrations as high as 100 μM [65].



34

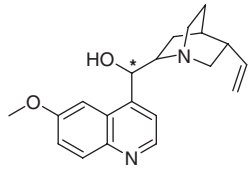


35

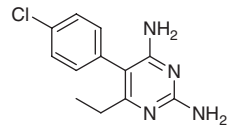


Alkaloids **39** and **40**, which are enantiomers of one another and are both used as antimalarial agents, inhibited GST P1-1 in the single-digit micromolar range ($IC_{50} = 4$ and 1 μM, respectively), indicating that the chirality of the secondary alcohol does not play a significant role in this activity. Pyrimethamine (**41**),

another antimalarial agent, also inhibited GST P1-1 in the single-digit micromolar range ($IC_{50} = 1\,\mu M$) [66].



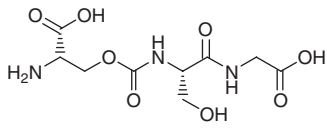
39: (*R*)
40: (*S*)



41

2.2.2 Glutathione-based inhibitors

Among the strategies used for the development of GST P1-1 inhibitors is the modification of the GSH backbone to leverage its inherent affinity for GST P1-1. One approach centered on the incorporation of a carbamate group as an isosteric replacement of the γ-carboxylic Glu linkage in GSH. Synthesis and *in vitro* testing of **42** and **43** showed that this carbamate-replacement approach was not well tolerated [67].



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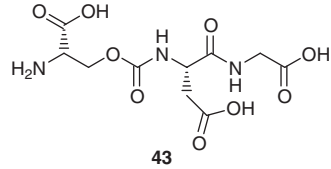
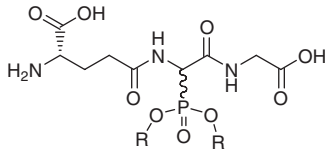
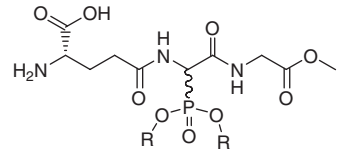


43

A similar approach consists of replacing of the methylene thiol group of the cysteine residue in **1** with phosphonate esters. Phosphonodiacid analogs **44–46** inhibited GST P1-1 ($IC_{50} = 145$, 61, and 15 μM, respectively) more potently than their corresponding monomethyl esters **47–49** ($IC_{50} = 288$, 201, and 98 μM, respectively). The SAR of these compounds indicates that an increase in the lypophilicity of the phosphono ester groups increases potency against GST P1-1 (nBu > i-Pr > Et), and that the more hydrophilic dicarboxylic acid analogs are more potent than their less hydrophilic monomethyl esters. Cellular uptake experiments using HT29 and EPG-257 cell lines confirmed that diacid analogs **44–46** did not enter the cells (unchanged extracellular compound concentration), while the monomethyl esters **47–49** were transported into the cells where their corresponding free acids **44–46** were detected. These data indicate that **47–49** act as prodrugs of **44–46** for GST P1-1 inhibition. Metabolic stability experiments of **44–49** against γ-glutamyl transpeptidase (γ-GT) did not produce proteolytic products, which indicates that the phosphonate ester groups increase peptide stability against enzymatic degradation [68].

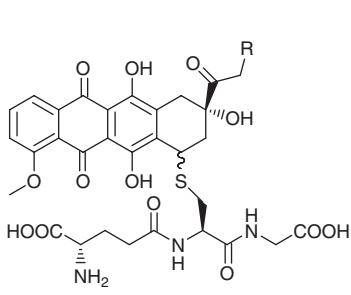


44: R = Et
45: R = i-Pr
46: R = n-Bu

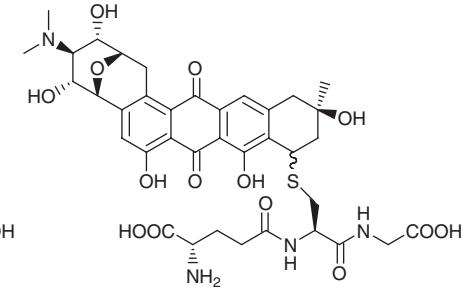


47: R = Et
48: R = i-Pr
49: R = n-Bu

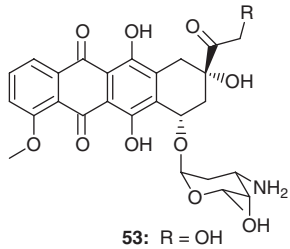
The most explored strategy for the development of GST P1-1 inhibitors involves the conjugation of the thiol group of the GSH cysteine residue with electrophilic moieties. Studies with anthracycline-based GSH conjugates **50–52** conducted in doxorubicin-resistant human breast cancer MCF-7/DOX cells, where the GST P1-1 activity is 14 times higher than in non-resistant MCF-7 cells, revealed that the epimers of **50–52** not only exhibited high GST P1-1 affinity ($K_i = 1.0–2.2\,\mu M$), but were also less cytotoxic than the respective anti-tumor anthracyclines doxorubicin (adriamycin, hydroxyldaunorubicin, **53**), daunorubicin (daunomycin, **54**), and menogaril (**55**). These studies also showed that GST P1-1 did not catalyze the conjugation of GSH to **53–55** under physiological conditions [69].



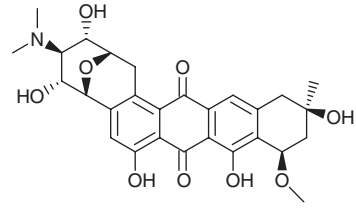
50: R = OH
51: R = H



52



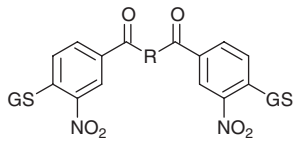
53: R = OH
54: R = H



55

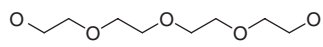
Another approach studied the influence of bivalent conjugates of GSH on GST P1-1 inhibition. It was observed that bis-glutathionyl nitrophenyl analogs **56–58** exhibited single-digit micromolar to submicromolar activity ($IC_{50} = 4.5$, 2.9 and $0.3\,\mu M$, respectively) against GST P1-1, but they were not as potent as analogs

36–38. Interestingly, the most potent analog **58** contains both the more rigid isophthalate linker and the longer propane-1,3-diol linker [65].

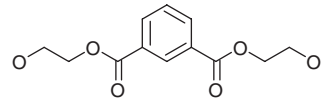


GS = Glutathionyl

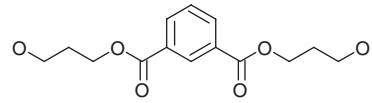
56: R =



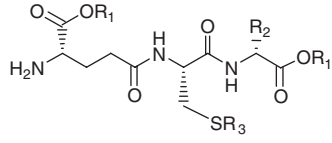
57: R =



58: R =



Another approach to the development of glutathione-based inhibitors involves analogs of GSH bearing different alkyl substituents on the cysteine sulfur. Compounds **59–63** revealed that inhibitory activity against GST P1-1 increased with the incorporation of linear alkyl chains on the cysteine sulfur (**59**, **60**: $K_i = 10$ and 1.9 μM, respectively), as well as bulky hydrophobic substituents on the glycine residue (**61**: $K_i = 0.85$ μM). When the *n*-hexyl group in **61** was replaced with a bulkier, more hydrophobic benzyl group, potency was increased 2-fold (**62**: $K_i = 0.42$ μM). Interestingly, it was found that the increase in hydrophobicity and bulkiness was moderately tolerated, as the incorporation of a naphthyl group resulted in only a 3-fold loss of activity compared with **62** (**63**: $K_i = 1.2$ μM) [70,71].

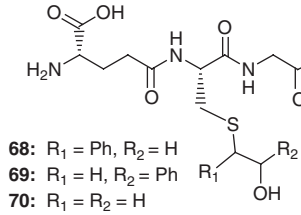


59: $R_1 = R_2 = H$, R_3 = n-hexyl
60: $R_1 = R_2 = H$, R_3 = n-octyl
61: $R_1 = H$, $R_2 = Ph$, R_3 = n-hexyl
62: $R_1 = H$, $R_2 = Ph$, R_3 = benzyl
63: $R_1 = H$, $R_2 = Ph$, R_3 = naphthyl
64: $R_1 = Et$, $R_2 = H$, R_3 = n-octyl
65: $R_1 = Et$, $R_2 = Ph$, R_3 = n-hexyl
66: $R_1 = Et$, $R_2 = Ph$, R_3 = benzyl
67: $R_1 = Et$, $R_2 = Ph$, R_3 = naphthyl

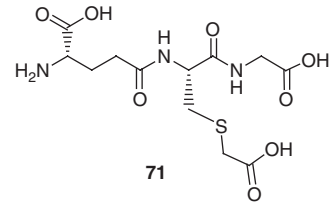
Attempts to increase hydrophilicity by incorporating a phenyl-containing 2-hydroxyethyl moiety on the cysteine sulfur resulted in further loss of potency against GST P1-1 (**68**, **69**: $K_i = 9$ and 4.7 μM, respectively). Removal of the phenyl group on the thioethyl chain resulted in a dramatic loss of activity (**70**: $K_i = 280$ μM). Replacement of both phenyl and hydroxyl groups with a more hydrophilic carboxylic acid group retained activity (**71**: $K_i = 5.5$ μM) [72]. Replacing the glycine residue of the peptidic backbone with a β-alanine residue was detrimental for activity (**72**, **73**: $K_i = 550$ and 710 μM, respectively) compared to the corresponding glycine-containing analogs (**59** and **62**, respectively).

Further studies with **60–63** showed that these compounds lacked cell membrane permeability. However, as observed in the previously discussed case of phosphonodiacid derivatives **44–46**, the diethyl ester analogs **64–67** possessed

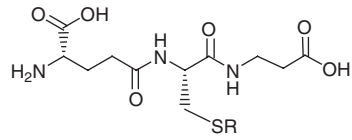
improved cell permeability. Cytotoxicity studies with **64–67** in HT-29 cells showed that these diethyl esters had IC_{50} values between 22 and 47 μM, while their corresponding diacid analogs **60–63** did not exhibit cell cytotoxicity at concentrations up to 200 μM [71].



68: R_1 = Ph, R_2 = H
69: R_1 = H, R_2 = Ph
70: R_1 = R_2 = H

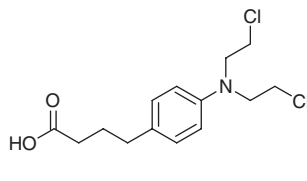


71



72: R = n-hexyl
73: R = benzyl

Owing to the favorable activity profile of **66**, which acts as a prodrug of the active species **62**, additional studies were conducted on **66** to establish its cell-based profile. It was determined that **66** potentiated chlorambucil (**74**) toxicity in cell lines expressing GST P1-1, namely HT-29, HT4-1, SK OV-3, and SK VLB. Also, while **66** alone did not prevent tumor growth in the HT4-1 xenograph model, **66** increased by 56% the tumor growth inhibitory effect of melphalan (**75**).



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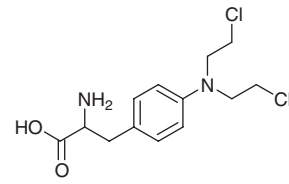
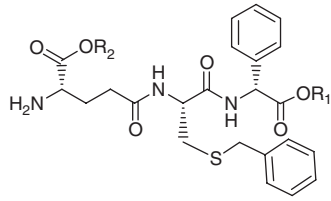


75

Metabolic stability studies with **66** in HT-29 cells showed that about 30% of **66** was converted into its phenyl glycyl monoethyl ester analog **76** after 20 min of incubation, and about 70% after 18 h. No traces of the glutamyl monoethyl ester **77** were detected at any time [71]. Although other diester analogs of **66** have exhibited promising profiles for cancer treatment (**78–82**, particularly **81**) [73], **66** (TLK199, TER199, Telintra®) is the only inhibitor in clinical trials. This compound is being tested for the treatment of myelodysplastic syndrome (MDS), a bone-marrow neoplastic disease that can eventually progress to acute myeloid leukemia (AML) [74].

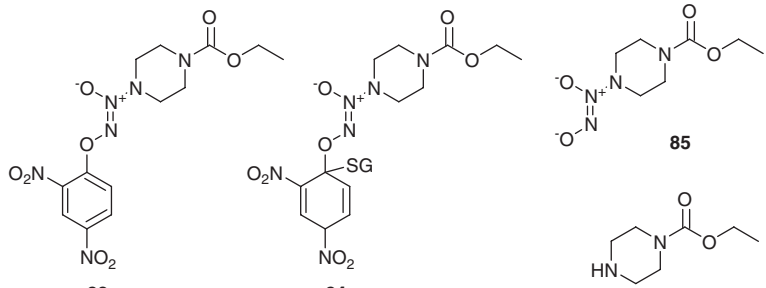


76: R_1 = Et, R_2 = H
77: R_1 = H, R_2 = Et
78: $R_1 = C_8H_{17}$, R_2 = Et
79: $R_1 = C_{12}H_{25}$, R_2 = Et
80: $R_1 = C_{14}H_{29}$, R_2 = Et
81 : $R_1 = C_{16}H_{33}$, R_2 = Et
82: $R_1 = C_{18}H_{37}$, R_2 = Et

2.3 GST P1-1-activated prodrugs

Rather than inhibiting the catalytic function of GST P1-1, GST P1-1-activated prodrugs undergo GST P1-1 catalyzed breakdown to release a molecule that is the active species responsible for the anticancer effect.

Based on the fact that nitric oxide (NO) induces apoptosis and inhibits growth in HL-60 cells, diazeniumdiolate analog **83** was developed as an NO prodrug. After establishing that **83** is stable at physiological pH, thereby ruling out the possibility of spontaneous degradation, conjugation of GSH catalyzed by GSTs (A1-1, M1-1, and P1-1) produced a Meisenheimer complex intermediate **84** that released the diazeniumdiolate intermediate **85** which, in turn, decomposed producing **86** and NO (two equivalents) [75].

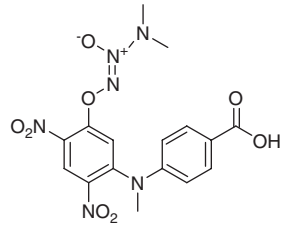


83 **84** **85** **86**

GS = Glutathionyl

Molecular modeling of **84** with GST A1-1, M1-1, and P1-1 indicated that while the complex could be accommodated in the catalytic sites of the A1-1 and M1-1 isozymes, steric constrains were expected in the catalytic site of GST P1-1. Measurements of NO release from **83** revealed that GST P1-1 weakly catalyzed the conjugation of GSH producing a slow NO release, confirming molecular modeling predictions. Cell growth inhibition experiments showed that **83** inhibited growth of HL-60 and U937 leukemic cells with submicromolar activity (IC_{50} = 0.5 and 0.3 μM, respectively). In a PPC-1 cancer cell xenograph model in NOD/SCID mice, **83** inhibited tumor growth when given at 4 μmol/kg, i.v., three times/week [75].

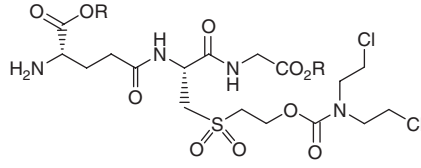
Molecular modeling of **84** suggested that it would be beneficial to replace the piperazine ring with a smaller amino group to improve a fit in the GST P1-1 catalytic site. With this in mind, analog **87** was developed. Measurements of NO release indicate that **87** releases more NO in the presence of GST P1-1 than in the presence of GST A1-1 (30% and 5%, respectively), validating the molecular modeling predictions. Compound **87** (3.36 mg/kg, twice/week) significantly delayed tumor growth when administered to SCID mice implanted with A2780 human ovarian cancer cells. After 45 days of treatment, no significant body weight loss was observed and renal activity remained normal as indicated by unchanged serum creatinine levels [76].



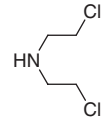
87

Another class of GST P1-1-activated prodrugs includes analogs of GSH with a β-linked alkylating agent-containing sulfonyl group. GST P1-1 recognizes the GSH backbone of this class of compounds (e.g., **88**, **89**, **93–96**). After being properly oriented in the enzyme, the Tyr 7 residue abstracts one of the alpha protons next to the sulfonyl group promoting release of the alkylating agent (**90–92**) and formation of a vinyl sulfone derivative of the parent compound [4,51,77–79] by a β-elimination/decarboxylation sequence.

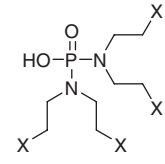
In vitro incubation of **88** with GST P1-1 and GST A1-1 showed that this compound was selectively metabolized by GST P1-1. Cell-based toxicity assays using GST P1-1 transfected MCF-7 cells (2-h exposure) revealed that the phenyl-containing analogs **94–96** were more cytotoxic (IC_{50} ~10 μM) than the more hydrophilic analogs **88** and **89** (IC_{50} = 86 and 69 μM, respectively) [4,51]. Cytotoxicity studies of **96** in 11 human malignant cell lines showed that this compound had IC_{50} values ranging from 6 to 67 μM [80]. Xenograph experiments using five cell lines in nude mice showed that **96** was more effective when administered at 200 mg/kg, q.d. × 5, than at a single 400 mg/kg dose. Tumor growth was delayed significantly in the MX-1 and DLD-2 cell lines [80].



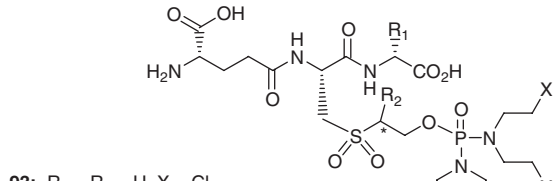
88: R = H
89: R = Et



90



91: X = Br
92: X = Cl



93: $R_1 = R_2 = H$, X = Cl
94: $R_1 = H$, $R_2 = Ph$, X = Cl (*)
95: $R_1 = Ph$, $R_2 = H$, X = Br
96: $R_1 = Ph$, $R_2 = H$, X = Cl

* stereochemistry not specified

From this pro-drug class, **96** (TLK286, TER286, Telcyta®) is in clinical trials for the treatment of cancer.

3. CONCLUSIONS

Cancer is one of the most devastating diseases. Even though a few cancer types are associated with a particular gender (e.g., ovarian cancer, prostate cancer), the onset usually takes place regardless of age, sex, and race. Although cancer prevention remains the ideal approach to eradicate the disease, the best second option is early detection.

The overexpression of GSTs in some cancer cells, particularly of GST P1-1, offers an opportunity to detect and treat some cancer types (e.g., ovarian cancer). Recent developments in the design of small molecules that either inhibit the catalytic activity of GST P1-1 or use GST P1-1 catalytic site to release the actual anticancer agent, have shown promising results in preclinical studies, with the graduation of **66** and **96** as potential anticancer drug candidates currently undergoing clinical trials.

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CHAPTER 21

Recent Advances in the Medicinal Chemistry of Histone Deacetylase Inhibitors

Dominic Brittain, Hilmar Weinmann and **Eckhard Ottow**

Contents

1. INTRODUCTION

Epigenetic changes, such as covalent modifications of chromatin, are increasingly implicated in the onset and progression of cancer and other diseases [1]. These covalent modifications are thought to constitute a histone code [2] or alphabet [3], which determine various regulatory functions, such as gene expression. As a result, significant work is underway to understand the regulatory role of such post-translational modifications and to determine ways of controlling them to offer new opportunities for therapeutic intervention [4,5].

One of the most-studied covalent modifications is the acetylation of the lysine residues of histone tails. The acetylation state of lysines of nucleosomal histones modulates chromatin structure and regulates gene transcriptional activity. The balance of lysine acetylation is controlled by the antagonistic action of two enzyme families: histone deacetylases (HDACs) and histone acetyltransferases (HATs). In humans there are essentially three main HDAC subclasses [6].

Bayer Schering Pharma AG, Medicinal Chemistry, D-13342 Berlin, Germany

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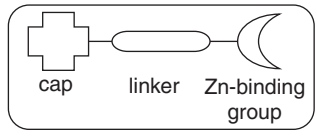
Class I enzymes, HDACs 1, 2, 3 and 8, range in size from 42 to 55 kDa, share homology in their catalytic sites, are ubiquitously expressed and are located primarily in the nucleus. Class II deacetylases (HDACs 4, 5, 6, 7, 9 and 10) range in size from 120 to 130 kDa, show more tissue specificity and can transfer in and out of the nucleus. The class IIa subset of HDACs 4, 5, 7 and 9 shares homology in the C-terminal catalytic domain and an N-terminal regulatory domain, while HDAC6, which makes up the class IIb subset along with HDAC10, has two deacetylase domains. HDAC11 is another member of the HDAC enzyme family which was recently cloned and characterized [7]. It bears low similarity with class I or class II and therefore is often classified on its own. Sirtuins 1–7 are the 7 human homologs of the yeast Silent Information Regulator (SIR2) family of proteins and constitute the third class of histone deacetylases, although deacetylase activity has not been reported for all members [8]. These class III enzymes are dependent on NAD^+ for activity, whereas class I and II HDACs and HDAC11 use zinc-dependent mechanisms.

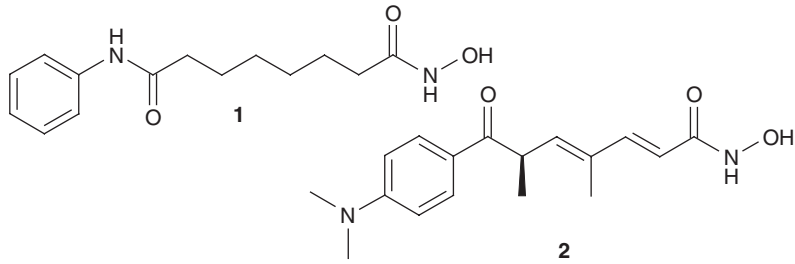
Although histones are the most common HDAC substrates, the number of acetylated non-histone proteins is rapidly expanding and is likely to offer further nuances in controlling biological effects [9,10]. A prominent example is HDAC6, which has been demonstrated to deacetylate tubulin [11] and is also involved in the deacetylation of other cellular targets like Hsp90 [12].

As a result of the role of HDACs in direct and indirect regulation of gene expression, the HDAC inhibitor (HDACi) field has continued to attract considerable research interest, particularly for oncology indications [13,14]. Numerous HDACis can be found throughout all stages of the development pipelines of pharmaceutical companies [15], culminating recently in the first approved HDAC inhibitor. The approval of SAHA (Vorinostat) **1** by the FDA for treating patients with cutaneous T-cell lymphoma (CTCL) on or following two systemic therapies in October 2006 marks a significant milestone for the HDAC inhibitor field and is certain to spur further activities [16].

2. ZINC-DEPENDENT HDAC INHIBITORS

Crystal structures of a histone deacetylase-like protein (HDLP) and HDAC8 have confirmed a general pharmacophore model for HDAC inhibitors, comprising a cap joined by a hydrophobic linker to a zinc-binding group (ZBG). This model is exemplified by SAHA and the natural product HDACi Trichostatin A (TSA) **2**.





Since the identification of hydroxamic acids as potent bidentate ZBGs, an enormous range of hydroxamic acid inhibitors based on this model has been developed and is described in numerous reviews and therefore will not be dealt with in depth here [17]. Instead, the focus of this report will be on efforts to improve on these ''1st generation'' inhibitors, specifically: to improve biological and physicochemical characteristics, such as pharmacokinetics and bioavailability; and to achieve isoform selectivity.

2.1 Improvement of biological and physicochemical characteristics

2.1.1 Non-hydroxamates

Since hydroxamic acids have been associated with problems such as poor pharmacokinetics and severe toxicity, there is much effort to replace this functionality in inhibitors [18,19]. In addition, the large contribution to total binding that the hydroxamate functionality confers may also increase the difficulty of generating isoform selectivity, as seen in the matrix metalloproteinase (MMP) field [20]. Computational efforts to rank ZBGs showed the difficulty of replacing hydroxamic acids [21]. The study indicated that the main problem for hydroxamate replacement is that bidentate zinc-binding is more advantageous than monodentate binding, but the geometric constraints for a bidentate binding mode are very stringent. Furthermore, despite the thiophilicity of zinc, the HDAC catalytic core behaves predominantly as a hard-interacting core, reducing the scope for simple sulfur substitution.

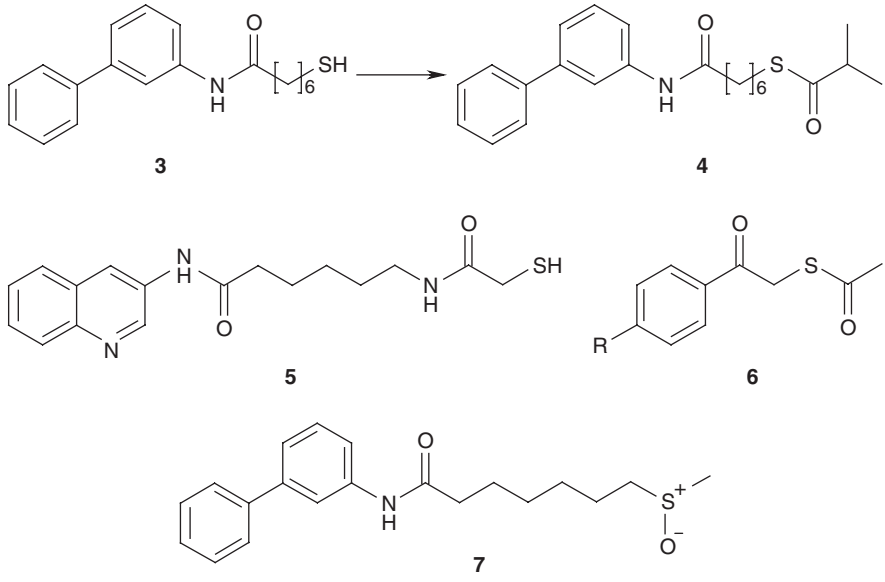
Nonetheless, analogously to the fact that sulfur-containing compounds are well known to bind to zinc-containing enzymes in the ACE and MMP fields, there is a growing number of sulfur-containing non-hydroxamate HDACis. During an early systematic study of ZBGs for HDACs to identify potent non-hydroxamate SAHA-like HDAC inhibitors, thiols such as **3** were found with an HDAC enzyme IC_{50} of 75 nM [22]. Although such compounds showed low activity against NCI-H460 cells, this activity could be improved by temporarily masking the thiol functionality as a thioester in order to improve cell permeability, as in **4** ($EC_{50} = 2.8\,\mu M$).

In a simultaneous attempt to screen for further non-hydroxamate ZBGs, this team focussed on potentially bidentate ZBGs and identified a SAHA-like compound with a mercaptoacetamide functionality, which had an HDAC enzyme

IC_{50} of 0.39 μM and was hoped to mirror the long-lived and low-toxicity mercaptoacetamides in the MMP field [23] The same mercaptoacetamide was also reported along with quinolinyl-capped mercaptoacetamides, such as **5** (IC_{50} = 48 nM) [24].

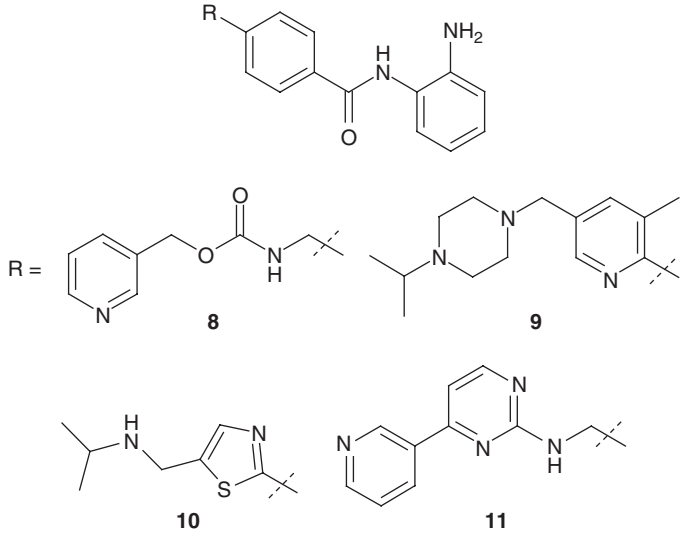
Interestingly however, a further publication on mercaptoacetamides reported an IC_{50} of only 1.1 μM for the same 3-quinolinyl-capped derivative **5**, in addition to similarly potent analogs in which the amide carbonyl group is transposed to give a mercaptoethylamide ZBG [25]. SAHA-like mercaptoacetamides were also found during a separate search for sulfur- and carbonyl-containing analogs, but here an α-mercaptoketone ZBG proved to be more active, with an HDAC enzyme IC_{50} of 0.15 μM. Surprisingly, the acylated derivative had a similar enzyme IC_{50} of 81 nM, for which the authors offer no explanation but state that it was not due to inadvertent hydrolysis [26]. A range of acylated and free α-mercapto ketones and acetates have also been claimed in various patents, such as analog **6** with typical HDAC enzyme IC_{50}s of < 1 μM [27].

Different oxidation states of sulfur have also been explored, particularly sulfones and sulfonamides as transition state analogs of lysine deacetylation, but without much success. The monodentate SAHA-like methyl sulfoxide **7** proved most potent, but still with an enzyme IC_{50} of only 48 μM and indications of HDAC/TDAC selectivity in cellular assays [28].

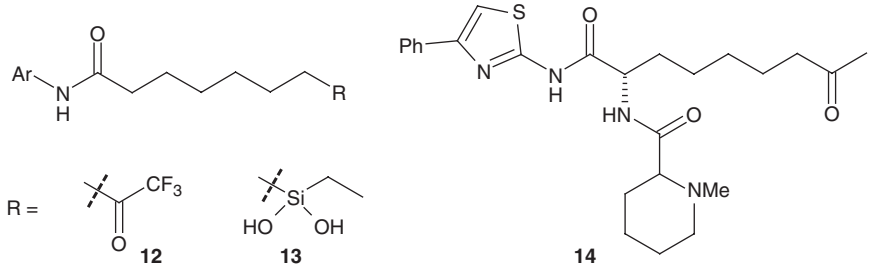


Benzamides e.g. MS-275 **8** are one of the more common hydroxamic acid alternatives but are often less potent. Recent exceptions to this pattern include substituted pyridyl [29] and thiazolyl [30] benzamides, such as **9** and **10** with HDAC1 enzyme IC_{50}s of 19 nM and 29 nM, respectively; and series of benzamides substituted with other heterocycles, such as indazoles [31] and benzo [1,2,4]thiadiazines [32], many with HDAC2 IC_{50}s of < 50 nM. The addition of

planar substituents (e.g. thiophene) *para* to the benzamide functionality has been claimed to improve potency, but the biological data provided states only that enzyme IC_{50}s are less than 1 μM [33].



By analogy to the initial work on trifluoromethyl ketones as ZBGs in their hydrated forms [34], as in compounds **12**, silanol versions **13** have been developed. HDAC1 and 8 assays are mentioned in the patent application, but activities are not stated, nor is there any indication of whether the compounds suffer similar metabolic liabilities [35].



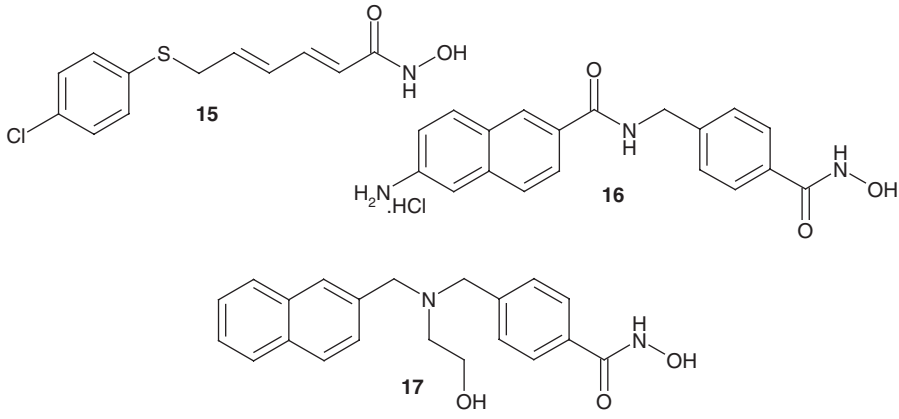
Various naturally derived cyclic tetrapeptide HDAC inhibitors have also been identified without hydroxamate ZBGs. FR 23522 contains an hydroxyketone as putative ZBG in the form of a 2-amino-9-hydroxy-8-oxodecanoic acid (Ahoda) sidechain [36] and the Azumamides contain carboxamides or carboxylic acids as ZBGs, the most potent of which has an enzyme IC_{50} of 45 nM [37]. Citing the main disadvantage of the cyclic tetrapeptides to be their chemical intractability, a series of simplified Apicidin analogues was developed. The most characterized compound, **14**, has a methyl ketone as a ZBG and an HDAC enzyme IC_{50} of 55 nM. Interestingly, profiling against a subset of HDAC isoforms indicated an

unusual selectivity (for HDACs 1, 2, 3 and 6 over 4, 5, 7 and 8) that does not match the typical classification of the isoforms [38].

2.1.2 Hydroxamates

Although still containing a hydroxamic acid ZBG, TSA-like mimics **15** in which the potentially cleavable amide functionality is replaced by an aromatic thioether were shown to be metabolically more stable than SAHA and TSA, but still with promising enzyme IC_{50}s of ca 310 nM [39]. Using the same logic in the hope of generating hydroxamates with further improved *in vivo* properties, replacement of sulfur with oxygen and saturation of the sidechain yielded simple analogs with enzyme IC_{50}s of ca 20 nM [40].

Believing that poor solubility could be a contributor to the toxicity observed after administration of hydroxamic acid **16** in mice, more hydrophilic analogs were prepared by replacement of an arylamide group with a (2-hydroxyethyl)(arylalkyl)amino group, analogously to that found in LAQ824. Analog **17** exhibited improved solubility without loss of potency but showed toxicity similar to the parent compound against a normal fibroblast cell line [41].

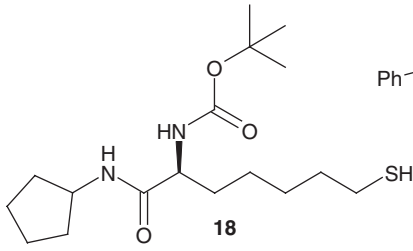


2.2 Isoform selectivity

The vast majority of HDACis show no selectivity between the class I, II and IV HDAC isoforms. Some notable exceptions include the largely class I-selective benzamides MS-275 and MGCD-0103 **11** [42], hydroxamic acid SK7041 [43] and disulfide FK228 [44]. In addition to those that show class selectivity, there is an even smaller but growing subset of HDACis that can distinguish between isoforms within one class. This subset is epitomised by the HDAC6 selective inhibitors, Tubacin [45] and the series of aliphatic thiols developed by Suzuki and Miyata [46], and the HDAC8-selective inhibitor SB-379872 [47]. The development of these and other isoform-selective compounds is slowly enabling the dissection of the multiple molecular functions of the HDACs. It is hoped that alongside

classical genetic approaches, such chemical genetic probes can enable the determination of desirable isoform inhibition profiles and provide a rationale for selective HDACi clinical candidates, such that for a given disease, only the most relevant isoforms need be targeted [48].

A greater pool of structural data should facilitate the pursuit of inhibitors with such class- and isoform-selectivity [49]. The crystal structure of a bacterial class II HDAC homolog confirms similarity with class I HDACs around the zinc atom in the active site but also reveals differences that might be exploitable. These include differences at the rim of the active site channel, two co-planar phenylalanine residues in the channel itself and an internal cavity that may serve as the acetate exit channel [50]. In the absence of structural data, homology modeling may also enable rational approaches to selectivity. Docking of known HDAC inhibitors into homology models of 4 class I HDACs confirmed that shape and charge differences around the opening of the active site were key to rationalising the inhibitors' selectivity profiles [51].



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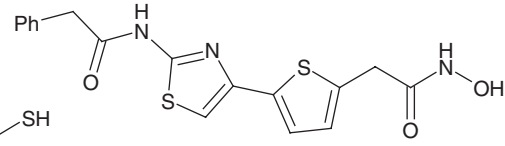

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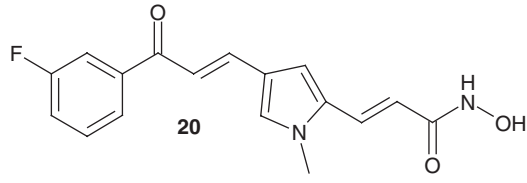

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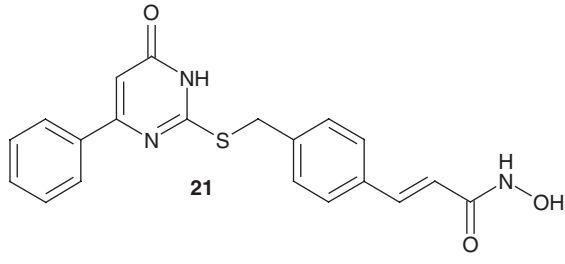

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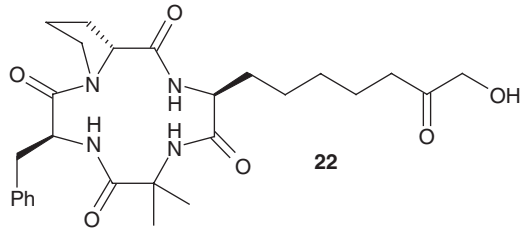


22

One of the most potent, selective inhibitors to date comes from a series of aliphatic sulfides which has been optimized for HDAC6 selectivity over HDACs 1 and 4. The best compound, **18**, exhibits an IC_{50} of 29 nM against HDAC6 and ca. 35–40-fold selectivity, but as with the original thiol compounds, the sulfur atom needs to be acylated to achieve cellular activity [46].

A range of potent biaryl hydroxamates has been claimed, certain of which exhibit excellent selectivities for HDAC8 versus HDAC1. These compounds include thiophene-based **19** with enzyme IC_{50}s of 41 nM versus $>100\,\mu M$, respectively [52].

Using a maize HDAC system, aroyl-pyrrolyl-hydroxamides (APHA) had been shown to exhibit 7–78-fold class IIa selectivity when appropriately modified. Continued fine-tuning of the APHA inhibitors, such as *meta*-fluorine-substituted **20**, enabled the generation of an even more selective HDAC inhibitor, with HD1-A IC_{50} of 0.22 μM, 176-fold selectivity and which inhibited human HDAC4 but not HDAC1 [53].

The same group also developed a series of uracil-containing hydroxamates, the most potent of which exhibited ca. 10 nM IC_{50}s against maize deacetylases [54]. However, highlighting the difficulties of evaluating isoform selectivities across species, while the compounds proved to be devoid of class selectivity in the maize HDAC system, two particular examples showed some selectivity for human HDAC1 versus HDAC4. For example, cinnamyl hydroxamate **21** showed 94% HDAC1 inhibition versus 14% HDAC4 inhibition, both at 5 μM concentration.

Making use of the cyclic tetrapeptide core seen in several naturally occurring HDAC inhibitors, such as Chlamodycin, the irreversibly binding epoxy ketone ZBG was replaced with a variety of functionalities [55]. Replacement with other potentially reactive functional groups e.g. epoxides, bromoketones and aldehydes yielded compounds with low- and submicromolar HDAC IC_{50}s, but most promising was hydroxymethylketone compound **22** with submicromolar IC_{50}s against HDACs 1, 4 and 6 and selectivity for HDAC4 over HDAC1 (ca. 4-fold) and HDAC6 (ca. 15-fold). Related compounds have been claimed in a patent by many of the same authors for the treatment or prevention of HDAC 1, 4 or 6-related diseases [56].

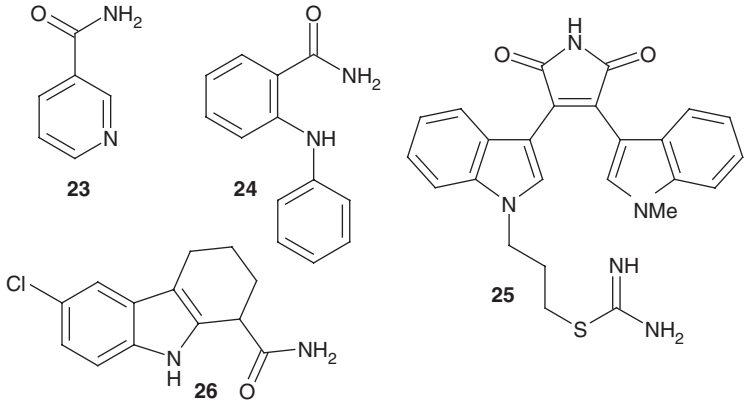
Using HDACs 1, 4 and 6 to represent the major HDAC classes, a series of hydroxamates bearing cyclic amide/imide caps was tested, typically showing submicromolar enzyme inhibition [57]. Early indications of a structure-selectivity relationship are presented, but some of the key data is missing due to a significant typographical error in one of the results tables. Nonetheless, by using compounds with different selectivities in a LNCaP cell growth inhibition assay, it was claimed that HDAC1 inhibition correlated most strongly with cell growth inhibition.

3. NAD-DEPENDENT HDAC MODULATORS

Although the localisation patterns of some of the Sirtuins (class III HDACs) and their unique NAD-dependent deacetylation mechanism are known [58], less is understood about their functions and targets when compared to other HDACs [59]. The field of small molecule Sirtuin modulators is also correspondingly less advanced, because this alternative mechanism renders the zinc-dependent

deacetylase inhibitor pharmacophore model inapplicable. The most extensively studied of the mammalian Sirtuins, SIRT1, modulates gene expression profiles in target tissues via regulation of transcriptional co-regulators or by directly interacting with transcription factors. It is currently believed that activating SIRT1 may lead to new therapeutic approaches for metabolic and age-related syndromes, Alzheimer's disease and stroke whereas inhibition of SIRT1 might play a role for future cancer therapies [60]. As with the other HDAC classes, the targets of SIRT regulatory deacetylation are not limited to histones For example, SIRT1 catalyses the deacetylation of p53 [61] and SIRT2 deacetylates α-tubulin [62].

In the deacetylation reaction of SIRTs, NAD^+ is hydrolysed to release nicotinamide **23** [63]. Based on this mechanism and the fact that **23** is the physiological sirtuin inhibitor (Sir2 and SIRT1 $IC_{50} = 50\,\mu M$), it has been proposed that **23** inhibits SIRTs by binding to a conserved pocket adjacent to the NAD^+ binding pocket, thereby blocking NAD^+ hydrolysis. **23** is under clinical investigation for cancer to determine whether it enhances response to radiotherapy in patients with cancer of the larynx or bladder [64]. Using **23** as a starting point, 2-anilinobenzamide **24** was found by screening a library enriched with nicotinamide and benzamide functionalities [65]. Benzamide **24** showed about 4-fold and 14-fold selectivity for SIRT1 over SIRT2 and SIRT3, respectively (IC_{50} for SIRT1 = 17 μM; SIRT2 = 74 μM; SIRT3 = 235 μM). In addition, **24** showed no inhibition of class I and class II HDACs at a concentration of 1000 μM. Focussing instead on the adenosine portion of NAD^+, ATP-competitive kinase inhibitors were screened to generate bisindolylmaleimides (BIMs) as potential sirtuin inhibitor leads [66]. BIM **25** proved to be the most potent with IC_{50}s of 3.5 and 0.8 μM against SIRT1 and 2, respectively. High-throughput screening for SIRT1 inhibitors yielded a series of indoles, most promising of which was EX-527 **26** with an IC_{50} of 0.038 μM. Further experiments indicated that **26** occupies the nicotinamide binding pocket and also posseses acceptable ADME properties [67]. Subsequent binding mode studies indicate the importance of the carboxamide and indole nitrogen for hydrogen-bonding interactions [68]. Various heterocycle derivatives, such as thiotriazoles, have been claimed as SIRT1 and 2 inhibitors by some of the same team that developed **26** [69].



A large number of patent applications has been filed, most recently describing imidazothiazoles [70], oxazolopyridines [71], benzimidazoles [72], benzothiazoles [73] and imidazopyridines [74] as sirtuin modulators, however it is not yet possible to determine which compound classes will prove most promising. Overall, due to their potential applications as new drug candidates for various indications, the class III HDAC inhibitors are currently a rapidly growing field of interest.

4. CONCLUSION

The recognition of the central importance of chromatin in orchestrating gene activation and silencing has fuelled an explosion in basic research into epigenetics. As understanding of the biological consequences of chromatin's chemical modifications has grown, so too have the pharmaceutical industry's efforts to translate this knowledge into medicines. The FDA approval of SAHA for CTCL has shown HDAC inhibition to be a valid approach in a clinical setting, spurring on progress in the field. Key to this progress will be greater understanding of the pleiotropic actions of HDAC inhibitors and the subsequent development both of compounds with optimized profiles and of markers that enable the measurement and prediction of their clinical potential.

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CHAPTER 22

New Developments in Antibacterial Drug R&D

Roger Frechette

Contents

1. INTRODUCTION

1.1 The urgent need for new antibacterial drugs

Bacterial resistance to antibiotics is an emerging public health crisis. The prevalence of pathogens resistant to currently available antibiotics continues to grow annually. Two million patients in the U.S. acquire an infection during a hospital stay and approximately 90,000 of these patients die each year as a result of the infection [1]. More than 70% of hospital-acquired infections are now resistant to

MaxThera, Inc., 100 Cummings Center, Suite 419E, Beverly, MA, USA

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commonly used drugs. The greatest medical need is for successful treatments of serious Gram-positive infections, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), with oral formulations; and increasingly prevalent resistant Gram-negative infections in hospitals due to *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. In early 2006, the Infectious Diseases Society of America (IDSA) released a ''hit list'' of the six top priority, most dangerous drug-resistant microbes [2]. First in order of these microbes is methicillin-resistant *S. aureus*, followed by *E. coli*, *Klebsiella* spp, *A. baumannii*, vancomycin-resistant *Enterococcus faecium* and *P. aeruginosa*. Gram-negative bacilli cause 60 percent of nosocomial pneumonias, comprising six of the seven most frequently identified pathogens: *P. aeruginosa* (17%), *S. aureus* (16%), Enterobacter species (11%), Klebsiella species (7%), *E. coli* (6%), *H. influenzae* (6%), and *Serratia marcescens* (5%) [3]. Of note, the proportion of *K. pneumonia* isolates resistant to oxyimino-β-lactams (third-generation cephalosporins) has risen by 47 percent between 2002 and 2003 [4]. *Acinetobacter* is a growing cause of hospital-acquired pneumonia and mortality rates range from 20 percent to 50 percent. The incidences of hospital-acquired pneumonia caused by *P. aeruginosa* have nearly doubled from 9.6 percent in 1975 to 18.1 percent in 2003. Yet, the IDSA report shows a steady 20 year decline in the number of new FDA-approved antimicrobials with only 10 new antibiotics approved since 1998 [1].

1.2 A brief history of antibiotics

Antibiotics played a major role in defining and developing the nascent pharmaceutical industry of the 1940s and 1950s to a highly successful, scientifically driven enterprise producing a steady stream of life-saving drugs and contributing significantly to expanding human life-expectancy and improving quality of life throughout the developed world [5,6]. From the early 1970s through the middle 1990s, the productivity of antibacterial drug R&D groups enabled the production of a steady stream of new antibiotics to treat bacterial infections. The most common strategy employed throughout this period was to make incremental changes to the chemical structures and formulations of existing drugs to produce next generation drugs with incremental improvements in properties. The changes typically addressed specific issues such as improved potency, spectrum of activity, toxicity profile or a more convenient dosing regimen. One of the important outcomes of this approach was the development of built-in obsolescence for new antibiotics. In addition to improving desired properties, each new product cycle afforded a brief delay before clinical resistance could be built up. Some companies met the resistance issue head on by combining an antibiotic with an inhibitor of a biological target responsible for resistance – with Augmentin being the most successful product derived from this approach [1]. This strategy extended the duration of full effectiveness in the clinic prior to significant resistance emergence, but few paired drugs have been successfully brought to market.

1.3 Antibacterial R&D has taken a downturn

In the face of the growing threat to human health from resistant bacterial infections, the need for new, effective treatments is critical [7,8]. However, the number of new antibacterial drug launches has dropped steadily since the 1980s. Only six new antibacterial agents were approved in the four-year period ending in 2002, with half as many new drug introductions expected in the subsequent four years ending in 2007 (see Figure 1).

The pharmaceutical enterprise currently has fewer than 30 new antibacterial drugs in clinical development for various indications, with most being revised versions of old drugs [1,9]. This is in stark contrast to development efforts in other disease areas such as cancer chemotherapy with nearly 650 drug candidates in active development [10]. There are also more than 145 drug candidates in development for cardiovascular diseases and nearly 80 for HIV/AIDS [8]. The low numbers of antibacterial drug candidates reflects patent expirations that have fueled the growth of generics in this market sector as many of the key players in the pharmaceutical industry have deemphasized or eliminated antibacterial R&D programs since 2000. There are approximately 25 publicly traded pharmaceutical companies worldwide with market caps ranging from < $10 million to $200 billion that have active antibacterial R&D programs [11,12]. Roughly 30 additional closely held companies also are working in this area. More than 90% of the new drugs being developed by these companies are based on older classes of compounds, and thereby failing to address the drug-resistance problem (see Table 1).

The near term prospects for new antibacterial product launches that could provide a durable solution to the problems of resistance are not encouraging [1]. Daptomycin, expected to launch in Japan in 2007, is the only new/late stage entry with a novel chemical structure and mechanism of action. While resistance emergence is not yet a major issue for this drug, it is limited to hospital infections of Gram-positive infections. A potentially interesting new candidate nearing NDA filing is oritavancin, a new glycopeptide. Although structurally related to

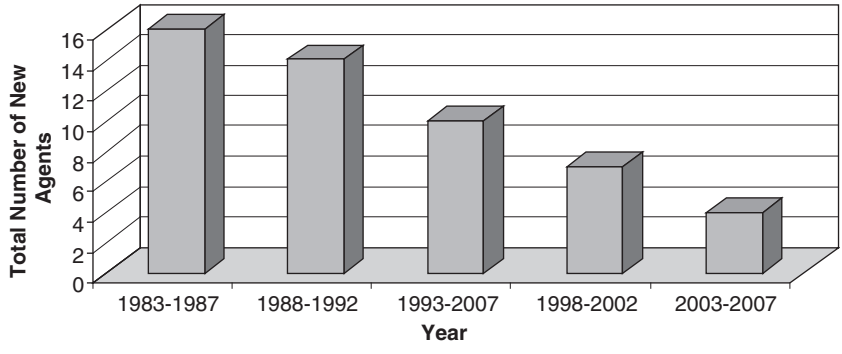


Figure 1 Approved antibacterial drugs: 1983–2007.
Source: David Shlaes in J.L. Fox, *Nat. Biotechnol.*, 2006, **24** (12), 1521

Table 1 Selected antibacterial drug candidates in late phase III clinical trials

Compound	Class	Estimated launch date			In development for
		US	EU	Japan	
Daptomycin	New	Launched	Launched	2007	SSSIs caused by G+ pathogens including MRSA
Tigecycline	Tetracycline	Launched	Launched	2007	SSSIs caused by G+ pathogens including MRSA
Doripenem	Carbepenem	2007	2008	Launched	Nosocomial infections caused by *S. aureus*, *P. aeruginosa*, *S. pneumoniae*
Dalbavancin	Glycopeptide	2007	2008	2009	SSSIs caused by G+ bacteria including MRSA
Ceftobiprole	Cephalosporin	2008	2009	2010	Nosocomial pneumonia and cSSSIs caused by MRSA
Cethromycin	Ketolide	2008	2009	2010	RTIs including those caused by macrolide and penicillin-resistant strains
Telavancin	Glycopeptide	2007	2008	2009	cSSSIs caused by G+ bacteria including MRSA
Iclaprim	DHFR inhibitor	2008	2009	2010	cSSSIs caused by G+ bacteria including MRSA

Source: Commercial Insight: Antibacterials DMHC2253© Datamonitor (Published 12/2006).
SSSIs – skin and skin structure infections; cSSSIs – complicated skin and skin structure infections; RTIs – respiratory tract infections.; G+ – Gram positive.

vancomycin, oritavancin appears to have a modified mechanism of action that currently confers activity against many resistant Gram-positive bacteria. Of the remaining candidates, assuming successful regulatory clearance for product launch, only Doripenem and Cethromycin are seeking indications for treating increasingly problematic Gram-negative infections (see Table 1).

With the growing rate of resistance, including increasing resistance to vancomycin, it is critical that this health risk be addressed with decisively new tactics. A substantial unmet medical need for new antibacterial drugs remains: to develop new drugs that function by inhibiting bacterial biochemical targets that have not been targeted previously. While the importance of new targets and new inhibitors has long been recognized, the discovery and successful development of truly novel antibacterial agents has proven to be extraordinarily difficult [13].

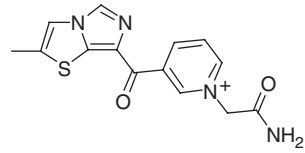
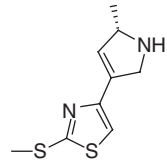
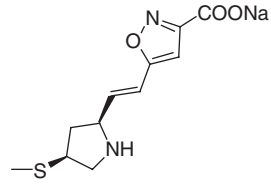
2. UPDATES OF ESTABLISHED CLASSES OF ANTIBIOTICS

A recent review has reported on the current status of antibacterial drugs in development specifically for Gram-positive infections [14]. This report provided an encouraging view of new treatments under development for this very important category of infectious disease. While acknowledging that the discovery of new pharmacophore templates has slowed in recent years, it has not stopped; and the development of new iterations of older drug classes continues to afford analogs having therapeutic advantages. In the antibacterial drug R&D realm, designing new versions of known drugs has been the time-tested approach to improving safety, spectrum of treatment and resistance avoidance. This approach to new treatments is being threatened increasingly by the spread of resistance both in hospitals and in the community, but remains a viable path to meeting the near term medical need. New analogs that drew attention at the 46th ICAAC in San Francisco in September 2006 are as follows.

2.1 β-Lactams

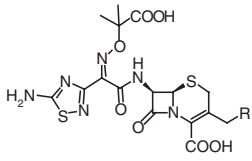
Penicillin, the first β-lactam antibiotic, marked the beginning of a revolutionary period of successful treatment of infectious disease and, in many respects, the beginning of the modern pharmaceutical industry. With more than 6 decades of history, the β-lactam core remains an important antibacterial pharmacophore.

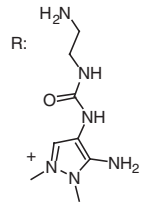
ME1036 (CP5609) **1** is a new broad-spectrum parenteral carbapenem which has excellent activity against multiple drug-resistant Gram-positive cocci, including methicillin-resistant staphylococci (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and drug-resistant *Enterococcus faecalis* [15]. Introduction of the imidazo[5,1-*b*]thiazole group in the 3-position of the carbapenem nucleus correlates with high affinities to penicillin-binding proteins, possibly accounting for the unusual activity against multi-drug-resistant cocci [16,17]. ME1036 was superior to vancomycin in a MRSA endocarditis model [18] and superior to both vancomycin and linezolid in a mouse abdominal infection modes infected with MRSA or vancomycin intermediate-susceptible *S. aureus* (VISA) [19].

		R
1	ME1036	
2	SMP-601	
3	P91022	

Another parenteral carbapenem, SMP-601 (PZ-601, SM-216601) **2**, has demonstrated broad-spectrum antibacterial activity with MRSA and PRSB MICs comparable to vancomycin, linezolid, meropenem and imipenem with activity similar to other carbepenems against vancomycin-resistant enterococci (VRE) [20–22]. Antibacterial activity was comparable to comparator carbapenems against Gram-negative species typically causative of community acquired pneumonia, complicated skin and skin structure infections and intra-abdominal infections [23]. Only imipenem was found to be superior against extended spectrum β-lactamase ESBL-producing strains. *In vivo* pharmacodynamic experiments concluded that the %time > MIC, the PK/PD parameter that best correlates with efficacy for carbapenems, is similar to other carbapenems as observed from efficacy data in numerous infection models [19,20,24,25]. A third new carbapenem, P91022 **3**, which had excellent antibacterial activity against respiratory tract pathogens, was designed for increased *in vivo* stability. The ethylene bridge between the pyrrolidine and isoxazole rings and the carboxylate salt on the isoxazole ring were incorporated specifically to improve stability to renal dehydropeptidase I, an important factor in hydrolytic inactivation of carbapenems [26]. PK experiments in rat, dog and monkey demonstrated PK profiles superior to meropenem. In addition, GABA receptor binding assay results provided evidence supporting the safety of P91022 against convulsive side effects often observed with carbapenems.

FR264205 **4** is a new parenteral cephalosporin designed specifically for anti-*Pseudomonas aeruginosa* activity [27]. The MIC_{90} against 193 *P. aeruginosa* clinical isolates was 1 μg/ml with a range of 0.25–4 μg/ml, 8–16-fold lower than for ceftazidime, imipenem, and ciprofloxacin [28]. FR264205 MICs were not affected by expression of efflux pumps and were similarly unaffected by β-lactamase-producing strains, except for ESBL producers. Resistance characterization demonstrated very low propensity for inducing resistance and efficacy in treating *P. aeruginosa* infections in pulmonary, urinary tract and burn wound infection models was significantly superior to the comparators. The excellent MIC results for FR264205, particularly against β-lactamase-producing strains was attributed to an extended SAR effort aimed at increasing the steric effect on the 3-position of the cephem nucleus culminating with the 4-position side chain on the pyrazolium ring. Key analogs (**5** and **6**) in the SAR progression illustrate the trend, with ceftazidime as a comparator, in an experiment with an AmpC β-lactamase over-producing strain (FP 1380) [29].





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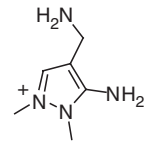

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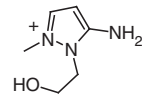


6 FK518

Ceftazidime

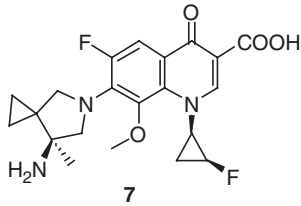
	Strain				
	FP 1380	2	8	16	128
MIC (μg/ml)					
	PAO1 Δ AmpD/ PAO1	1/0.5	5/0.5	4/0.5	64/2

FP 1380: AmpC β-lactamase over-producing clinical isolate
PAO1 Δ AmpD/PAO1: AmpD-deleted AmpC β-lactamase over expression strain

2.2 Quinolones

A session at the 46th ICAAC, comprised of 18 posters, was dedicated to the introduction of DC-159a **7**, a new oral respiratory quinolone [30]. The development of DC-159a is continuing the trend, with newer quinolone derivatives, of improving Gram-positive potency to achieved balanced broad-spectrum activity. In antibacterial tests, DC-159a had potent MICs against Gram-positive pathogens comparable to that of the newer fluoroquinolones and potent activity against multi-drug-resistant *Streptococcus pneumoniae* similar to that of gemifloxacin [31–35]. Time-kill experiments demonstrated more potent killing of quinolone-resistant streptococci relative to other quinolones [36]. Against pneumococci, the

killing rates for DC-159a were similar to four quinolone comparators, but with improved killing at earlier timepoints [37]. The antibacterial potency is correlated to potent inhibition of both DNA gyrase and topoisomerase IV, with $IC_{50} > 300\times$ for human topoisomerase [38]. In addition, studies have demonstrated reduced propensity for resistance selection [39,40], excellent pharmacokinetics and pre-clinical safety profile [41,42], and *in vivo* efficacy in various respiratory models of infection, including tuberculosis, comparable or superior to established fluoroquinolines [43–49].



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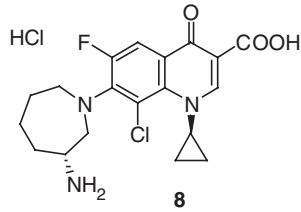

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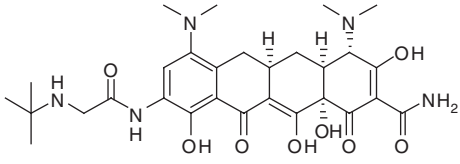
A new "fourth-generation" quinolone, SS734 **8**, has demonstrated broad-spectrum *in vitro* antibacterial activity with Gram-negative activity comparable to ofloxacin and superior activity against Gram-positive pathogens. SS734 is being developed for treatment of bacterial conjunctivitis [50,51].



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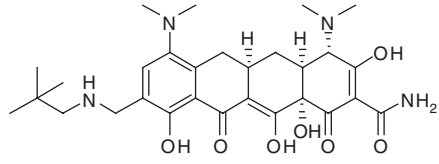

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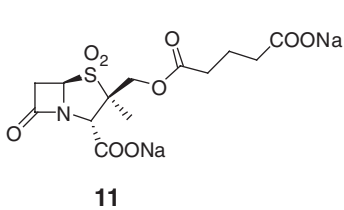
2.3 Tetracyclines

The tetracycline class of antibiotics has been a low priority for most drug discovery organizations for many years due to the synthetic challenges and the increasing prevalence of resistance caused by tetracycline-specific efflux [52,53] and ribosomal protection [54]. The launch of Tigecycline **9**, a semisynthetic tetracycline analog (termed a glycylcycline) [55] in 2005 may signify a new status for this class. Tigecycline evades the usual tetracycline-resistance paths to afford potent antibacterial activity against Gram-positive pathogens, including multi-drug-resistant strains and many Gram-negative strains [56,57]. This new drug was the subject of 35 posters at the 45th ICAAC and 37 posters at the 46th ICAAC in 2006. Another modified tetracycline, MK-2764/PTK0796 **10** is an amino-methylcycline with an antibacterial spectrum of activity similar to that of Tigecycline [58]. *In vivo* PK/PD studies against *S. pneumoniae* in a murine pneumonia model demonstrated a linear PK profile over a range of 0.5–10 mg/kg

doses and potent killing activity at 10 mg/kg [59]. A separate PK/PD study against a variety of Gram-negative and Gram-positive pathogens showed potency that was generally superior to that of Tigecycline [60]. The observed *in vivo* potency was in spite of superior MICs for Tigecycline for several of the pathogens tested, suggesting that MK-2764/PTK0796 has more favorable PK and/or PD characteristics.

2.4 Other modifications of established antibiotics classes

Numerous additional reports of new antibiotic analogs have been published recently with notable examples cited here. While not strictly speaking 'antibacterial agents' β-lactamase inhibitors have found utility, particularly when paired with a specific antibiotic (e.g., Augmentin) [61]. The penem sulfone SA-2-13 **11** was designed as a class A β-lactamase inhibitor using the crystal structure of tazobactam bound to the β-lactamase E166A SHV-1 as a guide. In tests, the *trans*-enamine intermediate formed by the SA-2-13 with sHV-1 was found to be 10 times more stable than the analogous tazobactam intermediate [62]. A new 6-alkylidenepenicillanic acid sulfone LN-1-255 **12**, a potent inhibitor of serine β-lactamases, was shown to dramatically lower the MICs of cefpirome and ceftazidime against highly resistant Enterobacteriaceae [63]. A novel non-β-lactam inhibitor NXL104 **13** has been shown to irreversibly inhibit both class A and class C β-lactamases [64,65]. In experiments with highly resistant Enterobacteriaceae, NXL104 restored rapid bactericidal activity to ceftazidime. NXL104 entered Phase I clinical trials in 2007.



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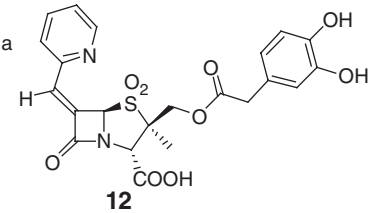

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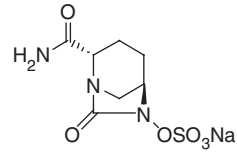

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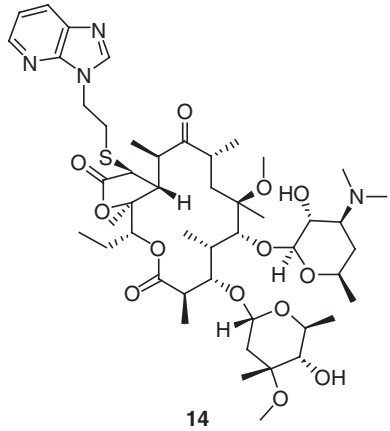

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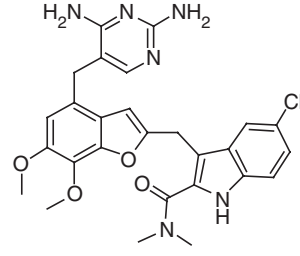

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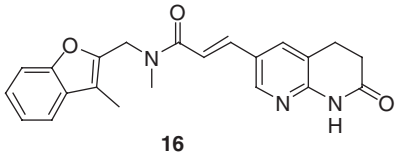
BAL19403 **14** is a new macrolide that is highly potent against erythromycin-resistant and clindamycin-resistant propionibacteria [66,67]. Potent anti-inflammatory activity and good pre-clinical safety make BAL19403 a good candidate for topical treatment of acne [68–70]. AR-709 **15** is a new diaminopyridine in Phase I clinical trials derived from a program aimed at optimizing dihydrofolate reductase (DHFR) inhibitors treating respiratory infections caused by multi-drug-resistant Streptococci, Pneumococci, and Staphylococci [71–73]. In enzyme assays using wild-type and mutant DHFR isolated from *S. pneumoniae* and *S. aureus*, the IC_{50} for AR-709 was 12 x-60 × lower than for trimethoprim (TMP) for the wild-type and 50–100 × lower for the mutant enzyme [74]. Accordingly, AR-709 has demonstrably more potent antibacterial activities than TMP against both TMP-S and TMP-R strains of *S. pneumoniae* and *S. aureus*. AR-709 is currently in a radiolabelled Human Microdose study where preliminary results indicate that the drug distributes well in the target lung tissue.

3. FUTURE ANTIBACTERIAL PIPELINE: INHIBITORS OF NEW TARGETS

3.1 FabI inhibition

Bacterial enoyl–acyl carrier protein (enoyl-ACP) reductase (FabI) is an essential enzyme that catalyzes the reduction of *trans*-2-enoyl-ACP to acyl-ACP in the final step of each elongation cycle in bacterial fatty acid biosynthesis and an attractive target for selective antibacterial therapy [75,76]. API-1252 **16** is a FabI inhibitor with highly potent antibacterial activity against Staphylococci, with MIC_{90}s 0.015 μg/ml for a large collection of *S. aureus* and *S. epidermidis* strains with susceptible and multi-drug-resistance phenotypes [77]. Against a group of 10 selected MRSA strains, including VISA and VRSA phenotypes from hospitals and the community, MICs for API-1252 were an order of magnitude lower than comparators vancomycin, linezolid, and quinupristin/dalfopristin [78]. *In vivo* pharmacodynamic model studies, infected with *S. aureus*, showed that API-1252 is a promising oral or intravenous treatment with once or twice per day dosing [79,80]. CG400462 **17** [81] and CG400549 **18** [82] are also potent FabI inhibitors. Both compounds exhibited MIC_{90}s between 0.5 and 1 μg/ml against *S. aureus* strains with MICs unchanged by a variety of resistance phenotypes. CG400462 had potent *in vivo* activity against MRSA in a systemic mouse model with subcutaneous dosing [83].



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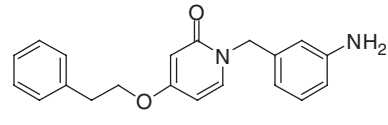

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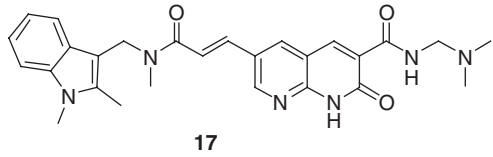

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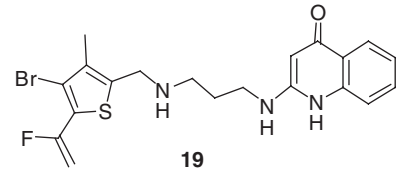

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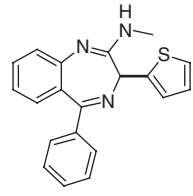
3.2 Methionyl-tRNA synthetase inhibition

Methionyl-tRNA synthetase is an essential target that is conserved in Gram-positive bacteria. A high throughput screening and medicinal chemistry effort against this target yielded a potent inhibitor: REP8839 **19** [84]. REP8839 has no Gram-negative activity but has potent bacteriostatic activity against important skin pathogens *S. aureus* and *S. pyogenes*, including MRSA and mupirocin- and vancomycin-resistant phenotypes with MICs ranging from $\leqslant$ 0.008 to 0.12 μg/ml. Resistance characterization experiments showed that resistance emergence was relatively facile, with spontaneous mutants elevating the MIC to 32 μg/ml (still within the range of efficacy for topical treatment), but that there was no cross resistance to mupirocin, a common topical antibacterial drug for which resistance is becoming increasingly prevalent [85]. REP8839 initiated Phase I clinical testing in July 2006.

H. pylori MurI IC_{50} = 1.7 μM
H. pylori MIC = 0.5 μg/ml
protein binding = 99%
solubility = 0.6 μM



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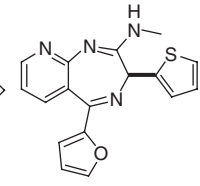

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H. pylori MurI IC_{50} = 2.0 μM
H. pylori MIC = 0.13 μg/ml
protein binding = 82%
solubility = 1360 μM

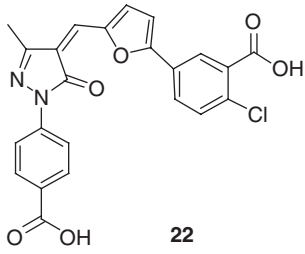
3.3 Cell wall biosynthesis inhibition

A high throughput screening effort directed at glutamate racemase (MurI), an essential enzyme in the peptidoglycan biosynthesis pathway, from *H. pylori* afforded the 2-methylamino-benzodiazepine hit **20**. The aryl (thiophene) group at C-3 and the aminomethyl moiety at C2 were found to be critical for MurI activity during optimization. A lead **21** was identified following the observation that heterocycles were preferred at C5 and that the fused aza ring retained potency while improving protein binding and solubility [86]. The lead series analogs represented by **21** are potent and selective inhibitors of *H. pylori* MurI. These inhibitors bind to an allosteric site and are > 200-fold selective for *H. pylori* MurI over MurI isolated from other Gram-positive or Gram-negative sources [87]. The compounds were cidal, with slow killing kinetics, against *H. pylori*, but inactive

(MIC > 64 μg/ml) against other bacterial species tested. MIC values were elevated in a MurI overexpressing strain but unaffected by overexpressing *H. pylori* efflux systems [88].

3.4 Antivirulence

Successful inhibition of virulence in bacteria might have the potential to eliminate antibiotic resistance – by eliminating the antibiotic. In effect, an antivirulence agent would hinder bacteria in their ability to actively infect the human host, but might otherwise leave them intact, and harmless [89,90].



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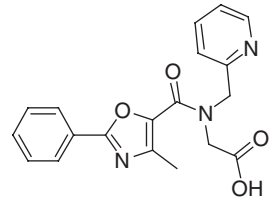

23

WaaC is a glycosyltransferase that is essential for inner core lipopolysaccharide (LPS) biosynthesis and that is conserved in Gram-negative bacteria. The fully formed LPS is needed to protect the cell against complement killing, and hence, contributes to virulence *in vivo*, although the full LPS is not needed for gut colonization. The consequence of WaaC deletion is a truncated LPS that leads to increased susceptibility to human serum and to loss of virulence in mice relative to the wild-type. MUT11931 **22** is a lead compound within a series of diarylpyrrazolone inhibitors of WaaC identified in a structure-based anti-virulence drug discovery program. MUT11931 is a competitive, reversible inhibitor with $IC_{50} = 3.1\ \mu M$ [91]. Proof-of-principle with this inhibitor series will likely be with treatment of an infected mouse with the optimized lead to look for the loss of virulence noted in the WaaC deletion experiment.

RfaE is an enzyme that catalyzes the phosphorylation of heptose-7-phosphate to form heptose-1,7-bisphosphate during the biosynthesis of ADP-heptose, a component of the inner core LPS. RfaE is conserved among Gram-negative bacteria and a target for anti-virulence. As with WaaC, deletion of RfaE results in bacteria with increased susceptibility to human serum and loss of virulence *in vivo*. MUT2585 **23** is the lead compound (IC_{50} 71 μM) in a series of aryl-oxazole inhibitors of RfaE that is undergoing lead optimization [92].

4. FUTURE OUTLOOK

The one certainty regarding the treatment of bacterial infections is that resistance will emerge sooner or later. The medical community is approaching a very difficult path as a result of the ever-increasing prevalence of resistance and a limited number of choices for effective treatments. With the introduction of new tools,

including Tigecycline, Cubicin, and Zyvox, the situation for Gram positives is improving. The numbers of new compounds in clinical development, assuming many achieve successful NDA filings, are encouraging, although most are intravenous Gram-positive treatments and most are new analogs of older drugs [2,11,14]. With the widespread distribution of resistance determinants for all of the major drug classes, many new analogs will likely have a relatively short duration of effectiveness.

Several new approaches, most in early stages of lead optimization, are cited here as clear evidence that new ideas are being directed at the challenge posed by antibiotic resistance. However, relatively few of the compounds in the pipeline, either new or old, are expected to address the growing problem of Gram-negative resistance, which may create a troublesome gap in available treatments if not adequately addressed soon. There remains a significant need for drugs with new mechanisms of action that will set back the clock of pre-existing resistance determinants. Treatments for serious Gram-positive infections with an oral dosing option to minimize hospital stays remain a high priority as do new treatments for multi-drug-resistant Gram-negative infections.

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CHAPTER 23

Small Molecule Inhibitors of AKT/PKB Kinase as a Strategy for Treating Cancer

Dirk A. Heerding, Igor G. Safonov and **Sharad K. Verma**

Contents

1. INTRODUCTION

AKT-1 kinase (also called protein kinase B or PKBα) is a serine/threonine kinase belonging to the AGC kinase family [1]. AKT was identified from a viral oncogene, v-akt, found in tumor lines established from spontaneous thymomas found in AKR mice [2]. Subsequently, two more AKT isoforms, AKT-2 (or PKBβ) and AKT-3 (or PKBγ) have been identified [3]. Reviews exist detailing the structural and cell biology of AKT and the reader is referred to these for further information [4,7,12].

AKT kinases participate in pathways that regulate several cellular processes, including survival, proliferation, tissue invasion, and metabolism [5–7]. Hyperactivation of the three isoforms of AKT kinases is a common finding in human malignancies. Increased AKT-1 activity has been observed in about 40% of breast and ovarian cancers and >50% of prostate carcinomas. Activation of

GlaxoSmithKline, 1250 S. Collegeville Road, Collegeville, PA 19426, USA

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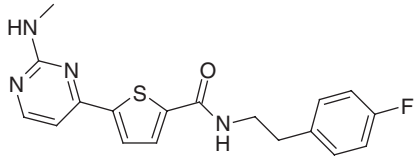
AKT-2 kinase has been observed in 30–40% of ovarian and pancreatic cancers. Increased AKT-3 enzymatic activity was found in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines [7,8]. Furthermore, preclinical data suggest that blocking AKT activity might inhibit the proliferation of tumor cells and either induce an apoptotic response or sensitize tumors to undergo apoptosis in response to other cytotoxic agents, making AKT kinases a good target for cancer therapy [9,10].

In this report, we will summarize information available on AKT inhibitors in order to update the reader with new material disclosed since the most recent reviews [11,12].

2. PI3K/AKT KINASE PATHWAY INHIBITORS IN PRE-CLINICAL DEVELOPMENT

2.1 ATP competitive inhibitors

The 2-pyrimidyl-5-amidothiophene **1** (AKT-3, $IC_{50} = 3.0\,\mu M$) was identified as an ATP-competitive AKT inhibitor [13]. Further optimization, guided by a homology model based on PKA, culminated in **2** (AKT-3, $IC_{50} = 3\,nM$). Compound **2** inhibited the growth of DOV13 cells ($EC_{50} = 1\,\mu M$) and the phosphorylation of targets downstream of AKT such as PRAS40, GSK3β and S6RP. The addition of the basic aminomethyl group to the amide side-chain was crucial to improving the AKT potency of this series, with the (*S*)-antipode approximately 100-fold more potent than the (*R*)-antipode. Good selectivity was achieved against kinases not belonging to the AGC family.



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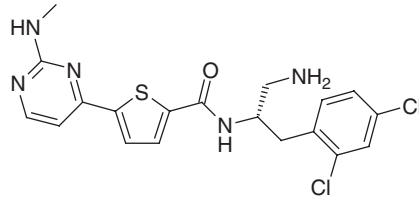
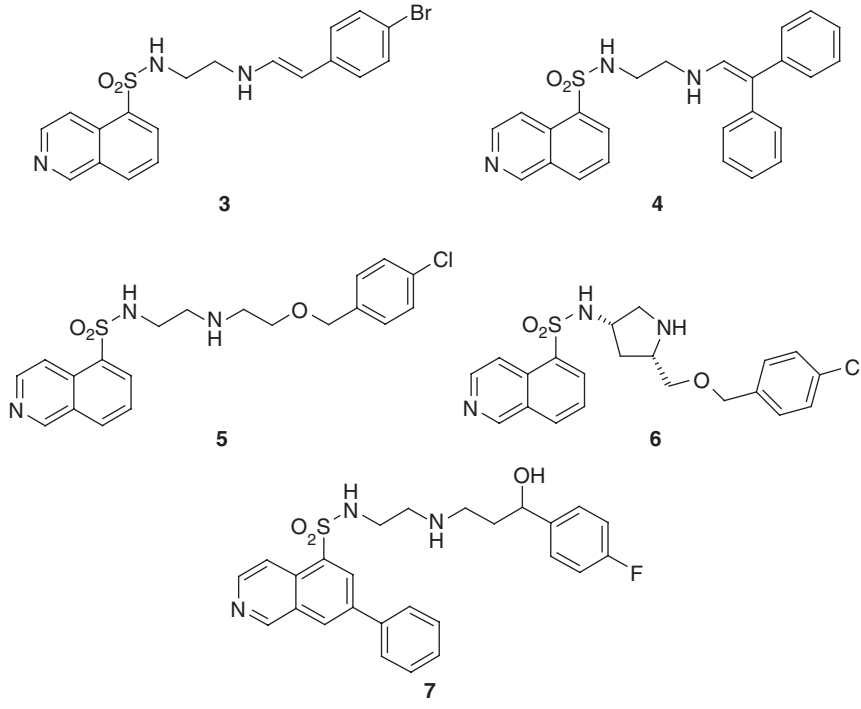
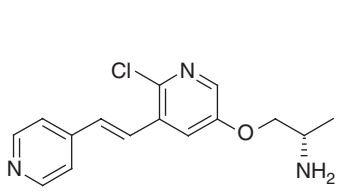


2

The AKT activity of the quinoline sulfonamide analog **3** (AKT, $IC_{50} = 4\,\mu M$; PKA, $IC_{50} = 9\,\mu M$) derived from the PKA inhibitor H-89 (**4**, PKA, $IC_{50} = 0.035\,\mu M$; AKT, $IC_{50} = 2.5\,\mu M$) has previously been disclosed [14]. Recently, a strategy for improving the AKT kinase and cell potency of this class of inhibitors was reported [15]. Reducing the lipophilicity and conformationally restricting the amine-containing linear side-chain led to **5** and **6** ($IC_{50} = 0.26$ and $0.78\,\mu M$, respectively). Both compounds inhibited cell proliferation and AKT-mediated Ser9 phosphorylation of GSK3β in PC-3 cells ($IC_{50} = 15$ and $17\,\mu M$ for **5** and **6**, respectively). Selectivity against PKA, however, still remains as a significant issue for both compounds ($IC_{50} = 0.17\,\mu M$ for **5** and **6**). 7-Aryl-substituted isoquinoline sulfonamides, such as **7**, have also been reported in the patent literature as AKT-1 inhibitors [16].



A series of 3,5-disubstituted pyridine compounds with activity against AKT has been reported [17–24,71]. Compound **8** was identified from high throughput screening and characterized as an ATP-competitive inhibitor of AKT-1 ($IC_{50} = 5.29\,\mu M$). Subsequent optimization of the ether-linked sidechain led to indole containing analogs such as **9** (AKT-1, $IC_{50} = 14\,nM$) and **10** (AKT-1, $IC_{50} = 2\,nM$) [17,18]. The (*S*)-antipode represents the active optical isomer, as the enantiomer of **9** is >25-fold less active against AKT. Although **10** was active in several mouse xenograft models, it had poor pharmacokinetic properties and was not orally bioavailable. This poor pharmacokinetic profile was attributed to rapid clearance due to oxidative metabolism at C-1 of the isoquinoline moiety [19]. Attempts to block this metabolic site while maintaining AKT activity were not successful. Ultimately, a 3-methylindazole was used to replace the isoquinoline as the hinge-binding motif, giving **11** and **12** (AKT-1, $K_i = 0.16$ and 11 nM, respectively) [20–22]. Reasonable selectivity was achieved with **11** and **12** against kinases from the CMGC, CAMK and TK families. Selectivity within the AGC family, however, was poorer, as exemplified by the inhibition of PKA by **11** and **12** ($K_i = 0.16$ and 16 nM, respectively). Both compounds were extensively evaluated as monotherapy, or in combination with known chemotherapeutics, in different mouse xenograft models and found to induce dose-dependent cytostatic responses in tumors [23,24]. In addition, **12** was found to be orally bioavailable in the mouse and dog ($\%F = 67\%$ and 63%, respectively). Drawbacks associated with these AKT inhibitors include a narrow therapeutic window in *in vivo* models with differing types of general and mechanism-based dose-limiting toxicities.



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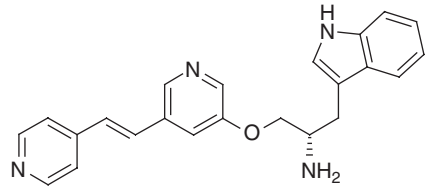

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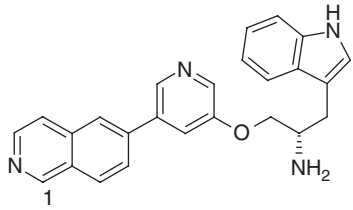

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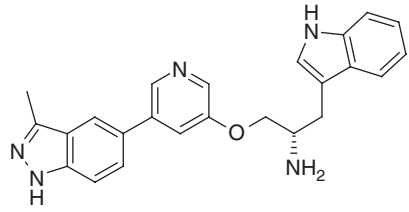

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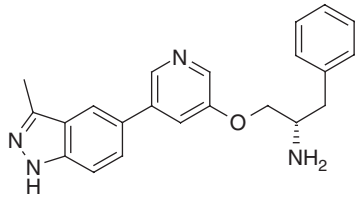

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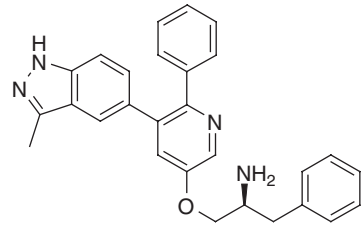
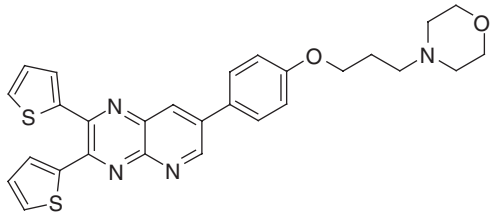


13

In addition, 2-aryl pyridyl indazoles have been reported in the patent literature [25]. For example, compound **13** is reported to inhibit AKT-1 ($IC_{50} \leqslant 0.18\,\mu M$). No kinase selectivity data were presented.

Compound **14** ($IC_{50} = 0.30\,\mu M$ against AKT-1) is representative of a series of pyridopyrizine AKT inhibitors [26]. This compound inhibited AKT-mediated phosphorylation of GSK3β in IGF-1-stimulated MCF-7 cells ($IC_{50} = 0.5\,\mu M$). No kinase selectivity data were reported.



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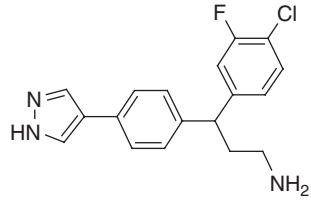
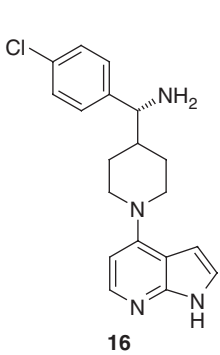


15

A series of pyrazoles was disclosed in a patent application claiming members of this series as AKT inhibitors [27]. The pyrazole **15** is representative of this series, with AKT activity reported as $IC_{50} < 0.1\,\mu M$. In the patent application, these compounds were specifically claimed to have oral exposure, reduced or no

hERG ion channel blocking activity and improved solubility characteristics. However, no data were presented to substantiate these claims.

A series of 4-(1-piperidinyl)-1H-pyrrolo[2,3-b]pyridines (**16**), 6-(1-piperidinyl)-1H-purines (**17**) and 4-(1-piperidinyl)-1H-pyrazolo[3,4-d]pyrimidines (**18**) were reported as AKT inhibitors [28–30]. Compounds **16** and **17** were described as having AKT $IC_{50} < 10\,\mu M$, whereas compound **18** was described as having AKT $IC_{50} \leqslant 50\,nM$.



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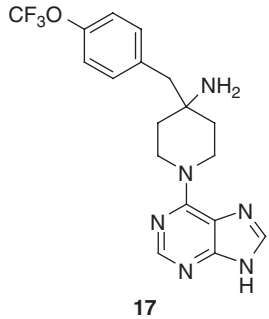

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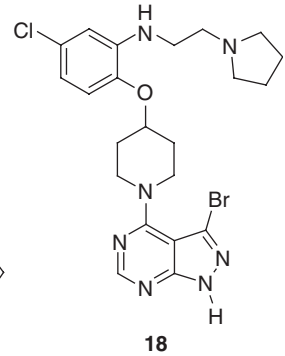
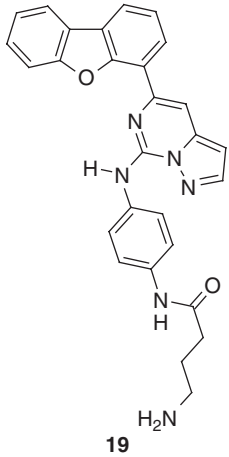


18

Pyrazolopyrimidines, such as **19**, have been reported as AKT-1 inhibitors. Three biochemical assays were used to determine inhibition of AKT-1 [31]: inhibition of PDK1-activated AKT-1 devoid of its PH domain ($IC_{50} \leqslant 1.75\,\mu M$), inhibition of PDK1-activated full-length AKT-1($IC_{50} \leqslant 4.03\,\mu M$), and inhibition of full-length AKT-1 where **19** is added prior to AKT-1 activation with PDK1 in the presence of phosphatidylinositol-3,4,5-triphosphate vesicles ($IC_{50} \leqslant 5\,\mu M$). No kinase selectivity data were presented. Compound **19** inhibited AKT-mediated phosphorylation of GSK3β in MCF-7 cells ($IC_{50} < 10\,\mu M$) and inhibited *in vitro* proliferation of MCF-7 and MDA-468 cells ($IC_{50} \leqslant 16.9$ and $13.6\,\mu M$, respectively). Imidazoquinoline derivatives (**20**) and aminofurazans (**21**) have also been reported as AKT-1 inhibitors ($IC_{50} \leqslant 0.5\,\mu M$) [32,33]. No kinase selectivity data were presented.



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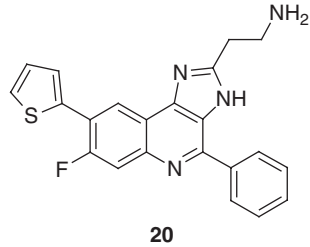

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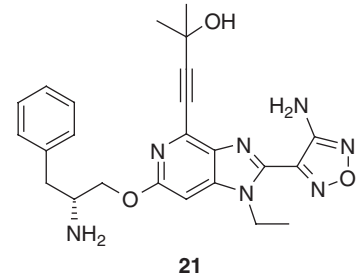

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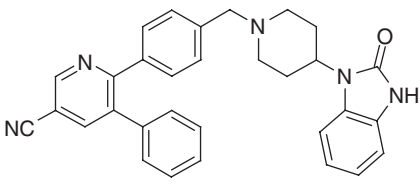
2.2 Allosteric inhibitors

A series of non-ATP competitive, PH-domain-dependent allosteric inhibitors selective for AKT-1 and AKT-2 have previously been described [34]. Recently, new analogs, such as **22** and **23**, were reported as potent and selective AKT-1 and AKT-2 inhibitors with improved aqueous solubility and cell permeability [35].



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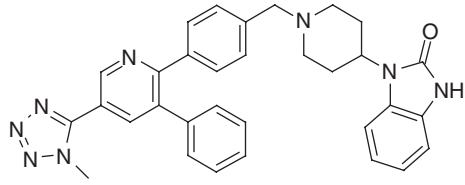
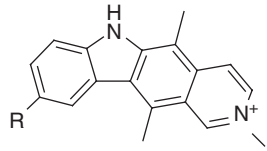


23

2.3 AKT pathway inhibitors with an undefined mechanism of action

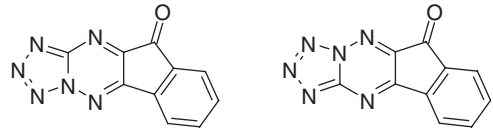
9-Methoxy-2-methylellipticinium acetate (API-59-OMe, **24**) has previously been reported as an AKT kinase pathway inhibitor in human endometrial cancer cells [36]. Similar results have been shown with **24** in ovarian cancer cell lines (A2780, MDAH2774 and OVCAR-8) [37]. The A2780 and MDAH2774 lines have high levels of activated AKT, while OVCAR-8 exhibits endogenous amplification and expression of AKT-2. Treatment of the cells with **24** significantly inhibited AKT-mediated phosphorylation of Bad at Ser136 and GSKα/β at Ser21/9 as measured by an IP kinase assay. Chloro-2-methylellipticinium acetate (CMEP, **25**) was profiled against a series of prostate cancer lines (Cl-1, LNCaP and PC-3) having high basal levels of AKT inhibition [38]. In these cell lines, **25** significantly reduced AKT-mediated phosphorylation as measured by an IP kinase assay. Compound **25** also induced apoptosis in these cells when dosed at 10 μM.



24: R = OMe
25: R = Cl

KP372-1 (**26**) consists of a 1:1 mixture of indenotetrazolotriazinones, representative of a novel class of fused polycyclic compounds that suppress AKT activity via an unknown mode of action. There have been several reports describing induction of apoptosis and inhibition of cell proliferation by **26** in cancer cells having high levels of AKT activation. For example, **26** induces apoptosis in thyroid cancer cells (NPA187, $IC_{50} = 30$ nM; WRO, $IC_{50} = 60$ nM) by blocking the phosphorylation and kinase activity of AKT [39]. Similar findings were observed with **26** in U251 and U87 glioma cells [40]. Compound **26** was also described as a dual AKT/PDK1 kinase inhibitor in leukemic cells [41]. Compound

26 almost completely inhibited AKT and PDK1 kinase activity *in vitro*. In addition, **26** caused mitochondrial dysfunction and led to apoptosis in several acute myelogenous leukemia cell lines, but not in normal hematopoietic progenitor cells.



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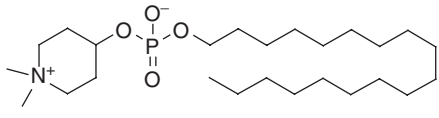
26

3. PI3K/AKT KINASE PATHWAY INHIBITORS IN CLINICAL TRIALS

Perifosine (**27**) is a synthetic, orally available alkylphospholipid, derived from alkylphosphocholine, which targets the PI3/AKT survival pathway. Although the molecular mechanism underlying the antineoplastic activity of **27** is not fully elucidated, studies suggest that **27** interferes with turnover and synthesis of natural phospholipids. This disrupts membrane signaling at several sites resulting in the inhibition of the PI3K/AKT survival pathway [42,43]. Recent preclinical evaluation in cultured human Jurkat T-leukemia cells has shown that adding low concentrations of **27** (5 μM) after treatment with the commonly used chemotherapy drug etoposide, induced cell death in a synergistic fashion. The observed increase in cell death is attributed to an inactivation of the AKT survival pathway, as treated cells showed a complete dephosphorylation of AKT [44]. Compound **27** also inhibited baseline phosphorylation of AKT in multiple myeloma cells in a time- and dose-dependent fashion [45]. Reduced tumor growth and increased survival was also observed in a murine multiple myeloma mouse model. In this study, administration of **27** at low concentrations (2.5–5 μM) with subtoxic concentrations of the conventional therapeutic agents dexamethasone, melphalan and bortezomib resulted in an enhanced cytotoxic effect in a dose-dependent fashion. Compound **27** is currently among the most clinically advanced small molecule AKT inhibitors as it has progressed to clinical evaluation for the treatment of many human cancers. Recently reported results from clinical trials involving **27** (either as single agent or in combination) have demonstrated clinical responses and antitumor activity in multiple myeloma, hormone-sensitive prostate cancer and non-small cell lung cancer (NSCLC) [46–50]. Adverse events associated with **27** include gastrointestinal irritation, fatigue and rash.



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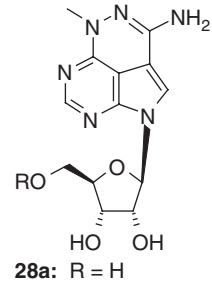
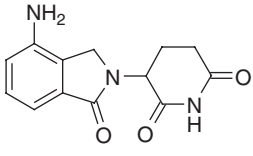


28a: R = H

28b: R = P(O)(OH)$_2$

Triciribine (API-2, TCN, **28a**) is a tricyclic nucleoside first reported in 1980 [51]. While **28a** and the corresponding 5-phosphate derivative (triciribine phosphate, TCN-P, **28b**) have demonstrated antitumor activity and progressed to clinical evaluation for the treatment of several advanced solid tumors, severe side effects (e.g. hepatotoxicity and hyperglycemia) related to dosing levels ultimately limited their use [52–59]. More recently, screening of the National Cancer Institute Diversity Set identified **28a** as a highly selective inhibitor of AKT, wherein cell growth was suppressed at a concentration of 50 nM [60]. While it is known that **28a** blocks the AKT pathway, the mechanism by which it prevents AKT activation has not been established. Additional preclinical findings report that **28a**, at a dose of 5 μM, effectively and selectively induced apoptosis and cell growth arrest in tumor cells in which AKT was aberrantly expressed or activated, while cancer cells without this trait were not affected [50,61]. In xenograft studies, no detectable side effects were observed in mice treated with **28a** at 1 mg/kg/day, a dose in which tumor growth was significantly inhibited in cancer cells overexpressing AKT. Another preclinical study demonstrated that treatment with **28a** reduced melanoma cell survival in a time- and dose-dependent manner. Phospho-AKT levels were decreased in these cells in response to **28a** [62]. Treatment of the human melanoma cell lines, MMRU and MMAN, with **28a** (5 μM for 48 h) inhibited cell survival by 35% and 45%, respectively, when compared to vehicle control. In a mouse tumor xenograft model, low concentrations of **28a**, in combination with a recombinant adenovirus containing human PUMA cDNA (ad-PUMA), resulted in enhanced cooperative growth inhibition of human melanoma cells.

Thalidomide has been re-evaluated in recent years for the treatment of a broad spectrum of diseases despite its known teratogenic properties [63]. The thalidomide analog and immunomodulatory compound CC-5013 (**29**) is in phase III clinical evaluation for the treatment of patients with relapsed and refractory multiple myeloma [64]. Although the exact mechanism of action in a neoplastic setting is unknown, it has been suggested that the anti-tumor effect is related to anti-angiogenic potency, through inhibition of growth-factor-induced AKT phosphorylation [65]. When orally administered in rats, **29** inhibited bFGF induced phosphorylation of AKT in a dose-dependent manner [65]. Using an *in vivo* rat mesenteric window assay, **29** was shown to inhibit growth-factor-induced angiogenesis by inhibiting vascularization in a dose-dependent manner. Adverse side effects were not noted in this study. A pharmacokinetic study revealed that a single oral administration of **29** at 50 mg/kg produced plasma drug concentrations comparable to levels that are required to inhibit angiogenesis in the human assay *in vitro*.



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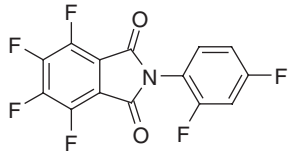

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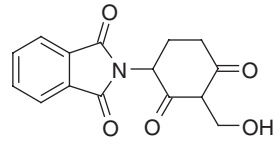

31

The thalidomide analogs CPS49 (**30**) and CPS11 (**31**) have been reported to inhibit PI3/AKT signaling in multiple myeloma cells via an anti-angiogenic effect. These compounds are devoid of the teratogenic properties seen with thalidomide and are currently in preclinical development [66]. Compound **30**, and to a lesser extent **31**, induced a dose-dependent inhibition of proliferation in several multiple myeloma cell lines and reduced phospho-AKT levels [66]. These compounds also inhibited DNA synthesis in cell lines resistant to conventionally used anti-multiple myeloma drugs (e.g. dexamethasone, anthracyclines and melphalan) in a dose-dependent manner.

In addition to small molecule therapies, oligonucleotides as AKT inhibitors are being investigated [67,68]. The 20-mer antisense oligonucleotide RX-0201, which is complementary to AKT-1 mRNA, has been reported to block AKT-1 activity and suppress cell proliferation in a number of carcinomas [69]. RX-0201 is active against 10 different AKT-1 overexpressing cell lines and demonstrated *in vivo* efficacy in mouse tumor xenograft models as measured by decreased tumor weight and increased survival time. No adverse side effects were noted. RX-0201 is currently in clinical trials for the treatment of patients with advanced or metastatic solid tumors [70], and has received orphan drug status from the FDA for the treatment of renal cell carcinoma, glioblastoma, ovarian, stomach and pancreatic cancers.

4. CONCLUSIONS

The central role that the PI3K/AKT pathway plays in cancer continues to fuel excitement in this arena as a potential target for cancer therapy. Progress with small molecule AKT inhibitors continues to be made, as judged by the compounds described in this review. However, the full scope of the clinical effectiveness of targeting this pathway has yet to be proven, as most of the reported small molecule AKT inhibitors are still in pre-clinical development, with only a few examples in early phase clinical trials.

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CHAPTER 24

Spleen Tyrosine Kinase (Syk) Biology, Inhibitors and Therapeutic Applications

Rajinder Singh and **Esteban S. Masuda**

Contents

1. INTRODUCTION

The need for new chemical entities directed towards treatment of inflammatory and autoimmune disorders such as asthma, allergic rhinitis, atopic dermatitis, seasonal allergies, rheumatoid arthritis (RA), multiple sclerosis (MS) and systemic lupus erythematosus (SLE) is imperative. These onerous, often debilitating, and occasionally fatal conditions afflict a wide range of groups in the population worldwide and have a major socioeconomic impact. The mandate of discovering safer, more efficacious, less frequently dosed and more cost effective therapies make the first steps in discovering new therapeutic agents critical. Attention must be drawn to the specific biological pathways, which then initiates the

Rigel, Inc. 1180 Veterans Blvd, South San Francisco, CA 94080, USA

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discovery of inhibitors for these selected pathways. One target in biological pathways with high potential for discovering novel inhibitors for treatment of the above-mentioned diseases is spleen tyrosine kinase (Syk).

2. SYK KINASE

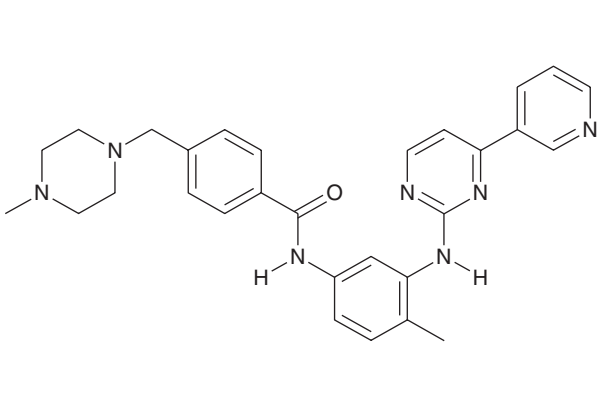
Syk kinase is a cytosolic 72-kDa member of the non-receptor tyrosine class of kinases and is most closely homologous to cytosolic 70-kDa ζ chain-associated protein kinase (Zap-70) [1,2]. These two kinases are expressed in diverse hematopoietic cell types. Syk is also expressed in non-hematopoietic cell lineages. Syk kinase is found in platelets, B lymphocytes, mast cells, basophils, neutrophils, dendritic cells, macrophages and monocytes, while Zap-70 is restricted to T lymphocytes and natural killer (NK) cells. Syk kinase plays a functional role in the signal transduction mediated by immunoreceptor tyrosine-based activation motifs (ITAMs) present in various hematopoietic cells that are causal agents in an assortment of inflammatory and autoimmune diseases [3–10]. From a structural point of view, Syk and Zap-70 kinases are unique in that they possess tandem N- and C-Src homology 2 (SH2) domains, and a carboxy-terminal catalytic kinase domain containing multiple phosphorylation sites. The presence of these multiple phosphorylation sites suggests the involvement of Syk kinase in numerous biological signal transduction pathways, and implies a mechanistic involvement in various diseases [3–10].

3. STRUCTURAL BIOLOGY OF SYK KINASE

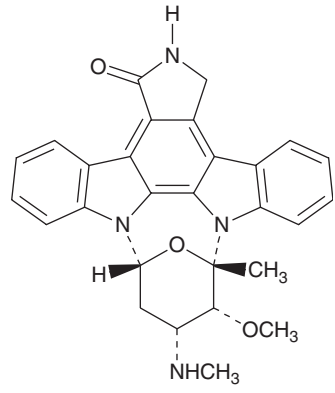
Determination of an enzyme's crystal structure can provide a very useful aid in designing virtual libraries, optimization of screening hits and ultimately improving design of more selective ligands. The crystal structures of the kinase catalytic domain and binding pockets of Syk kinase have been determined in conjunction with Syk_{cat}-peptide PT426-adenylyl imidodiphosphate complex (AMP-PNP), and staurosporine as co-crystallized ligands at a resolution of 2.4 Å and 1.65 Å, respectively [11].

Complementary to the aforementioned work, the crystal structure of the unphosphorylated form of the kinase catalytic domain of Syk kinase has been elucidated, and co-crystal structures were determined with Albelson tyrosine kinase (Abl) inhibitor STI-571 (Gleevec®) **1**, and staurosporine **2** [12]. The focal point of this structural work was centered on the fact that the conformation of the Syk unphosphorylated activation loop is reminiscent of activated and hence phosphorylated forms of other tyrosine kinases. STI-571 binds to the unphosphorylated form of Abl kinase, and is in fact a considerably weaker inhibitor of the phosphorylated form of Abl. Given this point and the fact that the *in vitro* enzymatic activity of Syk kinase does not require phosphorylation, STI-571 was utilized in crystal work on Syk kinase to gain insight towards the binding mode. In summary, this study demonstrated that STI-571 adopts a *cis*-conformation **3** when binding with Syk, $K_i = 5.0\,\mu M$, compared with a *trans*-conformation **1** adopted for binding with unphosphorylated Abl.

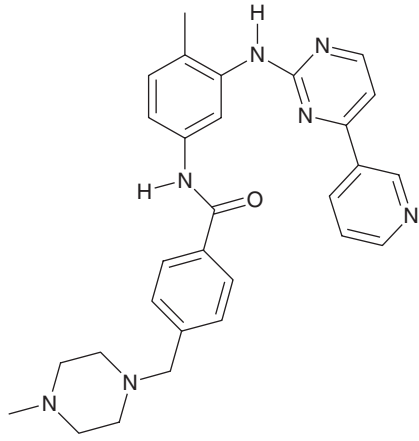
Intriguingly, the *cis*-conformation **3** overlaps remarkably well with the structure of staurosporine, which potently inhibited Syk with an $IC_{50} = 0.012\,\mu M$. This work can be used as a starting point for the optimization of more potent STI-571-like inhibitors for Syk kinase. The distinction between the *cis*-conformation **3** being adopted in Syk binding and the *trans*-conformation **1** for Abl kinase activity yielded an STI-571 analog showing a $K_i = 0.16\,\mu M$ for Syk binding [12].



1
STI-571, Imatinib
trans-conformation



2
Staurosporine



3
STI-571,Imatinib
cis-conformation

The crystal structure of the tandem Src-homology 2 (SH2) domain from Syk kinase in complex with a double phosphorylated ITAM peptide has been determined at a resolution of 3 Å [13]. The two adjacent Syk C-terminal and N-terminal SH2 domains are required for localization to the membrane and are fundamentally important in the activation of Syk kinase. If the interaction of the

natural peptide is competitively blocked, the diphosphorylation of the ITAM in the γ-chain of the FcεR1 receptor will be inhibited and thus the activation of Syk kinase. This approach offers the possible advantage of greater selectivity, relative to other kinases, by not targeting the ubiquitously present ATP pocket. Peptidomimetic approaches offer the opportunity to design novel ligands to understand the biological function of Syk kinase and ultimately for inhibitor design [14,15]. Peptidomimetic methodologies however, often fail to afford drug-like molecules, due to the difficulty of designing analogs that possess significant *in vivo* potency, readily permeate cell membranes, and have suitable DMPK and other desirable pharmaceutical properties.

4. SYK KINASE AS A THERAPEUTIC TARGET FOR DISEASE

Several studies in the field of immune cell signaling support the role of Syk as a key mediator of acute and chronic inflammation [3,16]. Biological data clearly implicate antibody-mediated signal transduction as a significant factor in the pathology of several allergic and inflammatory diseases. Owing to the role of Syk kinase in these signaling pathways during activation of leukocytes including mast cells, macrophages, granulocytes and dendritic cells, there is increasing excitement for development of Syk as a novel therapeutic target for the amelioration of inflammatory responses. Due to its upstream proximity to the immune receptors, where it plays a key signaling role, and its activation of several cellular players in the inflammatory cascade, Syk is an attractive target for inhibition of multiple downstream pathways, and thus diminution of several nodes of the inflammatory cascade.

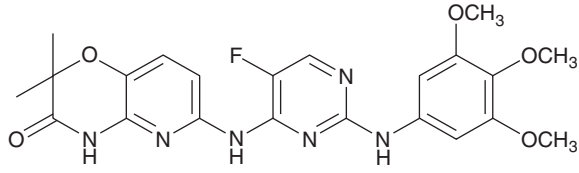
4.1 Allergic disorders

The most understood function of Syk kinase activity is in ITAM-dependent activation of immunoreceptors, including Fc receptor complexes (FcR) that bind the invariable Fc portion of the different immunoglobulin isotypes and the immunoglobulin B-cell-antigen receptor (BCR) complex [17,18]. Allergic disorders are characterized by hypersensitive type I immune responses, mediated by immunoglobulin isotype E (IgE), to foreign antigens. Allergen-specific IgEs bind tightly ($K_a = 10^{10} M^{-1}$) to their Fc receptors, FcεRI, and populate the cell surface of circulating basophils and of mast cells that are strategically located in mucosal and epithelial tissues. Subsequent encounters with allergens crosslink and activate IgE-FcεRI resulting in rapid and massive release of preformed granule contents, responsible for acute allergic symptoms. Trailing this event is generation and secretion of eicosanoids, chemokines and cytokines that help amplify and orchestrate the chronic inflammatory cascade and ensuing tissue remodeling [18,19]. Inhibition of Syk could block all inflammatory mediators given its proximity to the FcεRI receptor signal generation.

4.1.1 Allergic rhinitis, asthma and atopic dermatitis

Allergic rhinitis is characterized by IgE-mediated hypersensitive immune responses to seasonal or perennial allergens in the upper airways. Current therapies include anti-histamines and anti-cysteinyl leukotrienes, but most effective, albeit showing slow onset of action, are locally applied corticosteroids that owe their broad anti-inflammatory effects to their potent blocking of chemokine and cytokine production [4]. It has been reported that the glucocorticoid dexamethasone could inhibit Syk via inhibiting IgE-dependent cell activation by inducing the expression of Src-like adapter protein (SLAP), a negative regulator of Syk action [20]. Syk inhibitors are expected to elicit broad symptom control since genetic or pharmacologic Syk inhibition blocks the production and release of all IgE-mediated inflammatory mediators by mast cells and basophils [21,22].

The inflammation of the lower airways in allergic asthma is strongly associated with atopy, the tendency to produce hypersensitive reactions against innocuous substances mediated by IgE [23]. Anti-IgE therapy, omalizumab (Xolair®), for asthma inhibits both acute airway constriction and later inflammatory eosinophilia [24]. Omalizumab treatment also results in lower FcεRI expression in mast cells, basophils and dendritic cells [23,24]. Inhibition of Syk is expected to produce broad effects since Syk is expressed in various hematopoietic cells and occupies a central role in immune cell activation mediated by all immunoglobulin isotypes including IgE, IgG, IgA and IgM [3]. A small molecule Syk kinase inhibitor **4** has been shown to inhibit both IgE-dependent and IgE-independent mouse models of airway inflammation and hyperresponsiveness [25,26]. It appears that Syk inhibition also alters the function of dendritic cells [25], which express a variety of cell surface molecules containing or coupling to ITAM-bearing signaling motifs, such as FcRγ and DAP12 [27,28]. These ITAM motifs are capable of engaging and activating Syk, and Syk inhibitors can have far reaching anti-inflammatory activities beyond those mediated by IgE.



4

Atopic dermatitis has been proposed to be the cutaneous manifestation of IgE-mediated hypersensitive reaction to allergenic substances [29]. Conceptually, antagonizing IgE emerges as a logical therapeutic option. Systemic treatment with omalizumab, however, appears to be less efficacious in the skin than in the airway mucosa [23]. It is possible that small molecule Syk inhibitors may offer a more suitable mode to reach and prevent activation of sensitized dermal mast cells and dendritic cells.

4.2 Autoimmune disorders

Several autoimmune responses result in the generation of autoantibodies, which can promote cell and tissue destruction and chronic inflammatory responses via Fc receptor activation on leukocytes [17]. Autoantibodies against antigens on the cell surface of blood cells, for example, can result in the rapid destruction of these cells by macrophages localized in the reticuloendothelial system of the spleen and liver. Immune thrombocytopenia purpura (ITP) and autoimmune hemolytic anemia (AIHA) are caused in large part by self-reactive antibodies against platelet antigens (e.g., GPIIb-IIIa, GPIb-IX, GPIb, GPIIIa) and erythrocyte antigens (e.g., Band 3) [30]. Experimental mouse models of ITP and AIHA are dependent on FcγR-signaling, which in turn depends on Syk kinase activity for activation of phagocytes. Indeed, treatment with a Syk kinase inhibitor reduces platelet loss in an experimental murine model of ITP [31].

Other examples of organ-specific pathogenic self-antigens are the α3 domain of basement membrane collagen type IV, causing glomerulonephritis in Goodpasture's syndrome and desmoglein in pemphigus vulgaris, a blistering skin disease. A direct correlation between anti-desmoglein IgG4 levels and disease activity was recently reported, strengthening the idea that the autoantibody is the causative agent for tissue damage [32]. In this case, monocytes, neutrophils and mast cells activated via their Fcγ receptors are the likely triggers and mediators of tissue damage. Syk inhibitors should block these responses and thus potentially ameliorate clinical symptoms.

4.2.1 Rheumatoid arthritis (RA), multiple sclerosis (MS) and systemic lupus erythematosus (SLE)

The pathogenesis of RA is still incompletely understood but has been associated with rheumatoid factors, recognizing Fc portions of IgGs, and a battery of autoantibodies directed against joint-antigens, including type II collagen and several citrullinated proteins [6]. Syk was detected in the synovial intimal lining in the synovial tissue from RA patients, and significantly greater amounts of phospho-Syk expression were observed in RA synovial tissue as compared with osteoarthritis synovial tissue [33]. Moreover, Syk and FcγR-signaling play critical roles in the activation of immune cells elicited by these autoantibodies and their immune complexes (ICs) [3]. Syk inhibition blocks activation of mast cells, macrophages, dendritic cells, neutrophils and B cells via B cell receptors. Overall, Syk inhibitors can attenuate vascular leakage, leukocyte infiltration, disease propagation, release of reactive oxygen intermediates, nitric oxide, proteases and pro-inflammatory cytokines that cause adjacent tissue damage and osteoclast activation leading to bone destruction. These salutary effects, observed in Syk-deficient and/or FcRγ-deficient mice, have been phenocopied using a small molecule inhibitor of Syk [34]. In a rat model of RA, Syk inhibitor treatment drastically reduced bone erosions, as well as the influx of inflammatory cells into the synovium [35].

MS is a neurological chronic and progressive disease likely caused by an autoimmune response to various antigens present in the myelin sheath. MS is

characterized by focal demyelination and lymphocytic infiltration in the central nervous system and the brain [36]. The involvement of autoantibodies is not clear as reflected by the fact that induction of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, can occur in B-cell deficient animals. Remarkably, despite the apparent lack of participation by autoantibodies and immune complexes, FcRγ-deficient mice are resistant to EAE suggesting that the FcRγ signaling chain is coupling to receptors other than antibody-Fc receptors. In fact, FcRγ-deficiency actually protects mice from the neuropathogenic effects of adoptively transferred anti-myelin specific lymphocytes [37]. Other possible receptors linking to murine FcRγ, and by extension Syk, include NKR-P1 in NK cells, Pir-A (LILRA family of proteins in humans) on dendritic cells, mast cells, macrophages and platelets, and possibly the T cell receptor (TCR) complex in γδ T cells [37]. Thus, Syk inhibitors may also be able to dampen, directly or indirectly, the inflammatory action of autoantigen-specific effector T lymphocytes.

SLE is a chronic relapse and remitting autoimmune disease that affects multiple organ systems including the skin, joints, kidneys and nervous system. It initially manifests as fever, malaise, joint pains, myalgias and fatigue, and is virtually always accompanied by the presence of a diverse but characteristic set of autoantibodies [38]. In addition to organ-specific damage mediated by IC-triggered macrophage and neutrophil activation, Syk can potentially affect SLE pathophysiology in several other ways. The genetic contributions to disease susceptibility and severity are complex and involve multiple traits that implicate abnormalities of both the innate and adaptive immune systems [38]. Microarray studies with peripheral blood leukocytes from SLE patients have helped identify ''gene signatures'' typically found as a result of the action of type I interferons (IFNs) [39,40]. Interestingly, ICs containing nucleic acids, which are commonly found in SLE affected blood, were shown to be potent stimuli of IFNα production by plasmacytoid dendritic cells (pDCs) [41]. As expected, this IFNα production required intact FcγRIIa, which is known to signal through Syk. The importance of Syk function in SLE is further substantiated in the literature including reported affects on ITAM-bearing ezrin/radixin/moesin (ERM) signal transduction in T cell tissue invasion, and FcγRIIIa alleles predictive of progression to end-stage renal disease [38,42–44]. Treatment with **4** an oral small molecule inhibitor of Syk demonstrated delays in disease progression and improvements in survival in NZB/W lupus prone mice [45].

4.3 Oncology

In the proper cellular context in B cells, NK cells and mast cells, Syk kinase has been shown to transduce cell growth and survival signals by activating PI3K/Akt and Ras/mitogen-activated protein kinase pathways [3,46]. When deregulated, Syk activity can promote myelodysplasias, leukemogenesis, and perhaps viral-mediated cellular transformations [47,48]. By contrast, Syk has also been proposed to function as a tumor suppressor [49]. This is however, based on

functional studies and associations of debatable significance, and has been addressed in more detail elsewhere [50].

4.3.1 B-Cell lymphoma and leukemia

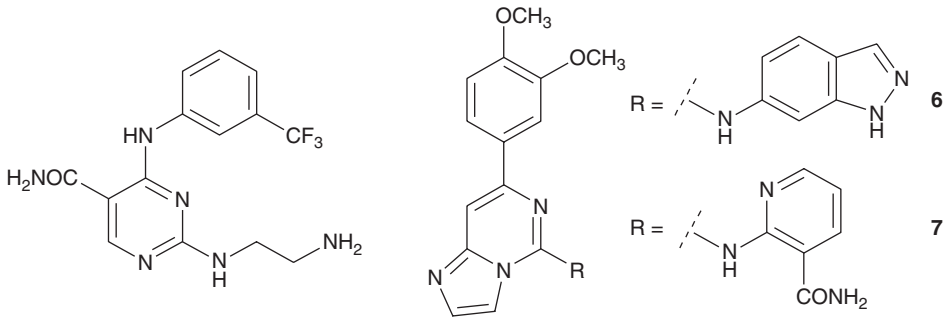
Several studies implicate Syk kinase in the transformation of lymphocytes. A TEL-Syk fusion demonstrating constitutive Syk kinase activity and capable of transforming B cells was isolated from a myelodysplastic syndrome patient, and an analogous ITK-Syk fusion was isolated from a T-cell lymphoma patient [51,52]. Constitutively active Syk was also observed and was required for the growth of various B-cell lymphoma cell lines and primary isolates [53]. Similarly, Syk-mediated activation of the mTOR pathway was required for proliferation of follicular lymphoma cells [54], and Syk DNA amplification and Syk protein overexpression were observed in mantle cell lymphoma [55]. On the other hand, overexpression of protein tyrosine phosphatase receptor-type O truncated (PTPROt), a negative regulator of Syk activity, inhibited lymphoma cell proliferation [56]. Pharmacologic Syk kinase inhibition was also sufficient to block c-Myc-mediated transformation of pre-B cells [57]. Thus, it appears Syk functions as a proto-oncogene in hematopoietic cells, and therefore offers a potential target in the treatment of certain leukemia and lymphoma cancers.

5. STATUS OF SMALL MOLECULE INHIBITORS OF SYK KINASE

5.1 Pre-clinical Syk kinase inhibitors

Only a handful of pertinent Syk kinase inhibitors have been reported. One such example has been a family of pyrimidine-5-carboxamides [58]. Structure activity relationship (SAR) studies around this series generated compound **5**, which potently inhibited Syk kinase with $IC_{50} = 0.041\,\mu M$, and showed selectivity over Zap-70, Itk, Btk, PKCε, and PKCβ2 with $IC_{50}s = 11.9$, 22.6, 15.5, 5.1 and 11 μM, respectively. Compound **5** inhibited 5-hydroxytryptamine (serotonin, 5-HT) release from rat basophilic leukemia (RBL-2H3) cells with an $IC_{50} = 0.46\,\mu M$. This activity can be construed as a measure of inhibition of basophil degranulation [58]. Investigation of **5** in the passive cutaneous anaphylaxis (PCA) reaction in mice showed efficacy in a dose-dependent manner with an $ID_{50} = 13\,mg/kg$ compared with an $ID_{50} = 10\,mg/kg$ for ketotifen administered via sub-cutaneous delivery as the positive control [58].

Compound **6** inhibited Syk kinase with $K_i = 0.01\,\mu M$, and less potently inhibited Lyn, Fyn, Src, Itk, Btk, PKCα and PKCθ with $K_i s = 0.75$, 2.05, 5.0, >9.5, >10, >15 and $>15\,\mu M$, respectively, and blocked antigen presentation of IC in mouse bone marrow-derived dentritic cells [59]. Inhibition of IL4 production ($EC_{50} = 1.08\,\mu M$) was measured to ascertain inhibition of antigen presentation of IC by dendritic cells to T cells.



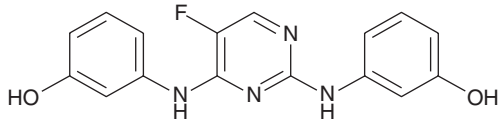
BAY 61-3606 **7**, is from the same class as **6**, with introduction of a nicotinamide group [60] in place of the 1H-indazolyl ring [59]. Analog **7** is an imidazopyrimidine derivative which potently inhibited Syk kinase with a Syk $K_i = 0.0075 \pm 0.0025\,\mu M$, and showed selectivity over Lyn, Fyn, Src, Itk and Btk with K_is > 5.4, 12.5, > 6.25, > 4.7 and $> 5.0\,\mu M$, respectively. Notably, in human cultured mast cells (HCMC), **7** inhibited FcεR1-mediated release of histamine, tryptase, PGD_2, $LTC_4/D_4/E_4$, and granulocyte-macrophage colony-stimulating factor (GM-CSF) with $EC_{50} = 0.0051$, 0.0055, 0.0058, 0.0033 and $0.2\,\mu M$, respectively. Oral delivery of **7**, in dose dependent fashion inhibited the PCA reaction on mast cell-mediated type-I allergic reactions in rats with an $ED_{50} = 8\,mg/kg$. The data shown for compound **7** is impressive, and IgG-mediated activity in addition to IgE suggests application for both allergic and autoimmune diseases. Even with these attributes, no further progress on **7** has been reported [60].

A number of other small molecule Syk kinase inhibitors have been reported in the literature [61–64]. Since there are no reports of detailed evaluation or animal efficacy data, some of these inhibitors may not have advanced further.

5.2 Syk kinase inhibitors which have progressed to clinical studies

R112 (**8**) is the first small molecule inhibitor of Syk kinase that has advanced to Phase 2 clinical trials [22,65]. Compound **8** inhibited Syk kinase with $K_i = 0.096\,\mu M$, selectively inhibited tryptase release from human mast cells induced by anti-IgE cross-linking, histamine from basophils, and dust mite allergen induced histamine release from human basophils with EC_{50}s = 0.353, 0.28 and $0.49\,\mu M$, respectively. One advantage of compound **8** was its rapid onset of action since rapidly effective treatment is highly desirable during an allergic attack. Compound **8** also inhibited leukotriene C4 (LTC_4), proinflammatory cytokines, including tumor necrosis factor α (TNFα), GM-CSF and IL-8, with EC_{50}s = 0.115, 2.01, 1.58 and $1.75\,\mu M$, respectively. Compound **8** was advanced to a Phase 2, double-blind, randomized, placebo-controlled, parallel-group seasonal allergic rhinitis trial in a park environment over a period of 2 days at two sites [65]. Patients received 6 mg of compound **8** b.i.d via metered spray pump delivery to each nostril. The results from these studies demonstrated that the

group receiving **8** experienced a 23% decline in clinical symptoms (stuffy nose, itchy nose, sneezes, cough and headache) as compared to the placebo control group, with overall p value of <0.0005. In order to assess activity with existing therapy, a second 7-day, Phase 2 study was conducted with placebo, beclomethasone spray and **8** at similar doses to the first study but with greater separation in dosing intervals. During this study, **8** did not differentiate from placebo control, mainly due to insufficient dose coverage believed to be needed to elicit efficacy. Based on the first study, topical intranasal delivery of a Syk kinase inhibitor to allergic rhinitis patients exhibited significant amelioration of symptoms, which included cough, facial pain, and headache as well as itchy and runny nose, sneezing and nasal congestion [65].



8

An orally bioavailable prodrug of **4** is currently in clinical trials for treatment of RA and immune thrombocytopenia purpura [33,34,66]. This compound potently inhibited all Syk-dependent cell-based assays, including Fc receptor signaling in human macrophages, neutrophils, mast cells and B-cell receptor signaling in human B cells. Selectivity was demonstrated by the examination of inhibition of phosphorylation in cells, and with a panel of off-target Syk-independent cell-based assays. Inhibition of Flt3, Jak and Lck, was also observed, which for inflammatory processes might be considered to be favorable [34]. Animal models were conducted that could be directly correlated to IC-mediated inflammatory processes proven to be dependent on activating Fcγ receptors [33–35]. Such models included the passive Arthus reaction and passive anti-collagen type II antibody-induced arthritis (CAIA) models [33–35]. Based on the compound's biological potency, selectivity, PK characteristics, safety and efficacy in IC animal models, it was tested in a double-blind placebo-controlled ascending single dose randomized study in normal healthy male volunteers [34]. Maximum concentrations were attained in a dose proportional manner up to a dose of 400 mg, and attained in 1.2–1.3 hours post-dosing, with a half-life of approximately 15 hours. During this first-in-human (FIH) study, basophil activation was measured as a biomarker by using heparinized blood from the volunteers. Stimulation *ex vivo* with anti-IgE and degranulation was measured as CD63 cell-surface upregulation on basophils by flow cytometry. The CD63 cell-surface expression was inhibited in a dose dependent manner, and the extent of the CD63 inhibition was directly correlated to increasing concentrations of the drug for as long as 24 hours post dosing. Basophil activation was reduced by 50% at a concentration of 496 ± 42 ng/mL which translated to an $EC_{50} = 1.06\,\mu M$ [34]. This preliminary data provided some initial confirmation of a PK/PD relationship in humans for this class of molecules [34].

6. CONCLUSION

Syk kinase as a therapeutic target appears to be relevant in a number of diseases and offers an opportunity for the discovery and development of novel therapeutic agents [3–10,16]. There is ample potential for the discovery of efficacious small molecule antagonists of Syk kinase, but an individual molecule may need to achieve significant results in the clinic before others enter the field [34]. To this end, Phase 2 results are eagerly anticipated for a prodrug of compound **4** in clinical trials for RA and immune thrombocytopenia purpura. The outcome of these clinical studies will provide further incentive to pursue inhibition of Syk kinase as a therapeutically robust drug discovery target.

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CHAPTER 25

Systems Biology and Kinase Signaling

Bruce Gomes and **David de Graaf**

Contents

1. COMPLEXITY AND SCOPE IN SYSTEMS KINASE BIOLOGY

Systems Biology is the integration of high throughput biology measurements with computational models that study the projection of the mechanistic characteristics of metabolic and signaling pathways onto physiological and pathological phenotypes. Traditional approaches to drug discovery use isolated cell systems that overexpress or knock-out single proteins to mimic some features of the disease phenotype. While this approach is a valuable tool, it underestimates the importance of the connections that the protein receives as the result of its presence in the cellular milieu. Systems Biology tries to provide a holistic picture of a biological system, at the cellular, organ, or organism level, by integrating information from the network of signaling pathways used to transform stimuli into a phenotypic response. In the case of signal-transducing kinase enzymes, one of the fundamental motivations of systems biology is to understand kinases in the context of their surrounding connections. These connections arise from various factors, which can be posed as questions about the kinase: How do the immediate preceding and succeeding proteins in the signaling cascade influence a particular kinase in a signaling pathway? How is the kinase influenced by the rest of the signaling pathway (i.e., feedback)? What connections to other

Systems Biology Group, Research Technology Center, Pfizer Inc., Cambridge, MA, USA

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pathways (i.e., cross talk) modify the flux of information transfer? How do the transcriptional events that are result of the signal transduction modify the persistence, amplitude and intrinsic time constants of the signal? How does intracellular compartmentalization change kinase dynamics? How do different cells differentially modulate these processes? How does acute or chronic disease change the components of signaling?

The first level of complexity in the understanding of kinase signaling is to correctly assign the pathway topology of connections in the pathway. In most cases, researchers build kinase signaling pathway models based on historically well-defined canonical pathways (An example of a database available to the public is the Signal Transduction Knowledge Environment [STKE] at stke.sciencemag.org). The details of the models, such as kinetic parameters and protein concentrations are either determined experimentally or gleaned from the literature. However, it is extremely rare to find literature-derived models that are built from information that comes from a single cell type or consistent cellular phenotype. This can hamper the overall certainty of a model.

Computational methods have been applied to determine the connections in systems that are not well-defined by canonical pathways. This is either done by semi-automated and/or curated literature causal modeling [1] or by statistical methods based on large-scale data from expression or proteomic studies (a mostly theoretical approach is given by reference [2] and a more applied approach is in reference [3]). Many methods, including clustering, Bayesian analysis and principal component analysis have been used to find relationships and ''fingerprints'' in gene expression data [4].

In theory, this information could be used to understand how disease modifies these pathways and to predict the effects of therapeutic intervention with drugs. In practice, however, the high noise in patient data, poor correlation to protein expression levels, and a large background of ''housekeeping'' expression changes that are only peripherally important to the central disease process mitigate against this. Similar methods have been applied to proteomic data in order to ameliorate some of these problems. However, proteomic data require a much greater effort to produce and is generally not amenable to the kind of large-scale efforts that are routine in expression analysis.

2. POSITIVE AND NEGATIVE FEEDBACK LOOPS

Positive and negative feedback loops are a means of tuning the dynamics of the signal flux in signaling pathways, which must respond to external stimuli while operating in noisy environments [5]. The MAPK pathway, which transduces the binding of growth factors to their cell surface receptors through the cytoplasm to transcription factors in the cell nucleus, supplies several examples of feedback loops (see Figure 1). An example of a negative feedback loop is provided by the MAPK phosphatase, MKP. Activation of the MAPK signaling pathway is initiated by binding of growth factors to receptor tyrosine kinases. After a number of signaling steps, ERK is phosphorylated by MEK. Phosphorylated ERK, ERK-P, has a number of nuclear transcription factor targets, whose activation leads to

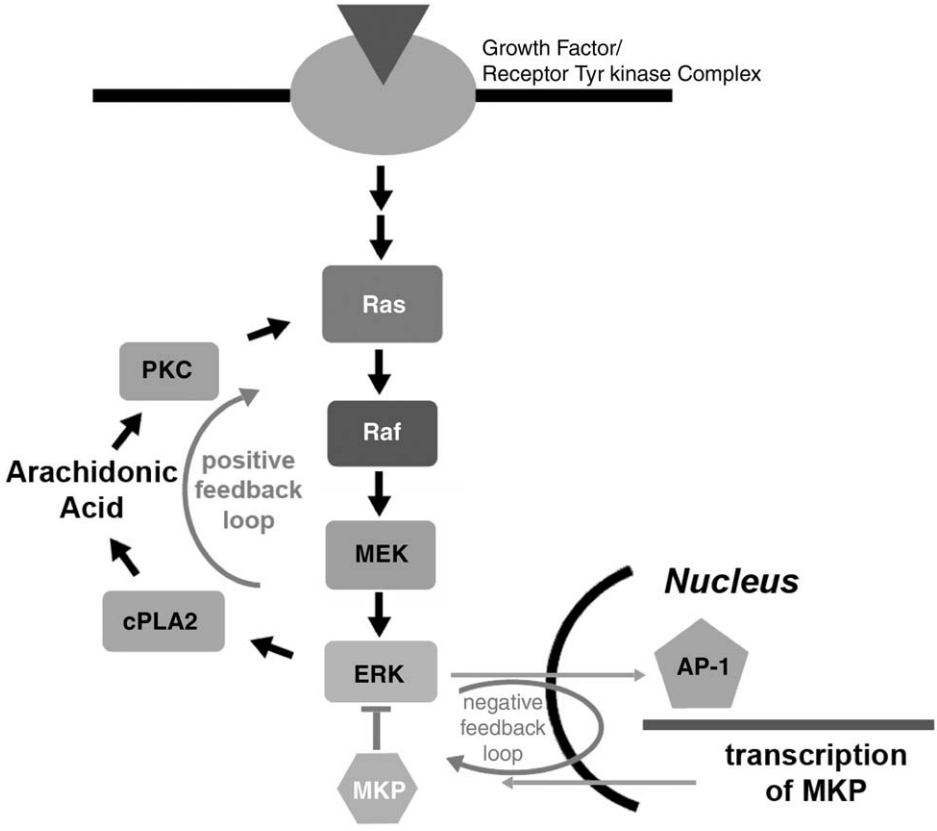

Figure 1 The MAPK pathway and its connections to other signals: A negative feedback loop connects the phosphorylated endpoint of the pathway ERK (Extracellular-signal Regulated Kinase) to the transcriptionally-driven synthesis of the phosphatase, MKP MAP kinase phosphatase. MKP then de-phosphorylates ERK to shut down the signaling cascade. The positive feedback loop again starts with the terminal kinase ERK which activates cPLA2 (cytosolic phospholipase A2). This leads to the synthesis of arachidonic acid, which, in turn activates protein kinase C (PKC). PKC is a positive regulator of RAS (Please see Color Plate Section in the back of this book).

transcription and translation of proteins, including MKP. MKP is both a tyrosine and threonine phosphatase for ERK-P. MKP phosphatase action deactivates ERK-P and hence is part of a negative feedback loop, whose function is to limit the MAPK pathway signaling [6]. The delay due to the transcription/translation machinery leads to a profound effect on the pathway dynamics and serves to limit the response once signaling through the receptor tyrosine kinase is curtailed.

The same MAPK network also contains an example of a positive feedback. ERK also activates cPLA-γ which generates arachidonic acid that in turn activates PKC. PKC stimulation of Ras, which is on the main trunk of the MAPK pathway, completes the positive feedback loop (see Figure 1). It has been suggested that

coupled positive and negative feedback loops with different response kinetics are an essential part of signaling pathways [7].

Both positive and negative feedback loops are common in many kinase pathways. For example, IκBα is responsible for strong negative feedback in the NF-κB pathway [8] and, in T-cell activation, IκB and Bcl10 represent a negative feedback loop [9]. Src, which participates in regulation of multiple kinase signaling cascades is a target for both positive and negative feedback control from PTPα and CSK, respectively [10]. The SOCS proteins are transcriptionally controlled by activation of the JAK/STAT pathway, and serve as a negative feedback loop. Positive and negative feed-forward loops also exist in kinase signaling, but these are less well characterized [11].

To understand the connectivity of signaling pathways, large scale, high-throughput assemblies of data are interrogated with probabilistic methods like Bayesian analysis [12]. Extracellular signals or cues, that may hit multiple canonical kinase pathways, are correlated with their influence on modulation of measured signals (i.e., phosphorylated kinases) and observed phenotypic responses (e.g., the extent of apoptosis, secretion of a cytokine, etc.). The Bayesian analysis then produces the most likely arrangement of connections in the network. In theory, this information could be used to build the minimum mechanistic model that could be used for quantitative prediction in drug design. It would, in effect, draw the boundaries of what kinase pathways explain the majority of the signals that gives rise to the phenotype. The PLS (partial least squares) method and PCA (principal component analysis) have been used to investigate the cue – signal – response paradigm of TNF-induced apoptosis [13,14]. These studies concluded that there were hidden autocrine reactions of TGFα and IL-1α that contribute to the apoptotic machinery for TNF-induced apoptosis. Without this kind of analysis these conclusions were non-obvious, and the contribution of these autocrine reactions was given a quantitative basis.

Once a mechanistic model of a pathway has been constructed, different methods of sensitivity analysis can be applied [15]. Sensitivity analysis is a means of showing the impact of each parameter in the model on output. It can also indicate where in the pathway the greatest effect of drug intervention would be expected, which could be beneficial to the drug discovery process.

3. AN EXAMPLE OF THE PRACTICAL APPLICATION OF SYSTEMS BIOLOGY: ErbB SIGNALING

The inherent complexity of the ErbB receptor signaling system and its strong linkage to cancer has turned this signaling pathway into a paradigm of Systems Biology research. The ErbB family consists of four tyrosine kinase receptors, each able to dimerize with itself as well as the other ErbBs. ErbB1 (EGFR, Her1) is activated by a family of growth factors and domains, including such molecules as EGF and TGF-alpha and can be activated through homodimerization, similar to the ErbB4 (Her 4) receptor (extensively reviewed in [16]) (see Figure 2). The ErbB4 receptor has a distinctively different pallet of growth factor activators, such as the nuregulin family of growth factors including heregulin. The ErbB2

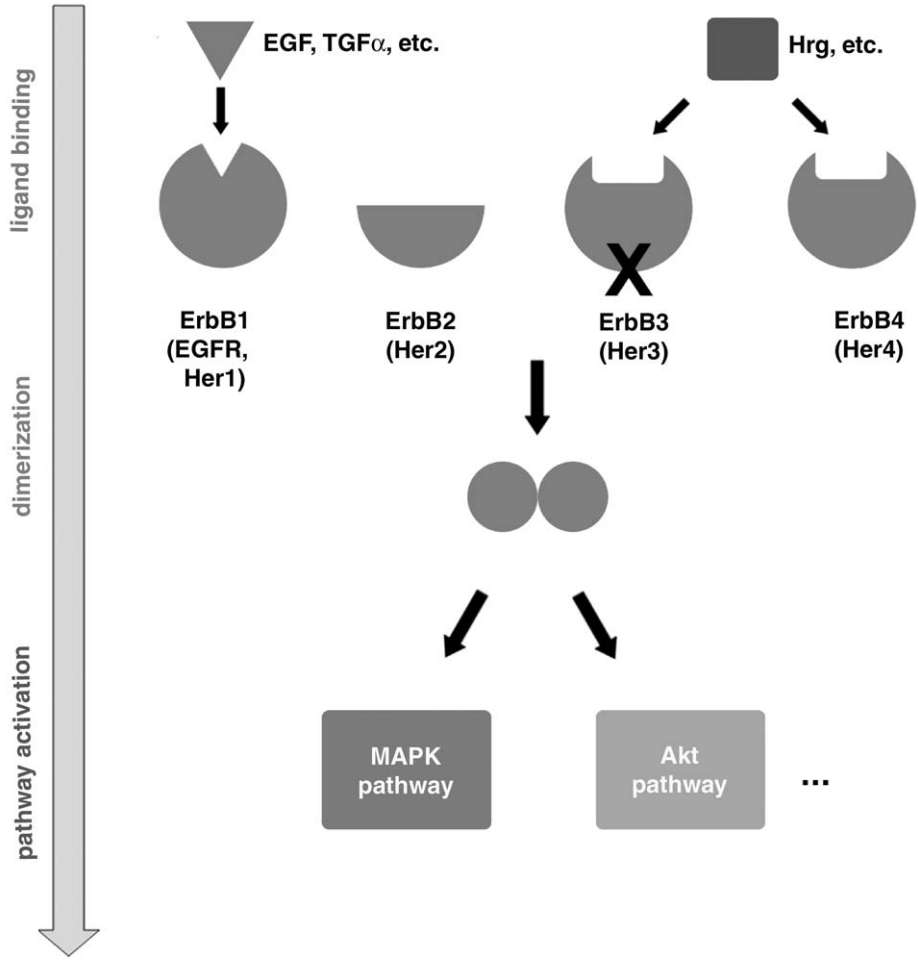

Figure 2 The ErbB signaling network. There are four forms of ErbB monomers. ErbB1 binds EGF and other ligands. ErbB2 has no binding site but does dimerize. ErbB3 binds ligands such as Heregulin (Hrg) but does not have any kinase activity (though it does dimerize). ErbB4 binds many of the same ligands as ErbB3, but does have kinase activity. After binding ligands all the monomers can homodimerize or heterodimerize. Dimerization leads to activation of multiple canonical signaling pathways (Please see Color Plate Section in the back of this book).

(Her2/neu) receptor does not seem to have ligand binding capability, but can function as a heterodimerization partner for other ErbB receptors, and the ErbB3 (Her 3) receptor has a highly impaired catalytic domain, although it can be activated through heterodimerization by the same ligands as the ErbB4 receptor. The combinatorial complexity of potential receptor dimers is driven both by the available ligands and by receptor expression which differs significantly between cell types in diseased versus normal tissue. In addition, mutations have been identified in these receptors in the diseased state and add to the combinatorial

complexity [17]. Once activated, homo- and heterodimers can drive distinct downstream signaling pathways leading to different cell behavior (reviewed in reference [18]).

Isolation of particular features of this cell regulatory system may provide a platform for decision-making in the context of drug discovery and development. This work is facilitated by the first generation of ErbB-targeted therapies (erlotinib, gefitinib, cetuzximab, herceptin, EGF-ABX) that have already reached the clinic [19]. As such, they provide a good case study for the predictive ability of these approaches through direct clinical validation.

In addition to the approaches covered in a recent review [20], the first comprehensive model covering receptor dimerization and internalization has been recently described [21]. In this work, the authors use direct time-dependent measurement of the phosphorylation of the four ErbB receptor species, as well as protein quantitation to develop a comprehensive mechanistic model of receptor dimerization and internalization. Once this comprehensive, quantitative framework was developed, the authors were able to show that receptor dephosphorylation, a key step in the downregulation of ErbB-driven signaling, was restricted to intracellular compartments.

It is still difficult, however, to put such effects as specific ErbB inhibition, pH dependence or receptor internalization in the context of their ultimate physiological consequence. A recent journal article [22] addresses this specific issue by comprehensively measuring cellular signaling under different ErbB-directed stimulation conditions and correlating these signals with cell migration and proliferation. Using combinations of ligands in cell lines with variables levels of the ErbB2 receptor, relevant intracellular signals were measured using a comprehensive phosphoproteomics-driven approach. With a variety of statistical modeling techniques, the individual contributory roles of the ErbB receptors were analyzed. Interestingly, specific dimerization events dictate phosphorylation events that correlate tightly with cell migration and proliferation, thus providing proof that cell-based phenotypes relevant for disease intervention can be tied to specific molecular events which in turn are amenable to drug-based interventions.

The relevance of cell-based phenotypes as markers for disease is still somewhat speculative. The only firm link between patient-directed therapeutic intervention and response was provided through genetics. Two ErbB1-targeted therapies, erlotinib and gefitinib, were found to have objective responses in the treatment of NSCLC (non-small-cell lung carcinoma), but those responses were relatively rare. Two groups published findings on a correlation between objective responses in NSCLC and mutations, mostly in the kinase domain of the ErbB1 receptor [23]. Although this finding provided a basis for deciding which patients should receive ErbB-based therapy, it did not explain how these mutations predisposed to drug response or why they originated. Hendriks *et al.* [21] addressed this issue, using a Systems Biology approach, by computationally identifying parameters that could explain the molecular features of the state shift between mutated and wildtype receptors as identified by Lynch *et al.* [24]. The authors then used imaging techniques to validate the modeling. Among others, they had

identified an attenuation of receptor phosphorylation upon stimulation and an attenuation of the AKT signal, a well-known inhibitor of apoptosis, or programmed cell death, commonly upregulated in cancer. Using these changes as a starting point, the authors identified receptor internalization as a critical parameter. Upon comprehensive testing of a number of cell lines with and without mutations in the ErbB1 receptor which predisposed to a response to gefitinib, Hendriks *et al.* [21] showed that the mutated receptors had a defect in ligand-induced internalization. Since these mutations exist before drug treatment, this may indicate that the mutation itself plays a role in the etiology of the disease, and may well involve AKT, a known pro-survival kinase [25]. By increasing signaling through AKT, which was generally shut off by internalization of the receptor, these cells became 'addicted' to this pro-survival signal (reviewed in [26]). Extrapolating from this work, one could surmise that other processes which impair ErbB1 receptor internalization may play a role in disease etiology. Indeed, heterodimers between ErbB1 and ErbB2 and between ErbB1 and ErbB3 are known to have much slower internalization kinetics and upregulation of ErbB2 and ErbB3 has been associated with gefitinib drug response in NSCLC and other cancers (reviewed in [27]). For kinase drug discovery, these papers provide a blueprint for a comprehensive *ab initio* assessment of drug intervention and its consequences for drug efficacy.

4. CONCLUSION

Systems Biology enables a better understanding of the complexity of cell signaling pathways and offers potential insight into targets for disease intervention. It has a solid foundation in both experimental cell biology and computational methods. It can explain the effect of feedback loops on signaling pathways and can be extended to modeling entire systems such as in the case of the ErbB receptors. Adoption of systems approaches in the wider scientific and pharmaceutical communities will find new applications and new methods to further enhance the value of this approach in drug discovery and development.

5. ACRONYMS USED

Akt	v-Akt murine thyoma viral oncogene homolog 1, protein kinase B
Bcl10	B-cell leukemia/lymphoma 10
cPLAγ	phospholipase A2, cytoplasmic phospholipase gamma
CSK	cytoplasmic tyrosine kinase or c-Src tyrosine kinase
ErbB	avian erythroblastic leukemia viral oncogene homolog, epidermal growth factor receptor
Erk	extracellular signal-regulated kinase 1
IκBα	nuclear factor of kappa light chain gene enhancer in B cells inhibitor
IL-1	interleukin 1
JAK	Janus kinase
MAPK	mitogen-activated protein kinase

MKP	MAPK phosphatase
NF-κB	nuclear factor-kappa B
PKC	protein kinase C
PTPα	protein-tyrosine phosphatase alpha
Ras	rat sarcoma viral oncogene homolog
SOCS	a family of proteins called suppressors of cytokine signaling
Src	avian viral oncogene homolog
STAT	signal transducer and activator of transcription
TGFα	transforming growth factor alpha
TNF	tumor necrosis factor

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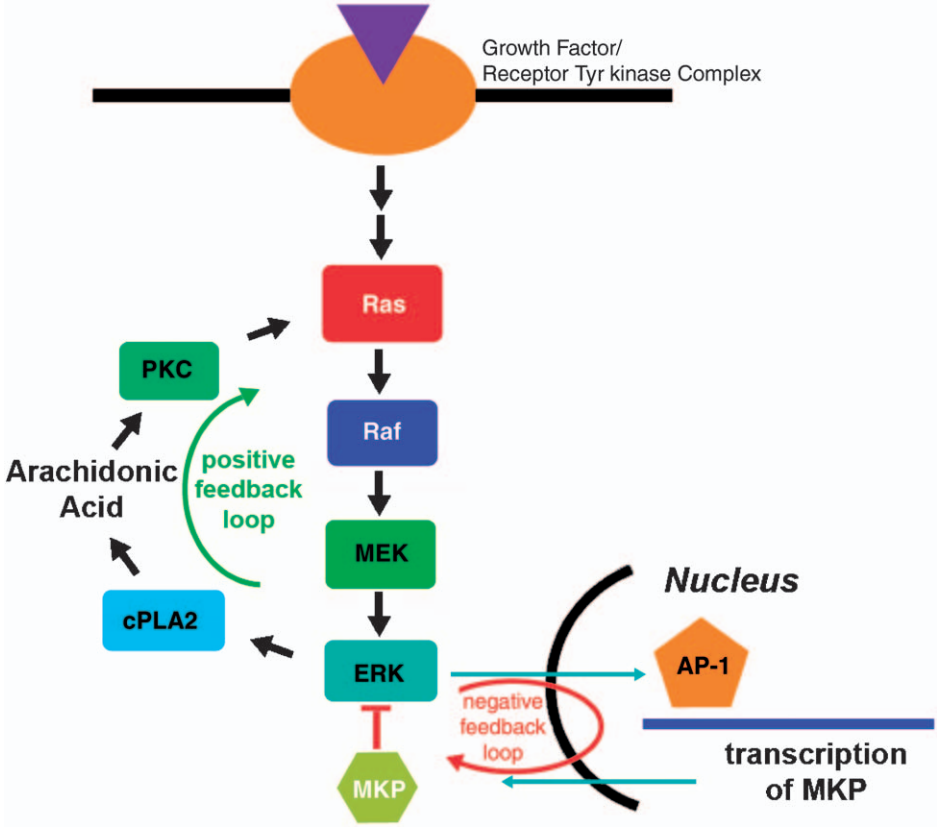

Plate 2 The MAPK pathway and its connections to other signals: A negative feedback loop connects the phosphorylated endpoint of the pathway ERK (Extracellular-signal Regulated Kinase) to the transcriptionally-driven synthesis of the phosphatase, MKP MAP kinase phosphatase. MKP then de-phosphorylates ERK to shut down the signaling cascade. The positive feedback loop again starts with the terminal kinase ERK which activates cPLA2 (cytosolic phospholipase A2). This leads to the synthesis of arachidonic acid, which, in turn activates protein kinase C (PKC). PKC is a positive regulator of RAS (For Black and White version, see page 395).

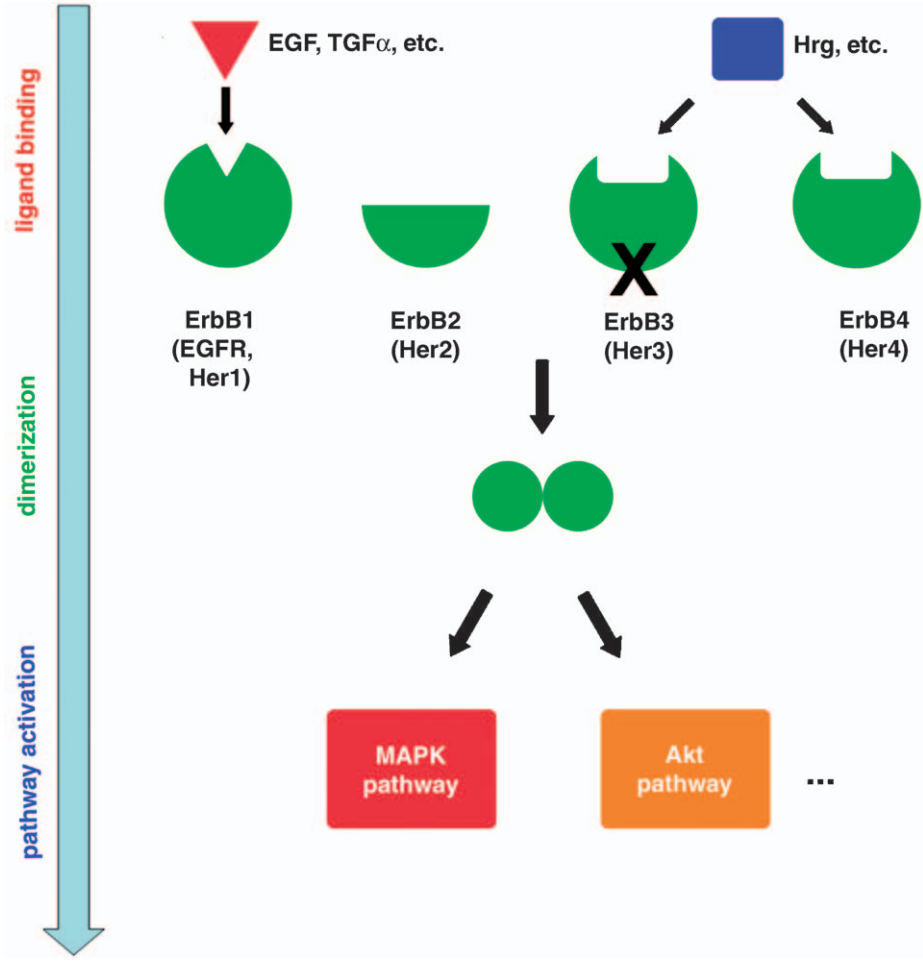

Plate 3 The ErbB signaling network. There are four forms of ErbB monomers. ErbB1 binds EGF and other ligands. ErbB2 has no binding site but does dimerize. ErbB3 binds ligands such as Heregulin (Hrg) but does not have any kinase activity (though it does dimerize). ErbB4 binds many of the same ligands as ErbB3, but does have kinase activity. After binding ligands all the monomers can homodimerize or heterodimerize. Dimerization leads to activation of multiple canonical signaling pathways (For Black and White version, see page 397).

CHAPTER 26

The Molecular Libraries Screening Center Network (MLSCN): Identifying Chemical Probes of Biological Systems

Donna M. Huryn* and **Nicholas D. P. Cosford****

Contents

* Pittsburgh Molecular Libraries Screening Center, University of Pittsburgh, Pittsburgh, PA 15260 and Penn Center for Molecular Discovery, University of Pennsylvania, Philadelphia, PA 19104, USA
** San Diego Center for Chemical Genomics, Burnham Institute for Medical Research, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA

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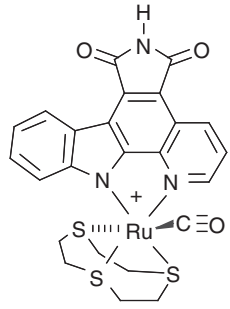
1. INTRODUCTION

1.1 Goals and overview of the MLSCN

The NIH Molecular Libraries Screening Center Network (MLSCN) is a subset of the Molecular Libraries Initiative (MLI) component of the NIH Roadmap for Medical Research [1,2]. It consists of a consortium of 10 centers, each having expertise in assay development, high-throughput screening (HTS), chemistry and informatics. Using a centralized screening library of approximately 100,000 small molecules and assays from the research community, scientists at each center optimize assays, carry out high-throughput screens, and deposit the results into PubChem (*vida infra*). Based on the data from those assays, chemists at each center further optimize the initial hits in order to develop unique, small molecule probes of biological systems [3]. Informaticists contribute to data handling and analysis throughout this process. The chemical probes developed are available to researchers (both public and private sectors) via data deposition into PubChem, and in the future, via access to samples and synthetic protocols to prepare the probes. These efforts support the ultimate goals of the MLSCN and the MLI, which is to ''expand the availability, flexibility, and use of small-molecule chemical probes for basic research'' [1].

A number of aspects of the MLSCN make this initiative unique from other academic screening centers [4,5], as well as from screening and lead optimization activities being undertaken at pharmaceutical and biotechnology companies. First, all researchers (public and private sector) have access to the screening centers through the NIH X01 and R03 funding mechanisms [6]. Second, due to the diverse source of assays and the wide expertise available within the MSLCN, specific biological systems investigated and screened will include: (a) ''high risk'' targets, that is proteins or biological systems whose function is unknown; (b) targets implicated in orphan diseases or diseases not typically addressed by the private sector; (c) novel or uncommon assay systems (e.g., zebrafish, high content screening); and (d) ''non-druggable'' targets, such as inhibitors of aggregation and protein–protein interactions. Third, the small molecule screening library contains structures not typically found in commercial collections or those housed in pharmaceutical companies. Sources of these unique structures include natural products and novel compound libraries prepared by academic investigators through the Pilot Scale Libraries (PSL) granting mechanisms [7], compounds generated by the Centers for Chemical Methodologies and Library Development (CMLD) (*vida infra*) [8] and those obtained through solicitation by the NIH [9]. Fourth, as the goal of the MLSCN is to develop selective chemical probes and small molecule tools that will interrogate novel biochemical pathways, the criteria for an acceptable class of molecules is broader for the MLSCN than for those involved in drug discovery and development. Therefore, chemical probes are not subject to the same constraints on physical properties, functional groups or metabolic profiles that are common in the pharmaceutical industry, and necessary for successful clinical candidates. An example of a compound that would fit the definition of a valuable chemical probe, but which would not

adhere to the commonly prescribed criteria of ''drug-like'' is a staurosporine-derived ruthenium complex, shown below, which is a selective, sub-nanomolar inhibitor of the kinase, Pim1 [10]. Fifth, and particularly important for this review, is the inclusion of integral medicinal chemistry within each MLSCN Center, that allows the network to produce chemical probes with particular properties, rather than simply identifying apparent activities from the screening collection. Finally, unlike all other screening efforts in both industry and academia, all data are available in PubChem with no delay in publication. In addition to HTS protocols and primary screening results, secondary assay data, ''profiling data'' (e.g., aggregation evaluation, Cytochrome P450 inhibition, spectroscopic profiling, solubility measurements), follow-up compound libraries and their associated biological data, and synthetic protocols are accessible.



Pim1 inhibitor (0.25 nM)

1.2 Description of MLSCN Centers

1.2.1 NCGC

The NIH Chemical Genomics Center (NCGC) [11] is an ultrahigh-throughput screening (uHTS) and chemistry center that applies the tools of small molecule screening and discovery to develop chemical probes for the study of protein and cell functions. Using a process called quantitative high-throughput screening (qHTS), chemical libraries are screened at multiple concentrations (typically seven) to generate a concentration–response curve for each compound that covers a range of five orders of magnitude (typically 1 nM to 100 μM). qHTS comprehensively and efficiently characterizes biological activities of large chemical libraries to yield high-quality datasets for chemical probe development and compound profiling [12]. This process has been applied successfully to both cell-free and cell-based assays. The Kalypsys robotic system uses multimodal detectors, the ViewLux and Envision systems, and a plate-based laser cytometry system (Acumen Explorer) for high-capacity screening (100,000+wells/day) in the reagent-sparing 1536-well plate format. Assay detection capabilities include absorbance, luminescence, fluorescence resonance energy transfer (FRET), time-resolved FRET (TR-FRET), fluorescence polarization (FP), fluorescence intensity (FI), and AlphaScreen^{TM}, as well as cell-based imaging assays that employ

fluorescent proteins such as GFP (green fluorescence protein). The NCGC also develops new paradigms for screening, informatics, and chemical probe development that extend the application of small molecule technology to new areas of the genome. Collaborations with academic investigators worldwide, as well as pharmaceutical and biotechnology companies produces public domain data, thereby allowing sharing of best practices to enable both chemical genomics and downstream drug development. The NCGC accepts chemical libraries for screening from academic and industrial investigators, and produces its own focused libraries for specific projects that are added to the screening collection. With its miniaturized qHTS process, as little as 0.1–0.5 mg of compound will support several years of screening against hundreds of diverse biological assays. The NCGC is part of the intramural NIH program of the National Human Genome Research Institute.

1.2.2 PCMD

The Penn Center for Molecular Discovery (PCMD) [13] approaches the high volume screening challenge with unique capabilities. A key technology for the Center is the ability to print thousands of molecules on a glass surface the size of a business card, and then rapidly test these molecules against proteases and other enzymes purified from human or animal cells, bacteria, parasites, insects, or viruses [14]. Scientists at the Penn Center are also able to test compounds in thousands of miniature wells each containing a millimeter-sized *Danio rerio* (zebrafish), an unlikely organism that has proven its worth in studies of heart and nerve function, as well as in cancer biology, because the transparent fish is easily imaged. The PCMD, based in Philadelphia, is surrounded by local HTS industrial labs including those of Merck, GSK, Wyeth, and Johnson & Johnson. These industrial connections help transfer HTS skills into the university environment of this center.

1.2.3 Emory

The Emory Chemical Biology Center in the MLSCN has the capability to adapt and optimize all target-based and phenotypic assays selected by the MLSCN, but has identified protein–protein interactions for small molecule probe discovery as the Center's theme. With two general screening platforms established, the Center is able to perform both HTS and high content screening (HCS) using a variety of *in vitro* biochemical assays, cell reporter assays, and cell phenotype-based assays. In particular, this center is experienced in assays for monitoring protein–protein interactions and enzyme activities with fluorescence-based assays, including FI, FP, and FRET. Examples of assays that are within this center's capacity include protein–protein interactions (FI, FP, FRET, AlphaScreen^{TM}), enzyme assays (FI, FP, and other coupled assays), receptor–ligand interaction assays (FI, FRET, Ca^{2+} imaging), reporter assays (luciferase, GFP, etc.), viability assays and protein translocation assays (e.g., receptor internalization and membrane and nuclear localization).

1.2.4 PMLSC

In common with the other MLSCN centers, the overall goal of the Pittsburgh Molecular Libraries Screening Center (PMLSC) [15] is to provide the scientific community access to a facility that is designed to optimize, validate, and implement assays for HTS based on optical-based detection methods to identify chemical probes, and to deposit these data into the PubChem database. The PMLSC has been designed for maximum flexibility with regard to target classes and assay formats. They develop and implement cell based, biochemical and model organism [*Danio rerio* (zebrafish) and *Drosophila melanogaster* (fly)] assays preferentially in the 384-well plate format. Existing optical-based detection capabilities include automated HCS imaging platforms, absorbance, FI, FP, time-resolved fluorescence (TRF), FRET, and luminescence. The PMLSC also examines the structure–activity relationships of active small molecules and synthesizes probe molecules that demonstrate significant potency and target selectivity.

1.2.5 SRMLSC

The Southern Research Molecular Libraries Screening Center (SRMLSC) is based at Southern Research Institute (SRI), where more than 20 anti-cancer agents have been discovered and entered into clinical trials, six of which received FDA approval and proceeded to market. The SRMLSC brings extensive drug discovery and development expertise to the network, especially in the areas of cancer, neurological diseases/CNS disorders, and infectious disease (HIV, hepatitis, TB, and emerging pathogens including influenza, H5N1 Avian flu, West Nile virus, and SARS coronavirus). The screening center has broad capabilities to implement any cellular, molecular, or target-based assay including those which require BSL-3 containment, and optimizes or miniaturized them as necessary. The HTS facility is equipped with state-of-the-art instrumentation to screen in up to 1536-well plate format, including two ORCA robotic rails, multiple plate readers, and two Biomek FX liquid handlers, a BioRaptr, and an Echo 550 for nanoliter volume dispensing. In addition, the Center uses a high-speed automated Evotec Opera confocal microscope for high-throughput imaging assays. Data analysis is performed by scientists with expertise in molecular modeling, predictive algorithms, and QSAR analysis, using a robust assortment of chemoinformatics software packages.

1.2.6 SDCCG

The San Diego Center for Chemical Genomics (SDCCG) [16], located in the biotech-rich heart of La Jolla, California, has broad expertise in biochemistry and the ability to run almost any assay type. Specific biological themes include targets involved in regulating cell death, using a variety of biochemical and cell-based assays, with particular emphasis on kinases, phosphatases and proteases. Another area of expertise is in phenotypic assays for stem cell differentiation, using fluorescent reporters. Two main technological themes are incorporated into the SDCCG. First, the Center has special expertise in high-throughput microscopy as a tool for performing high-content, cell-based screens where cellular phenotypes drive compound selection in an unbiased manner. Second, the

Center is unique across the network in having the capability to perform NMR-based small-molecule screening and optimization. NMR-based methods are exceptionally valuable when investigating molecular targets that are not easily tractable by other methods, such as protein–protein interactions and protein targets that cannot be formatted for the classical HTS environment.

1.2.7 Scripps

The Scripps Research Institute Molecular Screening Center [17] spans the Scripps campuses in La Jolla and West Palm Beach, Florida. Scripps has brought together an integrated combination of infrastructure, people and technologies that can support the identification of proof-of-concept small molecules in the academic setting. These small molecules comprise chemical probes of adequate potency, selectivity, physical properties and stability to show robust activities in cell-based assays and *in vivo*, allowing pre-competitive advancement to fields of breaking biology. The center is equipped with a fully automated Kalypsys screening system, with plate hotels and incubators, 200 nL to 20 μL volume dispensing 1536-well aspiration, and 1536 pintool heads for compound delivery for uHTS screening of larger compound decks. Plate readers and detectors include the ViewLux CCD-based plate reader and the EnVision multimode detector equipped with Alpha Screen™. Assay formats include TRF, FP, FI, FRET, luminescence, and absorbance. This center is vertically integrated with enterprise-scale data management and chemoinformatics, high throughput LC/MS for compound quality assurance and rodent pharmacokinetics, and facilities for downstream synthetic follow-up by modular, library or linear chemical approaches. The goal of the Scripps Center is the rapid, collaborative publication of interesting compounds that advance the understanding of biological problems, or illuminate new nodal control points in physiology by short-term chemical perturbation.

1.2.8 Columbia

The strength and experience of the MLSCN Center at Columbia University are in cell biology, high content/high-resolution automated cellular imaging and image analysis, and phenotypic assay design and implementation. The main imaging platform of this center is the INCell Analyzer 3000 (GE Healthcare), a state of the art high throughput cell imaging system. The INCell instrument uses three laser lines for excitation: a Krypton laser (647 nm) and an Argon laser (364 and 488 nm). Three fluorescence channels can be recorded by three independent high-speed 12-bit CCD cameras, and emitted light in the wavelength range from 420 to 720 nm can be captured. Connected to a Kendro Plate Hotel and a Mitsubishi robotic arm, the system can image and analyze 222 plates (96/384 well) without supervision. Depending on the specific assay, up to 50,000 wells can be processed per day. A whole array of different image analysis modules is available, and analysis is performed at high speed on the fly. This imaging system enables the center to screen and analyze a very broad variety of assays monitoring a wide spectrum of biological processes.

1.2.9 New Mexico MLSC

The New Mexico Molecular Libraries Screening Center (New Mexico MLSC) [18] has developed innovative flow cytometry tools for discovery research that enable homogeneous analysis of ligand binding and protein–protein interactions, high throughput sample handling, high content analysis, and real-time measurements of cell response. Using their novel HyperCyt® screening technology, virtually any molecular assembly or cell response can be displayed in a HTS format compatible with flow cytometry, and assessing both cellular and molecular activities of small molecules is possible. Moreover, by creating a suspension array of particles, assays and responses can be highly multiplexed or performed on complex cell populations without loss of throughput. It is likely that no single competing technology offers the versatility of flow cytometry for MLI screening or has the potential of being available to such a large number of laboratories that house flow cytometers (20,000 world-wide). The Center brings together expertise that spans biomedical, biophysical, chemical, computational, instrumentation, and engineering disciplines, and is particularly interested in enhancing the overall discovery process through the integration of physical screening and computational tools that include virtual screening, chemoinformatics, and data mining.

1.2.10 Vanderbilt

The goal of the Vanderbilt Screening Center for G-protein Coupled Receptors (GPCRs), Ion Channels, and Transporters [19] is to enable investigators to discover and develop a new generation of small molecule probes to promote our understanding of physiological and disease processes, with a particular emphasis on the structure and function of GPCRs, ion channels, and transporters. Measurements for biochemical, cellular and cell-free assays are made using a wide variety of commercially available and novel technologies. The suite of detection modalities includes two Hamamatsu FDSS kinetic imaging plate readers. These instruments are capable of collecting data from all wells of 96 or 384 plates simultaneously, and during integrated reagent addition at up to 10 frames per second over wavelengths from UV to far red with dual excitation, emission, and fluorescence polarization modes. The FDSS also supports ultra low-light detection for aequorin and other kinetic/flash luminescence formats. Additionally, the Vanderbilt Center supports high-content screening through the use of the BlueShift Isocyte, a laser scanning fluorimeter that generates two-dimensional anisotropy data. This combination of capabilities paired with robust automation provides tremendous flexibility for measuring the action of a test compound on a wide range of targets. The investment in infrastructure, the combination of basic and industrial research expertise, the dedication to translational and chemical biology, and the establishment and maintenance of a highly collaborative environment make the Vanderbilt Center well suited to support the MLSCN.

1.3 PubChem

PubChem is a comprehensive, publicly accessible database developed by the National Center for Biotechnology Information at the National Library of Medicine

that contains information on the biological activities of small molecules [20]. As of March 2007, PubChem contained more than 15 million records, 10 million unique structures, and data from over 400 assays. The database is linked to other Entrez databases such as PubMed and PubChem Central [21]. All data (e.g., assay results, secondary assays, structures of compounds synthesized) generated within the MLSCN is deposited into PubChem. Access to this range of data on a large library of diverse compounds has enormous potential for use by the Informatics community for the development of computational models, pharmacophore models, and other algorithms to predict biological activities and properties of small molecules.

2. ROLE OF CHEMISTRY IN THE MLSCN

Two distinct aspects of the MSLCN require participation by, and input from chemists: first, synthetic chemistry is a source of compounds within the screening library, and therefore of the assay hits; and second, expertise in synthetic and medicinal chemistry is required to optimize the hits into usable probes of biological systems.

2.1 Molecular Libraries Small Molecule Repository

The Molecular Libraries Small Molecule Repository (MLSMR), managed by Biofocus/DPI (under contract from NIH) collects, maintains, and distributes compound samples to the MLSCN [22]. Compounds, collected from a variety of commercial and non-commercial sources, such as PSL grantees, CMLDs (*vida infra*), and other academic investigators, are filtered through integrity, purity, and physical property criteria before being accepted into the MLSMR. Once accepted, samples are acquired and distributed to each MLSCN Center, and structures and associated data are deposited into PubChem. The approximately 100,000 compounds that currently comprise the MLSMR will grow to 300,000 during 2007, with a goal of reaching up to 1 million compounds over the next decade [23].

2.2 Chemistry cores

Each screening center has medicinal and synthetic chemistry expertise in order to optimize hits identified from HTS campaigns and develop them into chemical probes. Specific capabilities vary, however typical strategies employed include parallel synthesis, computational and informatics analysis, and analytical capabilities such as LC/MS techniques. The structures of novel compounds that are prepared, their synthetic protocols, analytical data and biological data are all available, and samples of final probes developed are deposited into the MLSMR. A Working Group comprised of chemists from each center meets regularly to share information, best practices, and insure optimal use of resources.

2.3 Pilot Scale Libraries and Chemical Methodologies and Library Development Centers

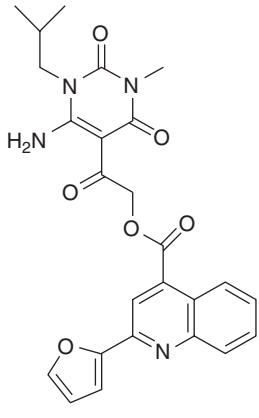
Another key component of the Molecular Libraries Initiative is the development of novel technologies for generating chemical diversity, the application of those

technologies to compound library synthesis or generation, and the deposition of those compounds into the MLSMR. Towards this end, NIH has established two funding opportunities. The objective of the Pilot Scale Library granting mechanism is to generate chemical libraries in order "to increase the diversity and the uniqueness" of compounds contained within the MLSMR [7]. Both synthetic and natural product-derived compounds are included in this initiative. Centers for Chemical Methodologies and Library Development [8] are multi-investigator research programs that develop state-of-the-art synthetic methodologies for the design and production of chemically diverse, small molecule libraries. Both initiatives contribute compounds to the MLSMR.

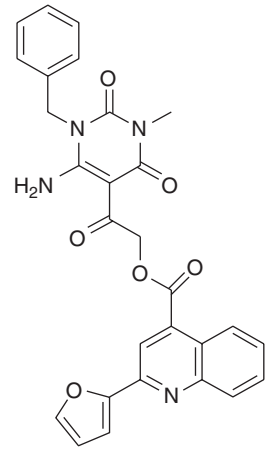
3. NOVEL PROBES FROM THE MLSCN

3.1 MKP-1 inhibitors (PMLSC)

The Pittsburgh Molecular Libraries Screening Center reported the identification of SID 3717140 as an inhibitor of Mitogen-activated Protein Kinase Phosphatase 1 (MKP-1) [24]. This initial hit exhibited only modest potency ($IC_{50} = 19.2\,\mu M$), however it appeared to display some selectivity against other phosphatases, and excellent selectivity against the 52 other targets tested [25]. A small library of compounds was designed and prepared in an effort to identify compounds with improved potency. Towards that end, several new uracil-based compounds, such as SID 14715524, exhibited improvements in potency. MKP-1 is a dual-specificity phosphatase involved in a number of processes related to cell proliferation. The availability of potent, selective and cell permeable probes would help enable a thorough understanding of the role this enzyme plays in cell cycle, signal transduction, oncogenesis, and apoptosis. While small molecule inhibitors of MKP-1 have been previously reported [26], they have been hampered by low *in vitro* potency, lack of cellular activity, and poor selectivity. As such, these uracil quinolines from the PMLSC represent a novel structural class which, based on their promising physicochemical properties, may provide an improvement over those inhibitors previously reported.



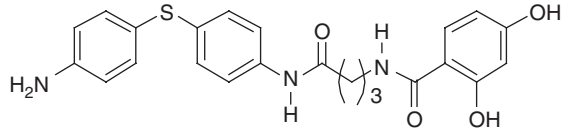
SID 3717140



SID 14715524

3.2 BID inhibitors by NMR-based screening (SDCCG)

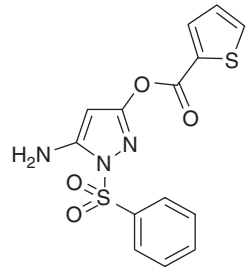
Bcl-2 family proteins play a crucial role in tissue homeostasis and apoptosis (programmed cell death). The BH3-interacting domain death agonist (BID) is a proapoptotic member of the Bcl-2 family, promoting cell death when activated by caspase-8, which cleaves BID to its truncated active form, tBID. NMR-based screening of a library composed of 300 fragments followed by SAR optimization by interligand NOE led to the identification of two chemical fragments that bind on the surface of BID. Covalent linkage of the two fragments provided high-affinity bidentate derivatives such as BI–11A7 [27,28]. *In vitro* and cellular assays showed that these compounds prevent tBID translocation to the mitochondrial membrane and the subsequent release of proapoptotic stimuli, and inhibit neuronal apoptosis in the low micromolar range. These compounds may lead to therapeutic agents with the potential to treat disorders associated with BID activation including neurodegenerative diseases, cerebral ischemia, and brain trauma.



BI-11A7

3.3 Cathepsin B alternate substrate (PCMD)

Screening of over 66,000 compounds from the MLSMR by scientists at the PCMD for inhibitors of Cathepsin B resulted in the identification and characterization of an alternate substrate, SID 16952359 [29]. This study also describes issues relating to the nucleophilicity of dithiothreitol (DTT) and cysteine, reductants frequently used in HTS protocols, and the potential for reactivity with electrophilic sites of probe molecules.



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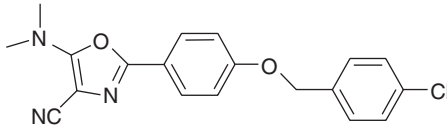
3.4 Pyruvate kinase probes (NCGC)

The development of quantitative high-throughput screening (qHTS) paradigms that provide concentration–response curves for large chemical libraries in a

single experiment is a major focus of the NIH Chemical Genomics Center [12]. This strategy was applied to a screen for inhibitors of the enzyme pyruvate kinase, and allowed SAR development directly from primary screening data, and rapid analysis and triage of active clusters. From this analysis, a class of cyanooxazole inhibitors, exemplified by SID 862236, was identified that exhibited activity in the nanomolar range ($AC_{50} = 30$ nM). Importantly, structurally related analogs exhibited a range of potency from the nanomolar to inactive ranges. Activators were also identified in the same HTS experiment: SID 3712493 activated pyruvate kinase at an AC_{50} concentration of 600 nM [30].



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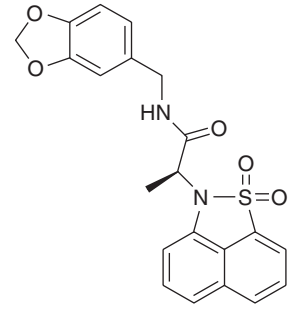
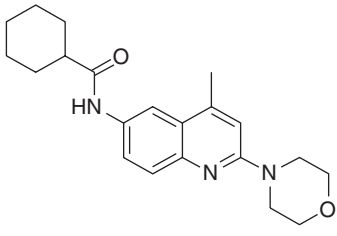


SID 3712493

3.5 Glucocerebrosidase inhibitor probes (NCGC)

Gaucher's Disease is an inherited disorder characterized by deficiencies of glucocerebrosidase activity. An assay to identify small molecule inhibitors of glucocerebrosidase was developed [31], and three probes were identified. NCGC00092410 was identified by testing a series of purchased analogs of an initial hit. SID 4264637 and SID 847960 were members of the initial screening library [32]. Several of these probes have been shown to restore glucocerebrosidase activity in cultured cells from Gaucher patients, a result consistent with the correction of trafficking of misfolded glucocerebrosidase.



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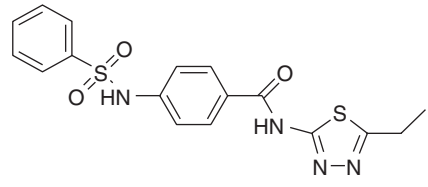

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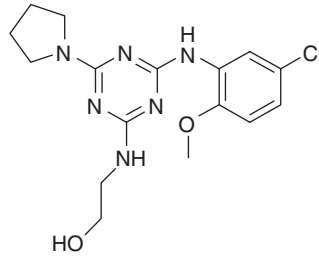

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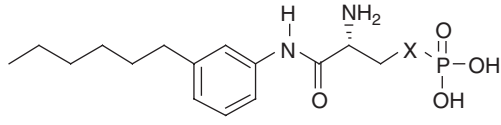
3.6 $S1P_1$ antagonist probe (Scripps)

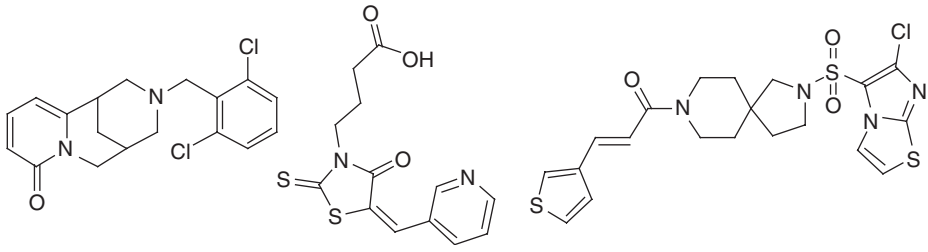
Sphingosine 1-phosphate (S1P) regulates vascular barrier and lymphoid development, as well as lymphocyte egress from lymphoid organs, by activating high-affinity $S1P_1$ receptors. Based on phosphate esters (such as the structure below where X = O), the reversible $S1P_1$ antagonist (X = CH_2) was designed to provide a non-reactive chemical probe with *in vivo* activity [33]. This compound was used to gain mechanistic insights into S1P systems organization not accessible through genetic manipulations and to investigate their potential for therapeutic modulation. Vascular (but not airway) administration of the preferred R enantiomer of this compound induced the loss of capillary integrity in mouse skin and lung, but did not affect the number of constitutive blood lymphocytes. Instead, alteration of lymphocyte trafficking and phenotype required supraphysiological elevation of $S1P_1$ tone and was reversed by the antagonist. *In vivo* two-photon imaging of lymph nodes confirmed requirements for obligate agonism, and the data were consistent with the presence of a stromal barrier mechanism for gating lymphocyte egress. Chemical modulation revealed differences in S1P-$S1P_1$ 'set points' among tissues and highlights both mechanistic advantages (lymphocyte sequestration) and risks (pulmonary edema) of therapeutic intervention.



3.7 SARS CoV inhibitors (SRMLSC)

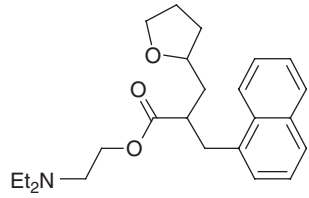
Researchers at SRMLSC recently developed a HTS that allowed the identification of potential inhibitors of the severe acute respiratory syndrome coronavirus (SARS CoV) from large compound libraries [34]. The luminescent-based assay, which measured the inhibition of SARS CoV-induced cytopathic effects (CPE) in Vero E6 cells, was validated with two different diversity sets of compounds against the SARS CoV. The hit rate for both libraries was approximately 0.01%.

The validated HTS assay was then employed to screen a 100,000-compound library against SARS CoV. The hit rate for the library in a single-dose format was determined to be approximately 0.8%. Screening of the three libraries resulted in the identification of several novel compounds that effectively inhibited the CPE of SARS CoV *in vitro*. Three hit compounds, shown below, were identified as promising lead candidates for further evaluation.



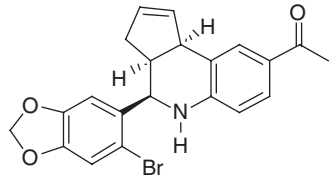
3.8 Pantothenate synthetase inhibitors (SRMLSC)

The team at SRMLSC also recently developed a screen for pantothenate synthetase (PS). PS (EC 6.3.2.1) is encoded by the *panC* gene and catalyzes the essential adenosine triphosphate (ATP)-dependent condensation of D-pantoate and β-alanine to form pantothenate in bacteria, yeast, and plants. Pantothenate is a key precursor for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP). Because the enzyme is absent in mammals, and both CoA and ACP are essential cofactors for bacterial growth, PS is an attractive chemotherapeutic target. An automated high-throughput screen was developed to identify drugs that inhibit *Mycobacterium tuberculosis* PS. The activity of PS was measured spectrophotometrically through an enzymatic cascade involving myokinase, pyruvate kinase, and lactate dehydrogenase. The rate of PS ATP utilization was quantitated by the reduction of absorbance due to the oxidation of NADH to NAD+ by lactate dehydrogenase, which allowed for an internal control to detect interference from compounds that absorb at 340 nm. This coupled enzymatic reaction was used to screen 4080 compounds in a 96-well format. This led to the discovery of a novel inhibitor of PS that exhibits potential as an antimicrobial agent [35].



3.9 GPCR30 antagonist (New Mexico MLSC)

Researchers at the New Mexico MLSC used a combination of virtual and biomolecular screening to discover a selective agonist of GPR30. Estrogen is a hormone critical in the development, normal physiology and pathophysiology of numerous human tissues. The effects of estrogen have traditionally been solely ascribed to estrogen receptor α (ERα) and more recently ERβ, members of the soluble nuclear ligand-activated family of transcription factors. However, it was recently shown that the GPR30 binds estrogen with high affinity and resides in the endoplasmic reticulum, where it activates multiple intracellular signaling pathways. To differentiate between the functions of ERα, ERβ and GPR30, the New Mexico MLSC team used a combination of virtual and biomolecular screening to isolate compounds that selectively bind to GPR30. Further studies led to the identification of the first GPR30-specific agonist, G-1 (shown below) capable of activating GPR30 in a complex environment of classical and new estrogen receptors [36].



G-1

4. FUTURE OUTLOOK AND CONCLUSIONS

The completion of the human genome project, a coordinated effort between government, academia, and industry, has prompted a vast expanse of medical research focused on the understanding of the fundamental causes of human disease. The Molecular Libraries and Imaging initiative is a natural extension of that groundbreaking effort. By providing access to assays, HTS capabilities, small molecule libraries and chemical optimization expertise, novel chemical probes are being developed that will allow the study of gene function, biochemical pathways, and cellular biology. It is also possible that through this initiative, starting points for new drugs, particularly of rare diseases, will be identified. The data deposited into PubChem via this effort will also serve as an unprecedented source of information for scientists in all biomedical disciplines. The availability of large datasets of biological activity on a common set of compounds should serve to stimulate advances in computational and predictive models of biological activities and chemical properties. This dataset should also expedite biomedical research through its use to evaluate selectivity, toxicity, and off-target activities of compounds. The value and success of this initiative may not be obvious or measurable for a number of years, but the adoption of some of the technologies

and approaches (e.g., HTS, hit optimization) typically available only inside the pharmaceutical industry should provide training, opportunities and inspiration to the wider scientific community.

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CHAPTER **27**

The Application of Transcriptional Profiling in Model Connectivity and Lead Assessment in Drug Discovery

Michael Neubauer and **Petra Ross-Macdonald**

Contents

1. INTRODUCTION

Transcriptional profiling, also known as transcriptomics or mRNA expression profiling, has several useful applications in the field of drug discovery. Most recently, there is recognition of its utility for making the connection between compounds that have been optimized through *in vitro* assays and their activity on the *in vivo* biology of the disease process. After outlining the state of the art in obtaining and interpreting transcriptional profiling data, two additional aspects of this tool will be reviewed here. First, we will discuss transcriptional responses in disease models as biomarkers, and their connection to potential pharmacological treatments for disease. Second, we will review the characterization of transcriptional selectivity profiles for potential drugs by comparison of their transcriptional changes. This review is not meant as a literature survey. Rather,

Bristol-Myers Squibb Research and Development, P.O. Box 5400, Princeton, NJ 08543

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the intent of this review is to illustrate with literature examples how transcriptional profiling of pharmacological response can be used to address important issues in drug discovery. Recent reviews have explored the use of transcriptional profiling in mechanism of action studies on mammalian models [1,2], yeast [3–5] and in antibiotics discovery and development [6]. The application of transcriptional profiling as a measure of response to toxicants in model systems, and the use of these responses to classify and predict toxicity in new drug candidates, is termed ''toxicogenomics'' and has also been extensively reviewed [7–11].

2. TRANSCRIPTIONAL PROFILING TECHNOLOGY: STATE OF THE ART, AND FORESEEABLE FUTURE

Transcriptional profiling is the study of changes in cellular mRNA expression by using one of several microarray technology platforms. When applied to cells from tissue culture models or body tissues, the entire transcriptome (all mRNAs known to be encoded in the genome) can now be measured simultaneously. Transcriptional profiling allows comparisons across multiple samples derived from compound treatment and/or genetic manipulation. It is generally recognized as the most advanced technology available for systematic measurement of cellular status on a genome scale [2,12]. One problem with studies that measure a large number of potential changes is that differences can be found just by chance (the multiple measurement issue). However, transcriptional profiling costs have dropped to such an extent that the number of samples that can be queried in a single study is now sufficient to achieve statistically robust conclusions for gene expression changes. Recently, it has become more common to employ sophisticated design of experiment strategies, such as random block design and mixed model ANOVA, to separate the observed responses from any potential artifacts of the experimental process [13]. The growing improvements in annotation of individual gene function and of relationships to cellular processes (gene ontology), combined with new software for literature mining and pathway analysis, are allowing scientists to connect the transcriptional response to the biological activity that is impacted by treatments [14,15]. Problems in pharmacology and toxicology often require a comparative analysis against well-characterized compounds, and internet resources are now available to aid the integration of transcriptional profiling data from different studies. These resources have been explored in a review [16] that focuses on integrating existing data into compendium studies to reveal gene-compound associations, and describes the myriad informatics challenges involved.

The near future will bring more in-depth applications of the technology. With any bioassay used to evaluate compounds, response curves have greater utility than single-point assays. The transcriptional response to a treatment shows the same dose-dependent behavior as most biological phenomena. Thus, it is possible for transcriptional profiling studies to generate pharmacological parameters (i.e., an EC_{50}) for each mRNA response [17]. In order to use dose-response transcriptional profiling on a routine basis to assess leads for a drug discovery program, it will be necessary to reduce the cost, improve sample management,

and increase the throughput of microarray analysis. One approach has been described using an ''array of arrays,'' which are physically formatted such that each microarray is positioned in a standard 96-well microtitre plate layout [18]. The advantage of this approach is that laboratory automation such as liquid and plate handling can be used to manage a greatly increased number of samples [19]. If a set of key gene responses can be identified for a particular drug development program, one could select representative mRNAs and use less costly technologies, such as quantitative PCR (qRT-PCR), to monitor pharmacology throughout hit-to-lead development. In this scenario, spot checks of key compounds with whole genome analysis might be sufficient to ensure that no new responses have been introduced into the lead series. One caveat is that lack of an observed transcriptional response does not prove lack of pharmacological activity, or that a pharmacological response might not occur in other models and tissues.

3. INTERCONNECTING DISEASE, TARGET VALIDATION MODELS, AND PHARMACOLOGICAL RESPONSE

In modern pharmaceutical discovery, compounds are largely selected and optimized through *in vitro* biochemical assays, followed by *in vitro* cellular assays. Only a handful of compounds will be evaluated *in vivo*. Connectivity between these three types of assays is crucial for selecting the right drug candidate. Transcriptional profiling has been demonstrated as a useful strategy for obtaining ''signatures'' or ''fingerprints'' of the cellular state associated with disease models. Comparison of signatures from the *in vitro* model with those from the *in vivo* disease state can reassure as to their similarity, or illuminate possible conflicts. Common signatures have the potential to be used as biomarkers. Such signatures can also be compared with the transcriptional changes in response to a drug to assess the overlap between the disease mechanism and the pharmacological effect. A significant overlap might be considered evidence for connectivity between the biochemical activity of a lead and the biological response observed. It has even been suggested [20] that transcriptional fingerprints can be used as part of a chemical genomics program to identify hits in screens and progress leads through optimization. Table 1 lists examples of studies that use transcriptional profiling to compare biological models and their responses, both to verify common mechanisms and to identify biomarkers.

Examining some of these studies in detail reveals the strengths and limitations of the transcriptional profiling technique. A good example is the study of adaptive responses to anti-depressant therapy [21]. Using cell-type specific labeling and microdissection, selected functional neurons were isolated for transcriptional profiling. The goal was to identify molecular markers for responses following treatment with the antidepressant amitriptyline. Functional dopaminergic neurons in mouse nucleus accumbens were identified by immunostaining fixed tissue sections with antibody to Mr32, a phospho-protein specific to cells undergoing dopamine signaling. A laser-assisted microdissection apparatus was used to isolate the specifically labeled neurons after 0, 4, and 28 days of

Table 1 Studies that use transcriptional profiling to compare biological models

System or disease	Models	Treatments	Observations	Ref.
Neurodegeneration Alzheimer's	Cortical neurons	B-amyloid	Markers of B-amyloid induced neurotoxicity	[27]
Neurodegeneration, microglial activation	BV-2 microglial cells	Lipopolysaccharide, HIV protein TAT, dopamine quinone	Gene ontology analysis of common pathway response	[28]
Peripheral nerve regeneration	Nerve lesion nerve development	Time course	Comparison suggests regeneration partially recapitulates development	[29]
Granulocyte differentiation acute promyelocytic leukemia	PBMCs (*in vivo*) Cultured bone marrow mononuclear cells (*in vitro*)	All-trans retinoic acid (ATRA)	Promoter analysis of ATRA response genes suggest molecular mechanism underlying ATRA-induced granulocytic differentiation	[30]
Epigenetic silencing, hepatic tumors	6 Hepatoma lines	5-AzaC	Comparison of gene ontology for responses suggests common modulation of cellular functions in models	[31]
Opioid addiction	Striatum from 129P3/J SWR/J C57BL/6J	Inbred mouse lines: opioid resistant, opioid sensitive	Markers for susceptibility to morphine addiction	[32]
Dopaminergic neurons depression	Mouse nucleus accumbens dopaminergic neurons	Amitriptyline time course	Markers for adaptive response to anti-depressant therapy	[21]
Depression	Seven different rat brain regions	Fluoxetine Sleep deprivation Electro convulsive	Comparison of responses to acute and chronic anti-depressant therapy	[33]
Anxiolytic	Rat preoptic area/mediobasal hypothalamus	progesterone	Markers for anxiolytic response	[22]
Fatty liver, nonalcoholic fatty liver disease (NAFLD)	Rat liver from Male (resistant to NAFLD), Female (sensitive to NAFLD)	Fish oil, dextrose	Comparison of liver response to high fat diet in male and female suggest pathways contributing to NAFLD	[15]
Osteoclast differentiation	Raw264, bone marrow macrophages, hematopoetic stem cells	Rankl	Comparison of response markers across models to identify common mechanisms	[23]

treatment. The transcriptional profile of these isolated neurons showed no change in mRNA expression levels at 4 days, with about 95 genes responding by 28 days. Genes showing regulation at 28 days included those encoding members of the dopamine-dependent signaling cascade, ion channels, and neuropeptides. The authors suggested that the time-course data supported a hypothesis that the therapeutic effect was due to an adaptive response to inhibition of neurotransmitter re-uptake. However, since this study was at the edge of feasible technology (as are many cited in the table), the results, although intriguing, should be treated with caution. For example, since only three animals were used per treatment group, the *p*-value threshold achieved was only 0.2 using a false discovery rate correction for multiple measurement testing. Also, the treatment may itself affect which subpopulation of cells are labeled and isolated for analysis, or there may be a variation in mRNA integrity after the 30 minute labeling and micro-dissection procedure.

Another study measured the transcriptional response associated with the anxiolytic activity of progesterone [22] in the preoptic area/mediobasal hypothalamus (POA/MBH) of the male rat brain. Focused microarrays were designed to measure mRNA for 109 target genes, all of which are involved in cAMP- and or calcium-dependent signaling pathways. These arrays were used to profile tissue samples from six treated and six control animals. Because a focused microarray was used, the changes in mRNA expression level observed in this study were considered significant if $p < 0.05$. Twelve such gene responses were found, of which four were selected and verified by qRT-PCR on the original samples. The four genes (Somatostatin, c-*fos*, Calreticulin, and Arc) were selected for this verification since they have been associated with arousal, stress response, and anxiety in previous studies. While it is important to verify measures of gene responses to ensure that data obtained with microarray technology is reproducible, it is equally important to verify the response in a replicate animal study, especially in genome-scale experiments where a multiple measurement correction is appropriate. A replicate study helps assure that the observation was not due to experimental procedures, or to random fluctuation in gene expression between the two groups, and was truly associated with the treatment. The authors suggested that these results supported a role for progesterone in male physiology and behavior, a poorly understood phenomenon. This potential signature for anxiolytic response, if confirmed in follow-up studies, could have utility in the exploration of novel mechanisms and models for drug effects on anxiety, stress, arousal, and analgesia.

The study of osteoclast differentiation is important for understanding potential new treatments for osteoporosis. Such therapies are typically explored in tissue culture models such as the Raw264 mouse monocytic cell line, which is capable of differentiation into functional multinuclear osteoclasts after treatment with the cytokine Rankl. Use of transformed cell lines raises the concern that results may not be extrapolated to normal tissue. To address this question, the transcriptional responses for Rankl treatment of the Raw264 cell line, and of two *ex vivo* primary cell systems (bone marrow macrophages, and hematopoetic stem cells) were compared using Affymetrix GeneChips [23]. The models proved to

have many response genes in common. Among them were three that encode rho GTPases, which were found to be upregulated by Rankl. The function of these three rho GTPase genes was explored by suppression in the Raw264 line, using short hairpin RNA interference (shRNAi) to selectively suppress the mRNA for each target gene. The function of the three rho GTPase genes was found to be essential for osteoclastogenesis. However they were not essential for viability in an NIH3T3 mouse fibroblast cell line, suggesting a selective role in osteoclastogenesis. By comparing responses from three osteoclast models, the authors were able to identify a common response. This comparison increased the confidence that the commonly used *in vitro* assay is relevant to *in vivo* osteoclast function and was not a potential idiosyncrasy of the Raw264 cell line. Responses in common suggested mechanisms that can be genetically manipulated in the Raw264 model which are physiologically relevant in the primary models. In this case, the response genes identified in the models comprised biomarkers that could be useful for exploring new mechanisms for inhibition of osteoclastogenesis, and potentially are drug discovery targets.

4. COMPARING SELECTIVITY OF PHARMACOLOGICAL RESPONSE IN LEAD ASSESSMENT

Lead development programs are often put in an untenable situation: new molecular targets are often poorly characterized while at the same time criteria for liability and tolerance of side effects are growing more stringent. A more complete descriptor of genetic and pharmacological phenotypes may aid in the comparisons among disease models and leads [24]. Transcriptional profiling is one such descriptor: it allows both a quantitation of the complex cellular phenotypes that result from compound treatment, and a comparison of these phenotypes across a series of compounds directed against a common target. One simple approach is to identify a treatment as a ''gold standard'' against which the profiles of new leads will be compared. Such a gold standard might be the control or ''normal'' sample in a disease model, where the goal is to identify a lead or drug that reverts the disease phenotype back to the normal phenotype. Other gold standards might be a known best-in-class drug, or a biologic drug that is to be emulated by a small molecule. In the case of antivirals or antibiotics, the gold standard would likely be untreated and uninfected host: in a study of drugs selective against viral targets, it is reasonable to expect minimal or no activity on cells that do not harbor the virus. In each of these cases, compounds can be monitored and compared for their on- and off- target activities by measuring changes in mRNA levels.

Table 2 identifies recent studies in the literature that compare pharmacological responses among compounds and models to support pathway mechanism, to investigate similarity or dissimilarity within a class of compounds, and to identify biomarkers.

In one of these studies, an effort was made to understand the observed dislipidemia and insulin resistance associated with long-term HIV protease inhibitor therapy [25]. Five HIV protease inhibitors (lopinavir, nelfinavir,

Table 2 Recent studies that compare pharmacological responses

Class	Representatives	Models	Observations or utility	Ref.
Vitamin E	RRR- α-tocopherol all-rac- α-tocopherol	HepG2 dose response	No difference in EC_{50} or IC_{50}	[17]
Estrogen, xenoestrogens	17β-estradiol genistein bisphenol-A PBC-54	MCF7 T47D	Highly similar responses among class members	[34]
HIV protease inhibitors	atazanavir, ritonavir nelfinavir	HepG2 3T3-L1	Divergence in class, dislipidemia mechanism	[25]
5-Lipoxygenase activating protein	MK886	A549 H720	Pathway analysis identifies mechanism of action	[14]
Vitamin D3	RO-438-3582	MCF10AT1 MCF10A1a	Compare premalignant and metastatic models	[35]
Short chain fatty acids	Sodium butyrate	MCE310	Pathway analysis shows activity on fatty acid biosynthesis	[36]
Fatty acid	Linoleic acid	CRL-1790	Model response compared to 10 colorectal cancer tissues fatty acid metabolism pathway	[37]
Retinoids and rexinoids	Targretin 9-cis retinoic acid 4-hydroxyphenyl-retinamide	Liver Lung Mammary (rat tissues)	Class comparison, pharmacodynamic biomarkers	[38]
Anti-tumor Natural products	Anoectochilus formosanus extract, plumbagin	MCF-7	Comparison of selectivity	[39]
Benzimidazole quinolinones Chk1 inhibitors	CHIR-A CHIR-B CHIR-C (inactive)	Camptothecin Pre-treated MDA-MB-435 breast carcinoma	Markers for CHK1 inhibition and cell cycle release identified and used in lead optimization of series	[40]
Androgen Anti-androgen	Dihydrotestosterone bicalutamide	LNCaP	Biomarkers for androgen signaling pathways	[41]

ritonavir, saquinavir, and atazanavir) were compared for activity on HepG2 hepatocyte and 3T3-L1 adipocyte cell lines. Ideally, these compounds should have minimal or no activity on these cells, which do not harbor the HIV virus. This comparison study used both transcriptomics and cellular assays for lipogenesis and glucose transport. The inhibitors were found to suppress triglyceride synthesis in 3T3-L1 cells and yet increase lipogenesis in HepG2 cells in a concentration dependent manner. In both cellular models, atazanavir was found to be the least potent at modifying lipid metabolism. The changes in mRNA induced by atazanavir, ritonavir, and nelfinavir were assessed by Affymetrix Genechip analysis of both cell line models. The transcriptional modulation by ritonavir and nelfinavir was consistently higher relative to atazanavir. A significant fraction of the genes modulated had functions in lipid and amino acid synthesis, and endoplasmic reticulum stress response pathways. The transcriptional and cellular assay data could be connected to known mechanisms, and suggested the proteasome as a potential off-target activity for some members of this class of anti-virals. This possibility was measured directly, and indeed potency of proteasome inhibitory activity correlated with the side-effect profile of these drugs. This study is a powerful demonstration of how a transcriptomics assay can be used to identify potential liabilities, to pinpoint off-target mechanisms, and to rank compounds based on their liabilities.

It is possible for transcriptional profiling studies to compare compounds in far more detail by generating pharmacological parameters (EC_{50}s) for each mRNA response, and on a genome-wide scale [17]. One of the first published studies to demonstrate this possibility was an exploration of transcriptional responses elicited by two preparations of α-tocopherol (vitamin E). The natural product RRR-α-tocopherol, which has three chiral centers, was compared with the synthetic racemate all-rac-α-tocopherol. The questions asked in this study closely mirror those asked in a typical lead evaluation study: for example, is there a difference in potency and selectivity between these two forms of the compound? The HepG2 tissue culture model was treated with four doses of each compound, and an untreated control for each compound preparation was also included. RNA derived from triplicate cultures for each treatment was analyzed using the Affymetrix GeneChip technology, and 215 responding genes were fitted to a standard four parameter logistic model for pharmacological response in order to estimate EC_{50} (up-regulation) or IC_{50} (down-regulation) values for each response gene. The results show multiple response modes, with response genes at EC_{50} values centered around 10 μM and 30 μM, and IC_{50} values for responses centered around 3 μM and 10 μM. Furthermore, no significant difference was observed between RRR-α-tocopherol and all-rac-α-tocopherol on EC_{50}/IC_{50} parameters obtained for response genes.

The power of a dose-response study design in transcriptional profiling, where fitted parameters can be compared, originates from the ability to compare the transcriptional response parameters with the estimated parameters for other cellular responses in order to establish connectivity. The selectivity of the response might be evaluated, not only by comparison with a ''gold standard,'' but by evaluating the number of response modes observed among the EC_{50} and IC_{50}

values of the responding genes. In the α-tocopherol study described above, response modes clustered at 3, 10, and 30 μM. These 3 modes suggest that there are at least 3 mechanisms for α-tocopherol activity in this model.

5. CONCLUSIONS

Transcriptional profiling is a new technology that has searched for appropriate applications in drug discovery. Figure 1 illustrates the transcriptomics study outcomes that can impact drug development program issues, and organizes these study outcomes from the perspective of context. If the analysis of study results requires only data derived from the study itself, the context is internal to the study. The internal context allows, for instance, comparisons between compounds within a particular drug class. In the external context, to a varying degree, data can be compared to results from other models to establish biomarkers, and to the literature to characterize pathways impacted. At an extreme in external context, the data might be compared to a compendium of drug responses for the transcriptome to identify similarities [26]. As use of high throughput technology becomes more commonplace in transcriptomics, we should see more high quality publications using study designs that allow direct comparisons of the pharmacology of transcriptional profiles with other pharmacological responses. Such data will help build the compendium into a set of genuine pharmacological response signatures, allowing a more robust characterization of leads. Ultimately such comparisons may effectively identify additional mechanisms, including liabilities, and even point directly to other targets interacting with a lead compound for development.

In summary, the technology and methodology of transcriptional profiling continues to improve. It is foreseeable that transcriptomics will eventually join

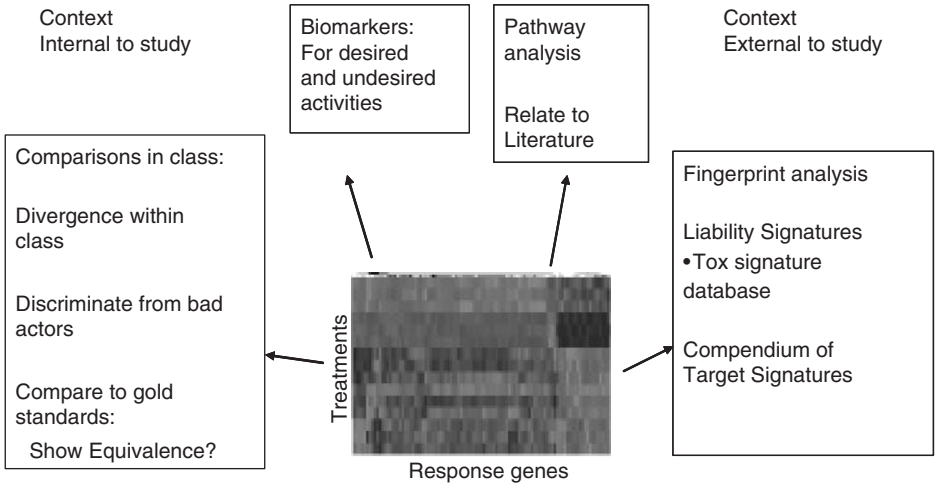

Figure 1 Transcriptomics study outcomes, and how they can impact drug development (Please see Color Plate Section in the back of this book).

other assays that are routinely used in lead profiling to evaluate efficacy, selectivity, and connection to disease biology.

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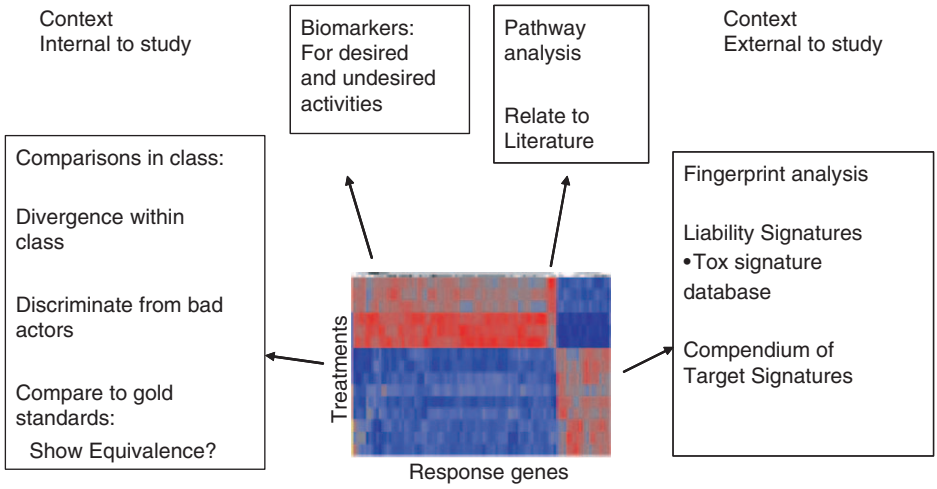

Plate 4 Transcriptomics study outcomes, and how they can impact drug development (For Black and White version, see page 425).

CHAPTER **28**

Fragment-Based Lead Discovery

Miles Congreve, Christopher W. Murray, Robin Carr and **David C. Rees**

Contents

1. INTRODUCTION

Fragment-based lead discovery (FBLD) is establishing itself as an approach that holds the promise of delivering leads with greater efficiency and speed when compared to high throughput screening (HTS). In FBLD, small libraries of low molecular weight compounds (typically 120–250 Da) are screened using sensitive biophysical techniques to detect weak binding. Lower absolute affinity of fragments is expected compared to much higher molecular weight hits detected by HTS due to their reduced size and complexity. Through the use of structural biology, it is often then relatively straightforward to optimise these hits to promising lead molecules.

Astex Therapeutics, 436 Cambridge Science Park, Milton Road, Cambridge CB4 0QA, UK

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There have been a number of recent reviews on fragment-based methods [1–15]. Two in particular were published in 2004 [3,13] that were aimed at medicinal chemists and gave comprehensive lists of examples of how lead molecules have been derived starting from fragments. This report focuses on examples that have been published since these two reviews and, in addition, where the starting fragment is less than 300 Da and has an affinity against the target of $>25\,\mu M$. The latter criterion means that it would have been difficult to identify the hits using conventional screening methods. In addition, the binding mode of the fragments must have been characterised experimentally underlining our view that efficient optimisation of fragments requires a structure-based approach.

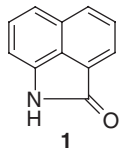
Section 2 briefly outlines the identification of fragments where the optimisation is either not described, or only a limited amount of optimisation was achieved. Section 3 shows examples where lead molecules ($<1\,\mu M$ potency) were successfully derived from fragments. Finally, in Section 4 we give a commentary of key concepts, impacts and challenges for the field.

2. EXAMPLES OF FRAGMENT HIT IDENTIFICATION

There have been a number of recent reports in which fragment screening has been employed for hit identification. Figure 1 illustrates the structures of representative fragments identified, the target, potency, ligand efficiency (LE) and screening method. LE is a measure of the free energy of binding per heavy atom count (i.e., non-hydrogen atom count) and is used to rank the quality of fragments [16]. If we consider as a target for a drug candidate a molecular weight of <500 Da and an IC_{50} <10 nM, then a minimum LE of 0.3 is required in a good fragment, assuming LE cannot be improved during the optimisation process. Throughout this review the units of LE are kcal/mol/heavy atom.

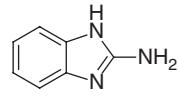
2.1 X-ray screening

Examples 1–5 have been identified by directly soaking fragments into protein crystals by protein–ligand crystallography. Compound **1** was one of four hits detected for nucleoside 2-deoxyribosyltransferase, a target from *Trypanosoma brucei*, the parasite that causes sleeping sickness. The approach used was screening of a library of 304 fragments by soaking of cocktails of nine or ten compounds into protein crystals of the target, and then confirmation in singleton experiments [17]. Similarly, compound **2** was identified as a binder to the S_1 pocket of the protease trypsin [18]. Eight targeted fragments were selected and all bound broadly as predicted. Exemplification of the Pyramid™ X-ray crystallographic approach has been outlined by describing soaking of mixtures of fragments into crystals of cyclin-dependant kinase 2 (CDK2), ribonuclease A and protein tyrosine phosphatase 1B (PTP1B) identifying fragment hits **3**, **4** and **5** respectively [19]. X-ray crystallographic screening has also been applied to human purine nucleoside phosphorylase (PNP), a target for inhibition to modulate the T-cell immune response [20]. As part of these studies, guanine and 8-azaguanine



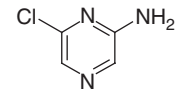
1

nucleoside
2-deoxyribosyltransferase
ED_{50} = 120 μM (LE = 0.41)
X-ray crystallography



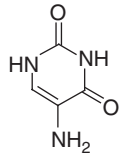
2

tryptase
K_i = 110 μM (LE = 0.54)
X-ray crystallography



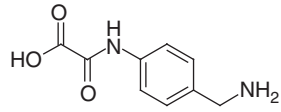
3

CDK2
IC_{50} = 350 μM (LE = 0.59)
X-ray crystallography



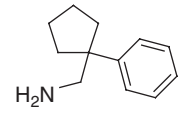
4

ribonuclease A
no potency given
X-ray crystallography



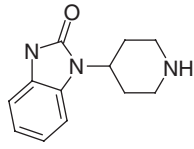
5

PTP1B
IC_{50} = 86 μM (LE = 0.40)
X-ray crystallography



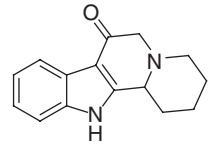
6

DPP-IV
IC_{50} = 40 μM (LE = 0.46)
bioassay



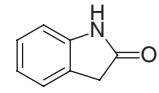
7

FXa
K_D = 110 μM (LE = 0.34)
NMR



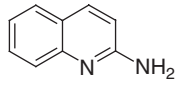
8

ZipA/FtsZ
62% inhibition at
50 μg/ml in FP assay
NMR



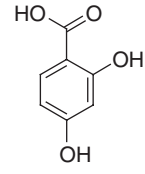
9

DNA gyrase
K_D = 10 mM (LE = 0.27)
NMR

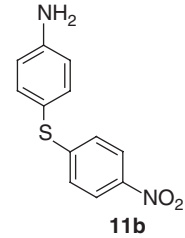


10

Tec SH3 Domain
K_d = 125 μM (LE = 0.48)
NMR

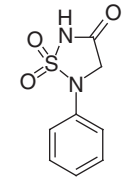


11a



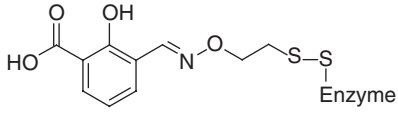
11b

Bid
K_d = 1mM (LE = 0.37) K_d = 1mM (LE = 0.24)
NMR



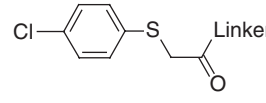
12

PTP1B
K_i = 2 mM (LE = 0.26)
NMR



13

IL2
Detected by tethering
to mutant enzyme



14

thrombin
Detected by SPR screening

Figure 1 Examples of fragment hit identification (showing fragment and target, bioassay activity and screening method).

fragments were found to bind in the same orientation in protein–ligand complexes as in larger ligands such as guanosine.

2.2 Virtual screening

A virtual screen of 10,000 primary amine fragments against dipeptidyl peptidase IV (a diabetes target) identified a number of hits, as determined by bioassay screening at 100 μM, e.g. **6** [21]. An X-ray structure of this fragment confirmed the predicted binding mode in the S_1 pocket and inspired a structure-based hypothesis that eventually led to identification of a potent series of DPP-IV inhibitors [22].

2.3 NMR screening

There have been a number of reports of NMR fragment screening. STD-NMR screening of 34 targeted fragments identified four 'strong' hits for the protease FXa, e.g., **7** [23]. ^{1}H-^{15}N HSQC NMR screening of 825 fragments (200–250 Da, cLogP <2.5) in mixtures of up to 6 fragments against the ZipA/FtsZ complex (an anti-bacterial target and protein–protein interaction) gave seven hits including **8** [24]. Binding was confirmed by the determination of a ligand–protein X-ray structure. *In silico* screening against DNA gyrase and characterisation of possible binders by ^{15}N HSQC NMR allowed identification of two fragment hits, including indolin-2-one **9** [25]. Follow-up screening of analogues gave one compound with improved potency of 25 μM (LE = 0.33). The mouse Tec kinase IV Src Homology 3 (SH3) domain has been studied as a model system to explore fragment binding to SH3 domains by NMR screening [26]. *In silico* screening identified candidate fragments with 2-aminoquinoline **10** (Figure 2) as the most potent hit identified from ^{1}H-^{15}N HSQC spectra with ^{15}N-labelled SH3 protein. Analogues of **10** were designed allowing identification of a hit with improved affinity ($K_d = 22$ μM). Fragments **11a** and **11b** were detected by NMR binding to Bid, a proapoptopic member of the Bcl-2 family [27]. The NMR method used was SAR by interligand NOE. Optimisation by linking of fragments gave a more potent analogue ($K_i = 1.5$ μM, LE = 0.26). Lastly, ^{1}H-^{15}N HSQC NMR was used to measure the affinity of fragments designed *de novo* as potential binders to the phosphate binding pocket of PTP1B, a target for treatment of diabetes [28]. Using a protein–ligand X-ray structure of **12** and docking studies, the fragment was optimised with the most potent analogue having $IC_{50} = 2.5$ μM (LE = 0.35). For a comprehensive review of protein–ligand NMR methods see Pellecchia [29].

2.4 Other screening methods

A technique called tethering, in which a library of di-sulfide containing fragments are reacted with active site cysteine-containing mutant target proteins in order to form covalent adducts, has been applied to the extremely challenging autoimmune disease target IL2 [30]. Ten cysteine mutants of the IL-2 protein were screened against 7000 disulfide fragments. Aromatic acids, such as compound **13**,

were detected to bind to a lipophilic region of the active site and this guided optimisation of a lead series. Fragment tethering has been successfully applied to a number of challenging targets, and the area has been reviewed [4]. Another example of fragments binding covalently to an enzyme has been reported for the protease thrombin [31]. Finally, compound **14** was identified as one of six hits from screening against thrombin of a library of 100,000 fragments covalently attached in a microarray format using surface plasmon resonance (SPR) [32]. Fragment **14** was a strong SPR hit when combined with a second guanidine-containing fragment in the array. This information was indirectly used to help design larger micromolar inhibitors of the enzyme. Applications and developments of SPR have recently been reviewed [33].

3. EXAMPLES OF FRAGMENTS PROGRESSED INTO NANOMOLAR LEADS

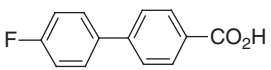
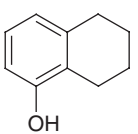
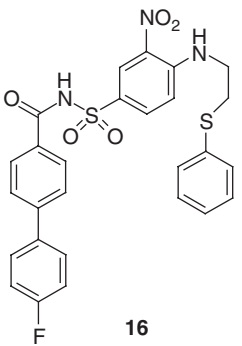
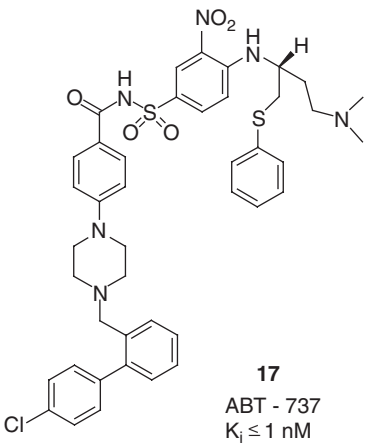
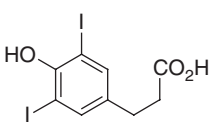
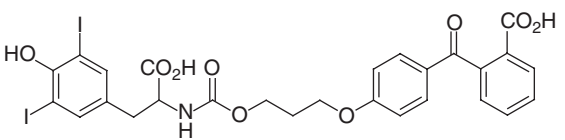
Over the last three years there have been a significant number of reports in which weakly active fragment hits have been identified and progressed into potent lead compounds (potency $<1\,\mu M$) and Table 1 summarises these reports. The table is ordered with the most challenging targets first.

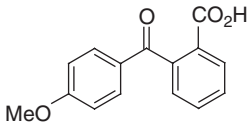
Bcl-X_L (Table 1, entry 1): Lead generation for the challenging protein–protein anti-cancer target Bcl-2 was explored using a high-throughput NMR-based method 'SAR by NMR' [34]. A chemical library of small molecules was screened for their potential to bind to the large highly lipophilic BH-3 binding groove of Bcl-X_L, a Bcl-2 family member. In this way, **15a** and **15b** were found to bind in distinct but proximal subsites within the binding groove. Using NMR-derived structural information and knowledge of key binding points for the native binding BAK peptide, the two fragments were linked and optimised for potency to give analogue **16** and subsequently further optimised for both potency and reduced protein plasma binding to give the preclinical candidate ABT-737 **17**.

HCV NS3/NS4A (Table 1, entry 2): The NS3/NS4A protease-cofactor complex is a challenging Hepatitis C target which has been the subject of intense study over the last 10 years. Using NMR-based screening of a customised fragment library against the NS3/NS4A complex, multiple fragment hits (K_i~100 μM–10 mM) were identified [35]. NMR chemical shift perturbation data indicated that the hits **18a** and **18b** bound at proximal S_1–S_3 and S_2' substrate binding sites. Using this structural information these fragments were linked together to identify a sub-micromolar lead compound **19**. Unfortunately, a protein–ligand crystal structure could not be determined to allow further optimisation of the lead molecule.

BACE-1 (Table 1, entry 3): The aspartyl protease enzyme β-secretase (BACE-1) is another difficult target and an approach to treating Alzheimer's disease. Mixtures of fragments have been screened using a high-throughput X-ray crystallographic approach. Fragments such as **20** were identified that bound with millimolar affinity to the catalytic aspartic acid residues in the active site through an amidine or aminopyridine motif [36,37]. This type of charged bidentate interaction expresses a newly discovered pharmacophore for this class of enzymes. Using the pharmacophore and structure-based drug design approaches,

Table 1 Weakly active fragments progressed into nanomolar leads

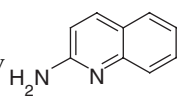
Target Method	Fragment(s)	Evolved fragment	Lead
1 Bcl-2 Bcl-X_L NMR	 **15a** K_d = 0.3 mM LE = 0.3  **15b** K_d = 4.3 mM LE = 0.29	 **16** K_i = 36 nM LE = 0.27	 **17** ABT - 737 $K_i \leq 1$ nM LE = 0.22
2 NS3/NS4A (HCV protease) NMR	 **18a** NS4A-NS3B Ki = 80 μM LE = 0.40 S1- S3 binding region		 **19** K_i = 800 nM LE = 0.21



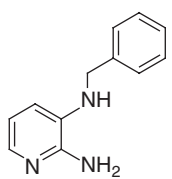
18b
K_i = 1 mM
LE = 0.22
S2' binding region

3 BACE-1

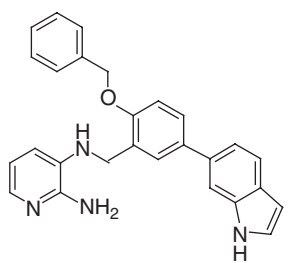
X-ray crystallography



IC_{50} = ~ 2 mM
LE ~ 0.33
20



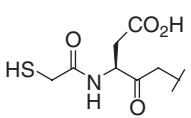
21
IC_{50} = 310 μM
LE = 0.32



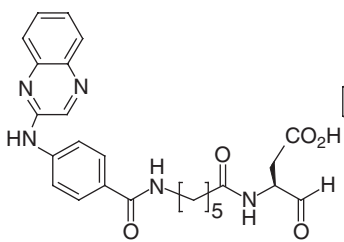
22
IC_{50} = 690 nM
LE = 0.26

4 Caspase 1

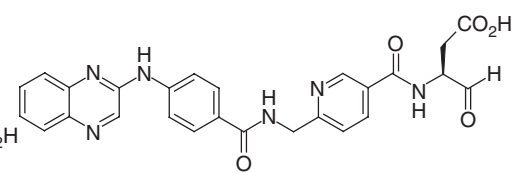
Tethering



23

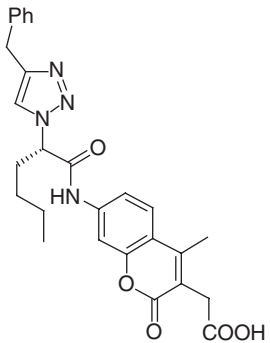
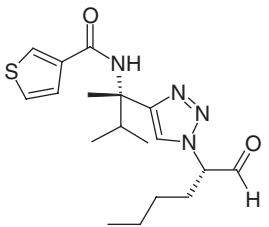
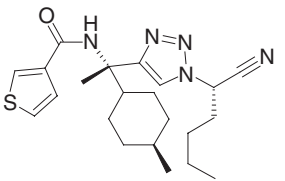
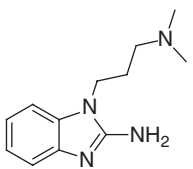
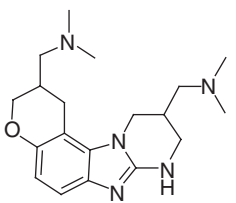


24
IC_{50} = 0.19 μM
LE = 0.26



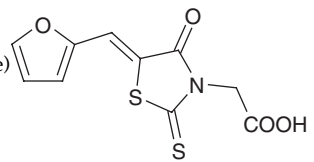
25
IC_{50} = 5 nM
LE = 0.31

Table 1 (*Continued*)

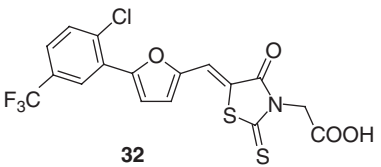
Target Method	Fragment(s)	Evolved fragment	Lead
5 Cathepsin S Substrate activity screening	 **26** Enzyme substrate	 **27** Cat S Ki = 9 nM LE = 0.44	 **28** Cat S Ki = 15 nM LE = 0.37
6 HCV IRES Mass spectroscopy	 **29** Kd = 100 μM LE = 0.34		 **30** Kd = 0.72 μM LE = 0.33

7 Anthrax lethal factor (metallo proteinase)

NMR



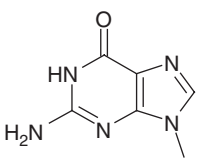
31
IC_{50} = 140 μM
LE = 0.31



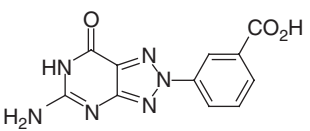
32
IC_{50} = 32 nM
LE = 0.36

8 Dihydroneopterin aldolase

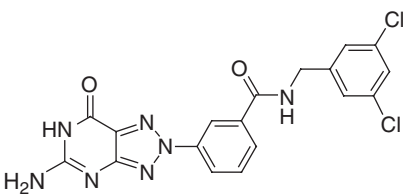
X-ray crystallography



33
IC_{50} = 28 μM
LE = 0.52



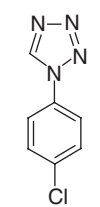
34
IC_{50} = 1.5 μM
LE = 0.40



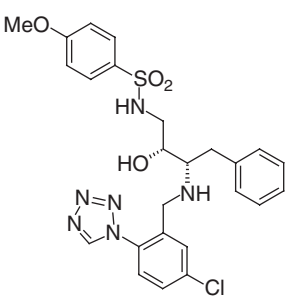
35
IC_{50} = 68 nM
LE = 0.34

9 Thrombin

X-ray crystallography

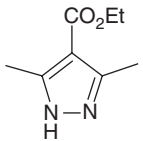
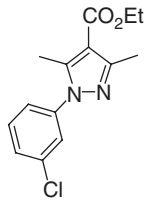
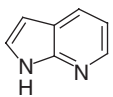
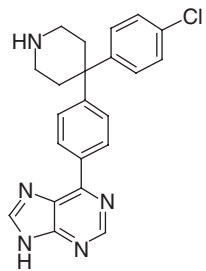


36
IC_{50} = 330 μM
LE = 0.40



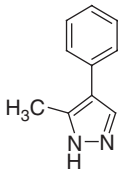
37
IC_{50} = 3.7 nM
LE = 0.31

Table 1 (*Continued*)

Target Method	Fragment(s)	Evolved fragment	Lead
10 PDE4 Biochemical assay	 **38** PDE4D IC_{50} = 82 μM LE = 0.46		 **39** PDE4D IC_{50} = 19 nM LE = 0.5
11 Akt (PKB) Biochemical assay	 **40** PKB > 100 μM inhibition of PKB		 **41** PKB IC_{50} = 20 nM LE = 0.37 Cell-based ELISA assay pGSK3β IC_{50} = 1.9 μM

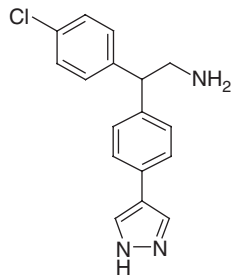
12 Akt (PKB)

X-ray crystallography



42

PKB IC_{50} = 80 μM
LE = 0.47

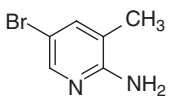


43

PKB IC_{50} = 34 nM (R-isomer)
LE = 0.48

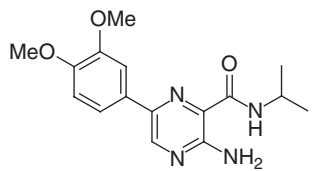
13 Syk

X-ray crystallography



44

"low mM" inhibition of Syk



45

Syk IC_{50} = 21 nM
LE = 0.46

potency was initially improved to the low micromolar inhibitor **21** and then to sub-micromolar lead **22**.

Caspase 1 (Table 1, entry 4): Another protease screened using fragments is caspase 1. Using a tethering approach (described earlier) active sub-site binders were identified and then linked to produce a sub-micromolar inhibitor **24**. Further optimisation by rigidifying the linker produced a ligand-efficient nanomolar inhibitor **25** [38].

Cathepsin S (Table 1, entry 5): Ellman has developed a novel fragment-based screening method called ''substrate activity screening'' for the efficient development of novel non-peptidic protease inhibitors, which does not require structure-based information [39,40]. The method consists of three steps: (1) a library of diverse low molecular weight *N*-acyl aminocoumarins is screened in a fluorescent-based assay to identify protease substrates (e.g., **26** for cathepsin S); (2) the aminocoumarins are then optimised using rapid analogue synthesis; (3) the optimised substrates are converted into inhibitors by replacement of the aminocoumarin with known mechanism-based pharmacophores (**27** and **28**). Although specific to proteases this may be a powerful new approach to fragment optimisation.

HCV IRES (Table 1, entry 6): A mass spectroscopy-based fragment approach was used to identify the weak binder **29** to the ribosome IIA sub-domain of hepatitis C (HCV IRES). Conventional optimisation led to a sub-micromolar lead **30** [41].

Anthrax lethal factor metalloproteinase (Table 1, entry 7): Anthrax lethal factor metalloproteinase is an integral component of the tripartite anthrax lethal toxin and is required for the onset and progression of anthrax. About 300 scaffolds were selected for an NMR-based assay leading to the carboxylic acid fragment **31** [42]. Subsequent synthetic elaboration led to nanomolar inhibitors such as **32**.

Dihydroneopterin aldolase (Table 1, entry 8): Inhibitors (such as **33**) of dihydroneopterin aldolase were identified using high throughput X-ray-based fragment screening of a 10,000 member random library [43]. Structure-guided optimisation gave potent leads such as **35**.

Thrombin (Table 1, entry 9): A small targeted library of fragments was screened by soaking into protein crystals of thrombin [44]. A number of neutral S_1 binders were identified and one of these **36** was linked to larger ligands occupying the S_2 and S_4 sub-sites, also identified by X-ray soaking. In this way, highly potent hybrid inhibitors such as **37** were discovered.

PDE4 (Table 1, entry 10): A library of about 20,000 ''scaffold'' compounds with molecular weights of 125–350 Da were screened in a combination of biochemical assays and crystallography studies to identify the PDE4 inhibitor pyrazole ester derivative **38** [45]. A 4000-fold increase in potency was achieved after only two rounds of chemical synthesis to give **39**.

PKB (Akt) (Table 1, entries 11 and 12): PKB (Akt) is involved in the PI3 kinase-PKB-mTOR cellular signalling pathway and is a target for anti-cancer drug discovery. Fragment screening (bioassay or X-ray) led to two different PKB binding fragments **40** and **42**, each of which was then rapidly elaborated using structure-based optimisation into nanomolar inhibitors (**41** and **43** respectively) that bound to the ATP pocket (Saxty *et al.*, and Collins *et al.*, *Journal of Medicinal Chemistry*, in press).

Spleen tyrosine kinase (Syk) (Table 1, entry 13): Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase implicated in the degranulation of mast cells in asthma. Crystallographic fragment screening using a structurally related kinase, Pak4, led to **44** which was then elaborated using a structure-guided strategy into potent lead **45** by synthesising fewer than 100 analogues [46].

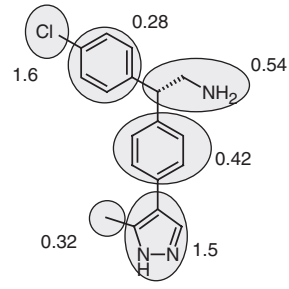
4. COMMENTARY AND DISCUSSION OF KEY CONCEPTS

4.1 Ligand efficiency

The free energy of binding of a ligand for a specific protein can be normalised to the size of the ligand to give a measure of LE as described earlier [2,16,47,48]. Kuntz *et al*. [48] in 1999 were first to compare normalised potencies of this type and also suggested that they would be useful in tracking potencies as molecules were built up from fragments. Practitioners of fragment-based discovery were monitoring potency and molecular weight during fragment optimisation, but it was not widely discussed until Hopkins *et al*. [16] coined LE in 2004. Since then it has been enthusiastically adopted by researchers in fragment-based drug discovery [2,5,49], and modified definitions of LE have been proposed [47,50]. Tracking of LE provides a conceptual road map for the fragment optimisation process [2].

4.2 Group efficiency

LE refers to whole molecules but does not offer insight in whether some parts of the molecule are more efficient than others. During a structure-based fragment optimisation project it is usually possible to track the progress of potency gains and to ensure that the developing series maintains the same binding mode. In such circumstances, comparison of matched pairs of compounds (i.e., a Free–Wilson analysis) allows one to assign a change in the free energy of binding to a particular group. Division of the free energy change by the number of heavy atoms in the added group yields a "group efficiency," and values greater than 0.3 indicate that the specified group is making an acceptable contribution to the potency (Saxty *et al*., in press and [51]). The figure below gives group efficiencies for a methyl analogue of compound **43** (LE = 0.48) in the kinase PKB (from Table 1, entry 12).



The group efficiencies can be compared with the known binding modes of compounds to infer which parts of the active site are responsible for contributing

most to the affinity of a lead series. They can also be used to elegantly illustrate how the addition of relatively large groups with moderate gains in potency is not necessarily helpful during optimisation (for example, the addition of a phenyl group should increase potency by at least fifty-fold). However, this utility comes with the caveat that the underlying assumption of the group-based additivity of free energies of binding is an approximation and will not always be true [52].

4.3 Current directions

Pharmaceutical companies are increasingly adopting fragment (or reduced complexity) screening as one of their core lead discovery technologies due to the substantial direct and indirect costs of HTS and concerns about its ability to deliver hits for challenging targets. However, companies face considerable logistical and cultural challenges when applying fragment screening across a substantial fraction of their research organisations. Fragment approaches must compete with the considerable historical investment in HTS, and despite its deficiencies, HTS does produce good quality lead molecules for many targets. HTS is also applicable to targets without any structural information, and improved strategies may deliver higher quality hits and increased hit rates in the future. The introduction of structure-based biophysical screening across a large proportion of research projects would require substantial up-front investment in infrastructure and may also cause disruption to the flow of projects because of the need to obtain a protein crystal structure and large amounts of pure protein prior to screening. Pharmaceutical companies have therefore so far tended to apply these techniques to projects where HTS has failed to deliver good quality hits, or via research collaborations with biotechnology companies.

If companies are to embrace fragment screening more widely, we predict that they will look to use high concentration screening (HCS) or ''reduced complexity screening'' as their method of choice [1,11,45] because this will most easily sit alongside their existing research infrastructure. However, there is a danger with HCS that it could easily evolve into what might be described as ''Fragment HTS'' where the original principles of the high sensitivity, design-intensive, information-rich fragments approach are compromised. The role of structure-based drug design in driving efficient fragment progression cannot be over-emphasised [13], so an application of HCS across many research projects is likely to require a substantial commitment to timely and robust crystallography. Additionally, the reduced sensitivity of HCS means that higher potency hits [1,11,45] (and therefore larger molecules) will be needed in the screening collection. In order to improve the sampling of the higher molecular weight chemical space, larger libraries must then be screened [1,11,45,53] (e.g., Plexxikon describes a library of 20,000 compounds [45] compared with libraries of a few thousand for biophysical approaches). Fink *et al*. recently attempted an enumeration of all ''sensible'' chemical compounds containing 11 heavy atoms (C, N, O and F atoms only) [54]. Their estimate suggests that there are about 13,900,000 such molecules and further addition of each heavy atom increases the size of the library by about a factor of 7. This implies a library that does a good job of sampling chemical space up to

11 heavy atoms will need to be 118,000 (i.e., 7^6) times bigger if it is to provide a similar sampling with molecules up to 17 heavy atoms (an increase in average molecular weight of about 80 Da). These data suggest even a small increase in the average molecular weight of a library might severely compromise the sampling benefits of fragment-based methods. Of course, it may often still be possible to find acceptable hits for tractable targets in such expanded fragment libraries, just as it is possible to find acceptable hits in HTS collections, but it may be less successful for more challenging targets.

4.4 Fragmentation of existing compounds

In fragment-based drug design, ligand efficient fragments are used to construct potent lead molecules. A related approach is to take an existing lead and break it into key fragments to see where they bind. We have routinely applied this procedure as a valuable source of focused fragments when screening targets where there is a pre-existing medicinal chemistry literature. A number of recent papers have explored the concept of fragmenting known binders [10,49,51,55,56]. Hajduk has analysed 18 highly optimised inhibitors and looked at their successive deconstruction to smaller component compounds [49]. In each case, a fragment-sized molecule was identified for which potencies were available. Interestingly, the fragments and final compounds had similar LEs indicating that fragment optimisation can realistically aim to maintain the LE of the starting fragment. This is consistent with our experience where we would place great emphasis on the LE of the initial fragment, and that unless its experimental binding mode suggests a poor quality interaction that can immediately be corrected, it is not reasonable to expect LE to increase during the fragment optimisation process.

Babaoglu *et al*. have considered four small and weak fragments (7, 10, 13 and 12 heavy atoms with potencies of 40, 19, 10 and 5 mM respectively) that are derived from a 1 μM inhibitor (22 heavy atoms-347 Da) of a β-lactamase [55]. They obtain experimental binding modes for all the fragments, but only one fragment binds in the manner that would be anticipated from the binding mode of the larger inhibitor. The explanation here is that not enough of the key interaction features were present in the other fragments to recover the binding mode observed in the larger inhibitor [57], whereas the successful fragment exhibited one of the minimal pharmacophores of the original inhibitor. The role of the ideal fragment library is to represent all the possible minimal pharmacophores that are available within a drug target and its inhibitors. A philosophical question brought up in this work is whether for some good inhibitors there is no minimal pharmacophore available that can be expressed by a fragment. This study has not really identified such a case, but it certainly remains an interesting possibility.

5. OUTLOOK

Perhaps, the most important question one can ask about fragment-based discovery is will it lead to the efficient identification of drugs? Although it is currently too early to answer this directly, it is now possible to assess if there are

candidate drugs derived from the approach that may ultimately reach the market place. Hajduk and Greer [8] have collated the clinical and pre-clinical candidates and programmes for which there is a statement in the public domain that the candidate drug has been derived using fragment-based discovery. At this time there are six compounds approved for clinical trials derived from fragments: ABT-263 (Bcl-2 inhibitor, Abbott), ABT-518 (MMP inhibitor; Abbott), AT9283 (Aurora kinase inhibitor; Astex Therapeutics), AT7519 (CDK inhibitor, Astex Therapeutics), PLX-204 (PPAR inhibitor; Plexxikon), and PLX-4032 (B-Raf inhibitor; Plexxikon). There are an additional 10 compounds or programmes listed in preclinical development, and it is likely that this number will increase over the coming months due to the popularity of the approach over the last 5 years. The extent to which fragments were used in these drug discovery projects cannot accurately be assessed at this point in time, but these data do support that the screening and optimisation of fragments is beginning to have an impact on the clinical pipeline of companies. It may still be the case that none of these candidates is ultimately successful in reaching the marketplace in the coming years, but it is our assertion that the growing impact of fragment-based discovery methodology is such that it is only a matter of time before the first fragment-derived drug becomes a reality.

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CHAPTER 29

Computational Models for ADME

William J. Egan

Contents

1. INTRODUCTION

"Current, major stumbling blocks in drug development are often the clumsy, empirical, and time-consuming efforts required to go from an exquisitely potent *in vitro* inhibitor to one with good bioavailability and an adequate duration of action. This is the unglamorous part of drug development but often separates highly successful ventures from those which lag behind them."

-Arthur A. Patchett, Merck
1993 E. B. Hershberg Award Address
"Excursions in Drug Discovery" [1]

"Models, of course, are never true, but fortunately it is only necessary that they be useful. For this, it is usually needful only that they not be seriously wrong."

-George E. P. Box
1979 Presidential Address,
American Statistical Association [2]

Novartis Institutes for BioMedical Research, 250 Massachusetts Avenue, Cambridge, MA 02139, USA

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The chances of success in drug discovery and development are slim. Only 11% of compounds selected to enter clinical trials became an approved drug during the 1991–2000 period. It is commonly accepted that thousands to tens of thousands of molecules must be synthesized before an acceptable clinical candidate is even found [3,4]. The most recent estimate for the cost of developing a new drug is between $500 million to $2 billion, depending on the indication and company [5]. Consequently, the pharmaceutical industry is intensely interested in methods which can increase productivity at all stages of the drug discovery and development process.

Nearly 15 years after Patchett's speech, we still face the same problems that he highlighted. Medicinal chemists often synthesize a potent molecule and find later that it has poor exposure *in vivo*, and thus poor efficacy. Poor exposure can be caused by many different factors. Most of the factors affecting exposure are commonly known by acronym ADME – absorption, distribution, metabolism, and excretion. A fifth factor, solubility, is also very important and is commonly considered to be part of ADME.

The prevalence of ADME problems is quite high, although the pattern has changed somewhat in recent years. Kola and Landis [4] reported that for 10 large pharmaceutical companies, ADME/formulation problems were responsible for ~40% of clinical failures in the year 1991 but only ~12% of clinical failures in the year 2000. Clinical safety and toxicity were responsible for ~22% of clinical failures in 1991 and ~33% of clinical failures in 2000. For comparison, clinical failures due to poor efficacy/PD were just under 30% at both time points.

Kola and Landis argue that the drop in ADME-related failures "…provide further compelling evidence that the industry can identify and remedy the causes of attrition." This statement is partially correct. What has happened is that pharmaceutical companies began to use *in vitro* ADME assays, created PK groups for early research, and initiated efforts to create and use computational models to help remove ADME and toxicity liabilities. These efforts in early research have allowed the industry to identify molecules with ADME deficiencies before they are nominated for clinical development or to catch them in preclinical development before entering the clinic, where they would have failed more expensively. There are fewer clinical failures from ADME problems because molecules are inspected earlier for poor ADME properties and because knowledge of how to improve ADME properties is improving, enabling medicinal chemists to design out ADME liabilities.

In drug discovery research, we ask two questions: "Is this molecule any good?" and "How can we make it better?" Computational ADME models can help answer these questions in several ways by: (1) helping chemists triage large numbers of molecules to select representative and interesting examples for testing, (2) providing alerts of ADME risks for otherwise promising molecules so that those risks can be addressed earlier in the drug discovery process, (3) helping chemists to interpret experimental ADME results, and (4) guiding decision making and prioritizing syntheses if predictions/analyses have been in good agreement with experiments so far.

The physiological and physicochemical mechanisms of ADME and solubility are amazingly complex and not fully understood even today. This is a fertile area for industrial and academic research due to its importance in drug discovery. This review will not attempt to provide comprehensive coverage of ADME and solubility modeling. Instead, recent research and issues will be discussed with the aim of alerting medicinal chemists to practical findings and insights.

2. BASIC COMPUTED DESCRIPTORS FOR DRUG-LIKENESS

One of the simplest and most common ways to evaluate a molecule for ADME properties is a qualitative examination of its basic descriptor values such as molecular weight (MW), ClogP for lipophilicity, polar surface area (PSA), counts of hydrogen bond donors and acceptors (HBD, HBA), and count of rotatable bonds (RB). This type of approach popularized by Lipinski's famous Rule of 5 was published a decade ago [6]. Lipinski *et al*. established cutoffs for MW (500), ClogP (5), HBA (10), and HBD (5). These cutoffs were based on the 90th percentile of distributions of molecules in the World Drug Index having USAN or INN names. The Rule of 5 considers a violation of any two of these cutoffs to be an alert for poor absorption or permeability.

More recent studies have expanded upon this type of analysis by subcategorizing descriptor distributions by oral vs. non-oral marketed drugs, temporal patterns of development candidates vs. marketed drugs, and target family differences. Wenlock *et al*. [7] compared the mean and standard deviations of MW, log P, log D7.4, HBD, HBA, and RB for orally administered clinical candidates entering and discontinued from Phase I–III clinical trials, preregistration, and a set of 594 marketed oral drugs. The results showed that the mean molecular weight declined consistently as drug candidates advanced through the clinical trial process, going from 423 at Phase I to 337 in marketed oral drugs. Mean lipophilicity, as measured by ACD log P, was roughly constant (2.6 at to 2.5) but the discontinued development candidates at each phase had higher mean log P values (3.5 at Phase I, 3.5 at Phase II, 3.2 at Phase III). These differences were statistically significant and indicate there is an increased chance of clinical failure for high MW and/or log P compounds. Vieth *et al*. [8] examined the distributions of computed descriptors for 1,729 marketed drugs, including 1,193 orally administered drugs. They tabulated means, min/max, and different percentiles for 12 descriptors by six categories. One interesting and statistically significant difference was that injectable drugs have higher MW, greater polarity, lower lipophilicity, and are more flexible than oral drugs.

Two studies examined the changes in computed descriptors over time. For oral drugs launched prior to 1983, mean MW, HBA, RB, and number of rings are lower than for drugs launched during 1983–2002, while mean %PSA, CLOGP, and HBD do not change significantly [9]. Similarly, Proudfoot [10] found that mean MW increased steadily from below 300 in 1950 to often above 400 in 1997, and that only seven drugs were marketed between 1937 and 1951 with MW >500 but that 32 drugs exceeding MW 500 were marketed 1983–1997. Lipophilicity did not increase. Increasing MW and steady lipophilicity causes an increase in

polarity which would lower the probability of absorption. Also, Proudfoot notes that less than 5% of oral drugs have HBD >4 which may be related to their propensity for phase II metabolism.

Studies of proteomic or target families show large differences in the distribution of computed descriptors between classes. Vieth and Sutherland [11] were able to assign a specific proteomic family to 642 of 1210 marketed oral drugs. Mean descriptor values were not statistically different from overall oral drugs for drugs in the CYP450, phosphodiesterase, kinase, and transporter families. Drugs targeting GPCRs and proteases had significantly greater means for one or more of MW, ClogP, HBD, or HBA. Drugs targeting ion channels were significantly smaller than the overall distribution. Morphy [12] analyzed the computed property distributions of a literature and internal compound database at Organon containing data on 1860 optimization projects. All target families showed increases in MW during optimization. Differences between families were due to differences in the properties of the leads. High property values were consistently observed for drugs targeting peptide GPCRs, integrin receptors, proteases, and transferases, while drugs targeting monoamine GPCRs, ion channels, oxidases, and transporters had lower property values.

Overall, several useful concepts emerge from these analyses. Different targets and routes of administration may require biased property distributions and screening libraries for successful lead optimization. This could influence the eventual chances of project success and should be taken into account early by project leaders. Once more, optimization focused on potency has been shown again to lead to larger molecules which increases the potential for poor ADME properties. The extent of any ADME issues would of course depend on the structure of lead molecule. Finally, larger, more lipophilic molecules historically have an increased rate of failure in the clinic.

3. SOLUBILITY

Solubility is a property that depends on many factors which must be specified carefully. It is particularly important to know precisely what form of the molecule was tested, what solvent system was used, and the performance characteristics of the experimental method. Molecules are commonly amorphous in form early on in the research process, less pure, and are dissolved in DMSO to create stock solutions for archival storage and high-throughput screening. DMSO stocks are then diluted with buffer for activity and ADME *in vitro* screening assays. In later stage research, larger quantities of promising molecules are synthesized with the aim of producing a crystalline solid suitable for formulation and dosing in animal pharmacology, pharmacokinetic, and toxicology studies. Salt forms, pH-dependent ionization, the existence of polymorphs and their varying solubilities, melting point of the crystal lattice, and the many available formulation solvents (water, PEG, methylcellulose, organics, etc.) all influence measured solubility. Solubility can be measured with varying degrees of accuracy ranging from cheaper and faster, but less accurate and more variable kinetic approaches using nephelometry or flow cytometry detection to 'gold standard' thermodynamic

solubility using shake-flask with HPLC-UV or LC/MS detection. These factors can cause a single molecule to have widely differing solubility values that are not comparable.

From a modeling standpoint, the prediction of a molecule's solubility is a very difficult task because of the issues listed above [13–15]. The problem of predicting solubility has been attacked with reasonable success with complex neural network models. While not interpretable, neural networks can function as an *in silico* assay. Other techniques which are more interpretable have also been applied to the problem.

A conceptually simple and elegant approach to estimating the effects of small modifications to molecular properties such as solubility was published by Leach *et al*. [16] The technique is called 'matched molecular pairs analysis.' First, a set of specific structural transformations are used to search a set of molecules having some type of property data. Subsets of almost identical molecules having each transformation are identified, e.g., all molecules differing by *p*-fluorine on a phenyl ring. The percentage of molecules with a positive property value change is computed, and the binomial distribution is used as a statistical test to ascertain if the change is significant. For example, the authors reported that when an amide is methylated, 112/142 pairs had increased solubility by an average of +0.64 log units. The percentage of pairs with increased solubility was 79% with a 95% confidence interval of 71–85%, indicating the effect is statistically significant. This technique is not limited to solubility but can be applied to any property of a molecule, ADME or otherwise. The authors also show examples of insights gained from matched molecular pairs analysis of data on protein binding and oral exposure in rats. The beauty of matched molecular pairs analysis is that it is clearly interpretable and as the authors state, ''can be used as a tool to test many of the ''rules of thumb'' that abound within medicinal chemistry.''

Huuskonen [17] assembled aqueous solubility data for 1297 organic molecules and modeled it using neural network and linear regression models trained on 55 connectivity, shape, and electrotopological state descriptors. Test set results were $r^2 = 0.92$ and standard deviation (s) $= 0.60$ for the neural network and $r^2 = 0.88$ and $s = 0.71$ for the linear regression model. Yan *et al*. [18] were able to build neural network and linear regression models of comparable quality for the Huuskonen dataset using only 18 topological descriptors primarily computed using the program PETRA. Test set results were $r^2 = 0.94$ and $s = 0.52$ for the neural network model and $r^2 = 0.89$ and $s = 0.68$ for the linear regression model. Further work by Yan *et al*. [19] modeled the aqueous solubility of a set of 2743 drug discovery molecules from Merck KGaA, resulting in a neural network model using 18 2-D topological descriptors with $r = 0.92$ and $s = 0.62$. The authors note that the Huuskonen set is limited in diversity in comparison to the Merck KGaA dataset.

One problem highlighted by several reviewers [14,20] is that datasets like the Huuskonen set cover unnecessarily large ranges of solubility. The Huuskonen set covers the range log S (log of solubility in mol/l) from -11.62 to $+1.58$, which converts approximately to 9.6×10^{-7}–1.5×10^{7} μg/ml for a MW of 400 Da.

Johnson and Zheng [14] recommend a pharmaceutically relevant range of 0.1–250 μg/ml as more appropriate.

However, the issue is more complex than a simple range. Lipinski [21] provides better guidance for minimum acceptable solubility based on maximal absorbable dose calculations. These take into account dose amount and permeability both of which have significant effects on required solubility. For example, the minimum acceptable solubility for a 0.1 mg/kg human dose (a 7 mg pill) of a high permeability molecule is 1 μg/ml, while the minimum acceptable solubility for a 10 mg/kg human dose (a 700 mg pill) of a low permeability molecule is 2100 μg/ml. This range is somewhat similar to the range recommended by Johnson and Zheng, but it is important for both medicinal chemists and modelers to be aware of the factors modifying the minimum acceptable solubility values within the solubility range relevant for drug discovery.

Goeller *et al*. [22] at Bayer modeled buffer solubility at pH 6.5 using a dataset containing 5000 molecules whose solubility was measured in a consistent fashion. The Bayer assay was a high-throughput assay starting from DMSO stock diluted to 1% DMSO in a PBS buffer at pH 6.5 and using HPLC detection. The log S range is approximately −6 to 3. The model used 65 VAMP/PROPGEN descriptors computed from 3-D structures plus eight common 2-D descriptors. These descriptors were used to train various neural networks. The best neural network had RMSE = 0.73 and 83% of predictions had < 1.0 log unit error on a test data set of 7222 molecules.

Recently, Gaussian Process nonlinear regression was used to model a set of combined literature aqueous solubility data and shake flask buffer solubility data for 632 molecules at pH 7.0–7.4 from Schering AG [23]. This machine learning algorithm has been used rarely in drug discovery modeling. Gaussian Process models have the advantage that they can provide error estimates for predictions. Results on a blinded test set of shake flask data were reasonable, with RMSE = 0.92. Analysis of the predicted error bars showed that for test sets matching the distribution of the training data, error bars were smaller, while for test sets dissimilar to the majority of the training, error bars were larger, as one would expect. This model has been implemented for routine use at Schering AG.

As mentioned, solubility in DMSO is important for compound storage and high-throughput screening efforts. Computational models for the prediction of DMSO solubility have been reported by Balakin *et al*. and Lu and Bakken [24,25]. Balakin *et al*. at ChemDiv, Inc. modeled a large set of 65,500 molecules with measured DMSO solubility. Molecules were classified as insoluble if they were not soluble at 0.01 mol/l. A 15 × 15 node Kohonen neural network was able to correctly classify 93% of compounds using only eight descriptors. Such models work by mapping the input data into a smaller dimensional space based on the nodes and making predictions based on node membership. In essence, a molecule is predicted as soluble or insoluble in DMSO based on the neighboring molecules in its assigned node. Surprisingly, a standard neural network performed worse on the same data, having approx. 75% accuracy. At Pfizer, 33,329 compounds dissolved in 30 mM DMSO stock solutions were visually inspected for precipitates. They computed 200 2-D descriptors (78 E-state keys and a set of

122 from the MOE software package) to build five models to classify compounds which showed precipitation vs. those which showed no precipitation. Test set accuracy was reasonably good across all five models: recursive partitioning 81%, random forest 81%, binary QSAR 74%, self-organizing map 69%, and linear discriminant analysis 76%.

Little work has been performed to model solubility while taking into account crystal packing. Johnson *et al*. [26] recently presented an interesting initial attempt using calculated intrinsic solubility corrected for effects of ionization, and crystal-packing forces derived from an escalating temperature molecular dynamics simulation. Although the model requires crystal structure information, it can be applied to analogues which do not have crystal structures simply by overlaying those analogues onto the known crystal form to begin the simulation. Results suggest this type of model could be useful to understand the solubility of late-stage optimization and early development candidates, although it is highly dependent on pK_a estimates.

4. INTESTINAL ABSORPTION

Theory and computational aspects of intestinal permeability have been reviewed in detail by Egan and Lauri [27]. Briefly, a drug must be somewhat permeable through the membrane of the intestinal tract if it is to be administered orally and achieve systemic exposure. The rate of membrane permeability is strongly related to the lipophilicity and hydrophilicity of the molecule. Thus, models with a small number of descriptors related to those two properties can provide useful predictions of drug absorption.

Egan *et al*. [27,28] demonstrated that a statistically based classification model built using only PSA and AlogP98 could predict the region of chemical space occupied by well-absorbed ($>90\%$ absorbed) molecules and exclude poorly absorbed molecules ($<30\%$ absorbed). Molecules with absorption in the range 30–90% were not used due to large data variability. Actively transported molecules were excluded. These results were validated on caco-2 permeability assay data from drug discovery projects at Pharmacopeia. The caco-2 permeabilities were shown to have a hill-shape in PSA-AlogP98 space. The sides of the hill declined rapidly at the edge of the well-absorbed region and less than 10% of highly permeable molecules were outside the well-absorbed region, while only 21% of poorly permeable molecules were inside the well-absorbed region.

In an excellent paper, Zhao *et al*. [29] assembled a carefully reviewed literature set of human absorption data on 241 drugs. They showed that a linear regression model built with 5 Abraham descriptors could fit percent human absorption data reasonably well ($r^2 = 0.83$, RMSE $= 14\%$). The descriptors are excess molar refraction (E), polarizability (S), hydrogen bond acidity (A), hydrogen bond basicity (B), and McGowan volume (V), all related to lipophilicity, hydrophilicity, and size. In a follow-on paper, data on rat absorption for 151 drugs was collected from the literature and modeled using the Abraham descriptors [30]. A model with only descriptors A and B had $r^2 = 0.66$, RMSE $= 15\%$.

All *in vivo* data, including the human and rat absorption data used by both Egan and Zhao *et al*., have considerable variability. Zhao *et al*. comment that measurements of percent absorbed for the same molecule may vary by 30%, and that the 95% confidence interval for a prediction is approximately 30% given a model RMSE of 15%. This is approximately the same as the normal experimental error for absorption values. This means that models predicting percent absorbed have to be carefully interpreted, i.e., a prediction of 30% absorbed really means the molecule is predicted to have absorption from 15 to 45%, and that classification models should work nearly as well as regression models.

A classification regression tree model (CART) was used to predict the fraction absorbed for a large set of 1260 drugs and drug candidates using 28 descriptors [31]. The training set was 899 molecules and fraction absorbed was split into six classes (0–0.19, 0.2–0.31, 0.32–0.43, 0.44–0.59, 0.6–0.75, 0.76–1). Predicted values were reported as the median of each class. Average absolute error (AAE) for the test set of 362 molecules was 0.169 and 80.4% of molecules were predicted within one class of their actual class. For 37 proprietary molecules having human data, AAE = 0.14 and 86.4% of molecules were predicted correctly within one class.

Descriptors such as PSA, ClogP, and the Abraham descriptors can be interpreted in terms of chemical structure without much difficulty. Jones *et al*. [32] showed that quantum mechanical descriptors can be used to successfully predict intestinal absorption and at the same time provide an interpretable model. They used the dataset of Zhao *et al*. [29] and computed molecular surface charges using density functional theory in COSMOtherm software. The model quality was almost identical to the Abraham descriptor model reported by Zhao *et al*. (RMSE = 15% for the same test set). The surface charges were mapped to the 3-D structure of drugs creating an easily intepretable image; Lamivudine was the example presented in the paper.

One of the more interesting aspects of membrane permeability is the effect that intramolecular hydrogen bonds can have. If a polar molecule can adopt a conformation which forms intramolecular hydrogen bonds, it will be able to present a more lipophilic surface to the membrane and solvent, and thus have greater permeability than standard measures of polarity would suggest. Rezai *et al*. [33,34] conducted two experiments testing this effect. The first experiment synthesized nine cyclic hexapeptide diastereomers and measured their PAMPA permeabilities. The least and most permeable cyclic hexapeptides had permeabilities differing by two orders of magnitude. NMR and molecular modeling studies suggested that the most permeable cyclic peptide exposed only one amide to solvent, while the least permeable cyclic peptide exposed 3–5 amides to the solvent. In the second experiment, virtual libraries of 128 hexapeptides and 320 heptapeptides were analyzed computationally using extensive conformational sampling in low (membrane) and high (water) dielectric environments. They hypothesized that the partition coefficient between two different environments (the free energy of insertion) of the lowest energy conformer in the low dielectric environment would be proportional to the PAMPA permeability. Eleven peptides with varied predicted properties were synthesized and their PAMPA permeabilities did have a high correlation ($r^2 = 0.96$) with the computed free energy of

insertion. The method does not take into account molecular size and the authors conclude that to do so will require estimating translational, rotational, and internal energy losses for membrane penetration. These approaches could give insights into the mechanisms of permeability of drug candidates with larger molecular weight and greater flexibility that are capable of forming multiple intermolecular hydrogen bonds.

Computational models are increasingly being added to drug discovery workflows. At Pfizer, computational models for passive permeability and active efflux were developed using internal caco-2 data on 3018 molecules [35]. Two models were built because the apical to basolateral measurements of permeability normally used to estimate passive permeability will be affected if a compound is an efflux substrate. Logistic regression was used to fit MOE 2-D graph fingerprints. Model predictions and results for similar compounds are reported to chemists. ROC curve analysis was used to evaluate model quality: AUC = 0.9 for the efflux model and AUC = 0.83 for the passive permeability model (perfect score is 1.0). Guidance is provided to project teams based on the predictions, e.g., molecules predicted to have low passive permeability without active efflux should be submitted to the cheaper PAMPA assay and not to cellular assays during lead optimization efforts.

5. BLOOD–BRAIN-BARRIER PENETRATION

Computational models for blood–brain-barrier penetration have been well reviewed in detail by Clark [36]. Penetration of the blood–brain-barrier (BBB) via passive diffusion is dependent upon the hydrophilicity and lipophilicity of a molecule. However, the BBB is a thicker, more lipophilic membrane than the intestinal membrane. Kelder *et al*. [37] showed that very few of 776 orally administered CNS drugs had PSA >90, while a substantial fraction of 1590 orally administered non-CNS had PSA >90. These results demonstrate the poor BBB penetration by hydrophilic molecules.

A simple two variable linear regression model using PSA and CLOGP was used to successfully predict logBB with $r = 0.887$, $s = 0.354$ ($\log BB = \log_{10}$ [brain]/[blood]) [38]. Lobell *et al*. [39] compared a set of 14 models designed to predict logBB and concluded two models had advantages. Lobell used a stepwise linear regression on 34 2-D and 3-D variables to produce a model with 5 terms plus intercept with $r^2 = 0.837$ and MAE = 0.26. This model was judged best for low–medium throughput applications. The 2-D Cerius^2 ADME model for predicting logBB was judged the best compromise between speed and accuracy for ultra-high throughput processing of large datasets. The 2-D Cerius^2 ADME model fit AlogP98 and 2-D PSA to predict logBB with a robust regression and uses an exclusionary region to prevent extrapolation.

The calculated cross-sectional area of a molecule (A_{Dcalc}) based on the internal amphiphilic gradient of a molecule has been used as the basis for a novel BBB model [40]. For each molecule, a conformational ensemble was generated and the smallest A_{Dcalc} was chosen. A simple bi-plot of log $D_{7.4}$ vs. A_{Dcalc} was sufficient to correctly predict the BBB penetration of 85.2% of 122 drugs.

Abraham *et al*. [41] modeled literature data of rat *in vivo* BBB penetration measured in blood, plasma, or serum. They concluded that the three types could be combined because the systematic differences were so small. A linear regression model built using the Abraham descriptors for 116 molecules had $r^2 = 0.73$ and $s = 0.34$, and performed well on a test set with AAE = 0.25 and $s = 0.31$. They note the experimental error (s) for logBB should be approximately 0.3 log units, which is the error of the fitted model. Work by Zhao *et al*. [42] further demonstrates the ability of models built using 1–5 descriptors (Abraham, PSA, HBA, HBD, RB, etc.) to provide useful predictions of BBB penetration. Models were built using a 1093 compound training set and tested on a 500 compound set. Models built using 1–5 simple descriptors had test set accuracies for +/− classifications in the range 96.5–99.8% for BBB+molecules and 65.3–79.6% for BBB- molecules.

A concern about the use of logBB values as the index of brain permeability/penetration has been raised by Pardridge [43]. He argues that logBB is a simplistic and incorrect distributional measure that does not take into account actual permeabilities. Pardridge advocates using the BBB PS product which is a measure of unidirectional clearance from blood across the BBB to the brain, and predicts the level of free drug in the brain. Modeling results for two small datasets of BBB PS data suggests that models similar to those discussed above can readily predict BBB PS. Liu *et al*. [44] measured the BBB PS and fit a linear regression model to predict logPS of 23 molecules with only three terms (logD, PSA, and van der Waals surface area of basic atoms) and $r^2 = 0.74$ and $s = 0.50$. Abraham [45] achieved similar results modeling literature data on logPS for 30 molecules using a linear regression model fit to 5 Abraham descriptors, with $r^2 = 0.87$ and $s = 0.52$.

5.1 P-glycoprotein efflux

P-glycoprotein is an ABC cassette transporter encoded by the MDR1 gene in humans that is responsible for the efflux of drugs from cells. It plays a significant role in limiting brain penetration and to a lesser extent limits intestinal absorption of drugs. For oral drugs dosed in quantities greater than 50 mg with reasonable dissolution rates, *p*-glycoprotein transport will be saturated and thus unable to limit absorption. It should be noted that drugs with poor solubility effectively have a 'low dose' and may have limited absorption due to *p*-glycoprotein efflux, e.g., paclitaxel. Unfortunately, the blood concentrations of drugs at the BBB do not achieve the levels found for most drugs in the intestines and so the *p*-glycoprotein transporter in the BBB cannot be saturated and will decrease the brain penetration of substrates. [46,47]

In a study of *p*-glycoprotein substrates vs. non-substrates, Varma *et al*. [48] concluded that substrate molecules with high passive permeability overwhelmed the transporter while substrate molecules with moderate passive permeability were more affected by *p*-glycoprotein. Approximately half of 63 *p*-glycoprotein substrates studied had MW >400 and PSA > 75 indicating that larger, more polar molecules are more likely to be *p*-glycoprotein substrates.

Several QSAR models have been used to predict whether a molecule is a *p*-glycoprotein substrate or not. Gombar *et al*. [49] modeled a set of 95 *p*-glycoprotein substrates and non-substrates using stepwise linear discriminant analysis. Class assignment was based on efflux ratios measured by an *in vitro* Madin–Darby canine kidney cell assay run at GlaxoSmithKline. The initial 254 descriptors were trimmed down to a set of 27 descriptors with an accuracy of 98.9% Performance on a test set was also good, with 50/58 (86.2%) correctly predicted. A single e-state descriptor, MolES, representing molecular bulk, was particularly good at discriminating substrates. For MolES >110, 18/19 molecules were substrates, and for MolES <49, 11/13 molecules were non-substrates.

Cabrera *et al*. [50] modeled a set of 163 drugs using TOPS-MODE descriptors with a linear discriminant model to predict *p*-glycoprotein efflux. Model accuracy was 81% for the training set and 77.5% for a validation set of 40 molecules. A ''combinatorial QSAR'' approach was used by de Lima *et al*. [51] to test multiple model types (kNN, decision tree, binary QSAR, SVM) with multiple descriptor sets from various software packages (MolconnZ, Atom Pair, VoSurf, MOE) for the prediction of *p*-glycoprotein substrates for a dataset of 192 molecules. Best overall performance on a test set of 51 molecules was achieved with an SVM and AP or VolSurf descriptors (81% accuracy each).

Analyses of molecules that are *p*-glycoprotein substrates have suggested a number of possible pharmacophores. For example, based on an analysis of 100 molecules, Seelig [52] proposed that molecules containing at least one Type I or Type II unit would be *p*-glycoprotein substrates, and their binding increases with the strength and number of these groups. Type I units contain two electron donor groups 2.5 ± 0.3 Å apart, and Type II units contain two or three electron donor groups whose maximum distance apart is 4.6 ± 0.6 Å. Pajeva and Wiese [53] proposed a pharmacophore containing two hydrophobic groups, three HBA groups, and one HBD group. They conclude that binding depends on the number of these pharmacophore points present and that different drugs interact with varied groups with multiple possible binding modes. This pharmacophore hypothesis was shown to agree with a homology model of *p*-glycoprotein created using *E. coli MsbA* as the template [54].

Two 3-D QSAR models were built using GRIND descriptors for *p*-glycoprotein substrate recognition. Cianchetta *et al*. [55] selected 100 proprietary molecules and 29 publicly available molecules having caco-2 A-B/B-A ratios >1 and screened them for inhibition of *p*-glycoprotein activity in a calcein-AM assay. The inhibition values were modeled using GRIND and VolSurf descriptors. The 3-D alignment independent GRIND descriptors fit the data well, with $r^2 = 0.83$. VolSurf descriptors produced a model that was slightly better than random. The pharmacophoric GRIND features suggested the following features were important for *p*-glycoprotein substrate recognition: two hydrophobic groups 16.5 Å apart, two HBA groups 11.5 Å apart, plus the size of the molecule (21.5 Å distance required between edges of the molecule). Crivori *et al*. [56] similarly compared VolSurf and GRIND descriptors for the prediction of *p*-glycoprotein substrates. Fifty-three drugs were classified as substrates or non-substrates by a cutoff of two for their caco-2 efflux ratio and modeled using VolSurf descriptors; the model

was 89% accurate. When tested on a proprietary dataset of 272 molecules, the VolSurf model correctly classified 72% of the dataset. Thirty of the 53 drugs were assayed in a calcein-AM assay and the data were used to select 9 substrates and 14 non-substrates for modeling with GRIND descriptors. The model was tested on a set of 125 drugs from the literature and accurately predicted 82% of them. Two GRIND features were important in the model: two hydrophobic regions 11.5 Å apart and two HBA groups 8 Å apart.

The effect of *p*-glycoprotein efflux limiting brain penetration has been examined by two analyses. A bagged recursive-partitioning model was built using the R software on 190 compounds with literature logBB data and three sets of descriptors [57]. The literature-based model was tested on 250 Pfizer compounds, of which approximately 60% showed significant *p*-glycoprotein-mediated efflux based on brain penetration experiments in knockout vs. wildtype mdr1a mice. Results were much worse for the Pfizer compounds than for the training set (Q^2 ~0.5 vs ~0.2), indicating the effect of *p*-glycoprotein efflux. Garg and Varma [58] used a prediction of *p*-glycoprotein efflux probability as an input into a neural network model with good results ($r = 0.89$, $s = 0.32$ for test set of 50 molecules).

Raub [47] has published an excellent review with examples discussing the SAR of *p*-glycoprotein substrate recognition. He notes that "…the SAR for P-gp is obviously complicated and poorly understood…" and "…no single functional group alone is recognized, but one group can accentuate the recognition points existing within a scaffold. It is likened to a rheostat, rather than an on/off switch, where addition or removal of a key group can increase or decrease the pumping efficiency." Raub concludes that the best approach to reduce *p*-glycoprotein efflux effects is to increase passive diffusion to overwhelm the *p*-glycoprotein transporter.

Raub's point is well made. P-glycoprotein transports many of the same substrates that the liver enzyme CYP3A4 metabolizes. CYP3A4 is responsible for the metabolic clearance of ~50% of marketed drugs. For the *p*-glycoprotein transporter to recognize so many different types of substrates, it requires multiple binding modes and/or multiple sites with wide tolerances. However, the 2-D and 3-D models reviewed above demonstrate that useful insights can be attained from computational models. For specific chemical series, local models may be required to best predict *p*-glycoprotein efflux.

5.2 Properties relating to duration of drug action

The half-life ($t_{½}$) of a drug is related to the volume of distribution (Vd) and clearance (CL) by the equation $t_{½} = 0.693 \times \mathrm{Vd}/\mathrm{CL}$. The volume of distribution at a steady-state (V_{ss}) is related to the volume of plasma, tissue, and fraction of the drug unbound in plasma and tissue.

5.3 Plasma protein binding

The binding of drugs to plasma proteins has a significant effect on pharmacokinetics and pharmacodynamics. The fraction of unbound drug, also called the

free fraction, directly affects Vd and thus half-life. The biological effect of a drug is due to the free fraction. The most abundant plasma proteins to which drugs can bind are human serum albumin (HSA) and α_1-acid glycoprotein.

The lipophilicity of molecules can strongly affect their plasma protein binding. Van de Waterbeemd *et al*. [59] showed that percent plasma protein binding had similar, but offset, sigmoidal relationships to log *D* at pH 7.4 for acids, bases, and neutral compounds. Molecules with log $D > \sim 3$ were greater than 90% bound. Yamazaki and Kanaoka [60] performed a more complete analysis of the relationship between lipophilicity and protein binding for 302 drugs. They successfully used a simple non-linear equation to predict the percent protein bound for neutral/basic/zwitterions using only log *D* at pH 7.4 ($r^2 = 0.80$, MAE = 10.4%). A similar attempt for acidic drugs gave a poorly fitting model. When a simple pharmacophore (any two of a hydrophobic, aromatic, or HBD group within 4–5 Å) was used to classify acidic drugs, the protein binding of the acidic drugs matching the pharmacophore could be fit using a simple-non-linear model. Kratochwil *et al*. [61] have reviewed the effects of lipophilicity on protein binding and conclude that for smaller data sets the correlation may depend on the nature of the datasets.

The log of the primary binding affinities for HSA for a set of 138 molecules were used to build a QSAR model for protein binding [62]. Moloc topological pharmacophore descriptors were subjected to dimensionality reduction and fit using partial-least squares. The model fit parameters were $r^2 = 0.72$, $s = 0.62$ and the experimental variability of the binding constants was estimated to be 0.54 log units. Validation results gave error estimates on the order of $s = 0.7$–0.9. The model is sufficiently precise to distinguish drugs bound 99% vs. those bound 99.9% to HSA. Interestingly, for a subset of 76 molecules, measured log *D* values had moderate-to-poor correlation with binding constants.

A thoughtful paper by Leeson [63] presented models of several large sets of protein binding data from GSK internal compounds using partial-least squares and 30 descriptors related to ionization, size, lipophilicity, and polarity. The percent protein bound values were converted into a pseudo-log equilibrium constant. For 1081 compounds measured in rat, the model performance was reasonable ($r^2 = 0.44$, RMSE = 0.62) with similar performance on test 347 test compounds. A model based on human protein binding data for 686 compounds had somewhat better results, $r^2 = 0.56$, RMSE = 0.55. For these large datasets, protein binding increased with increasing lipophilicity and acidity, while addition of a basic group decreased binding, as did increasing a basic pK_a. Leeson comments that models with this level of predictive error can be used to rank compounds, because the 95% confidence limits for predictions of protein binding less than 95% rule out the possibility of protein binding of greater than 99%, which is usually the level of protein binding causing the greatest concern.

A variety of other QSAR-type models for the prediction of plasma protein binding have also been published recently, including neural networks/support vector machines [64], 4-D fingerprints [65], and TOPS-MODE descriptors [66].

A crystallographic study of drug binding to HSA provides a valuable resource for structure-based design efforts to modify protein binding affinity of

drug candidates. Ghuman *et al*. [67] published 17 co-complexes of drugs and small toxins with HSA. Both binding sites of HSA were occupied by various compounds revealing specific binding interactions. The binding pockets were determined to be flexible, with distinct sub-spaces, and overlapped with binding sites for fatty acids, the endogenous ligand.

5.4 Tissue distribution

Three recent papers have presented computational models for the prediction of tissue distribution of drugs. Zhang and Zhang [68] modeled the distribution into brain, kidney, muscle, lung, liver, heart, and fat of 80 diverse molecules. A complex, non-linear regression model was fit to a set of physicochemical descriptors generated by the Hyperchem software package. The model also incorporated known weight fractions of lipid, protein, and water for each tissue type. The model performance on the training set of 67 molecules for the prediction of the log partition coefficient was $r = 0.877$ and $s = 0.352$, and on a test set of 13 molecules the model gave similar results, with $r = 0.844$ and $s = 0.342$.

Gleeson *et al*. [69] reported the first purely computational models for large datasets of volume of distribution at steady-state in rat and human. The rat dataset contained 2086 in-house measurements for AstraZeneca compounds and the human dataset contained data from 199 marketed drugs. Individual models for each species were built using Bayesian neural networks, classification and regression trees, and partial least-squares algorithms with physicochemical descriptors. Best performance on the test sets was given by a combined 3-way model: for rat, RMSE = 0.374 log units, and for human, RMSE = 0.479 log units. Lombardo *et al*. [70] also developed a model of human volume of distribution. Their model fit i.v. clinical data reported for 384 drugs using a mixture linear discriminant analysis – random forest model using 31 descriptors. For the training data, the geometric-mean-fold-error was ~2, and for a test set of 23 proprietary compounds, the geometric-mean-fold-error was 1.78.

5.5 Clearance

Hirom [71,72] demonstrated more than three decades ago that the route of excretion of xenobiotics is dependent upon MW by testing up to 75 compounds in rat, guinea-pigs, and rabbits. Lower MW compounds (<350) were mainly eliminated in the urine (>90%). As MW increased from 350 to 450, a sharp increase in the fraction of compound eliminated in the bile occurred, and for MW >450, compounds were eliminated 50–100% in the bile in all three species. Smith [73] correlated the log of free metabolic and renal clearance (ml/min/kg) with log *D*, and found a similar relationship. Metabolic clearance increases with increasing log *D*, while renal clearance decreases with increasing log *D*.

Percent renal clearance was modeled for a set of 130 compounds from the literature using partial least squares applied to 3-D VolSurf or 2-D Molconn-Z descriptors [74]. The model based on VolSurf descriptors gave the best prediction

quality: model $r^2 = 0.844$, training set $s = 11\%$, test set $s = 13.4\%$. Yap *et al*. [75] tested a variety of algorithms and descriptors to develop a model for total clearance using a large set of literature data on 503 drugs administered intravenously to males. General regression neural network and support vector regression algorithms performed best, particularly when using the full set of 645 descriptors. Average fold error was on the order of 1.6 × for the best models.

6. METABOLISM

Oxidative drug metabolism is extremely complex and possibly the most poorly understood ADME property. Rapid metabolism is unacceptable for drug candidates, except for drugs whose metabolite is the active moiety, because it causes duration of action to be too short. Considerable work has focused on the liver enzyme CYP3A4, which is responsible for the metabolic clearance of approximately 50% of marketed drugs. Recent approaches used to model and understand drug metabolism include database matching, quantum mechanics, QSAR, and structure-based analyses.

For a commercial database of known metabolic transformations, Borodina *et al*. [76] extracted all known sites of aromatic hydroxylations. These observed transformations were used to generate all possible transformations for each molecule, giving an estimate of the probability that each transformation would actually occur. The method was 85% accurate in predicting site of aromatic hydroxylation when tested against a second metabolism database containing 1552 molecules. Boyer *et al*. [77] took a similar approach using reaction center fingerprints to estimate the occurrence ratio of a particular metabolic transformation. The method successfully predicted the three most probable sites of metabolism in 87% of compounds tested.

Quantum mechanical approaches have been successfully used to predict hydrogen abstraction potentials and likely sites of metabolism of drug molecules [78–81]. AM1, Fukui functions, and density functional theory calculations could identify potential sites of metabolism. Activation energies for hydrogen abstraction were calculated by Olsen *et al*. [81] to be below 80 kJ/mol, suggesting most CH groups can be metabolized; which particular one depends on steric accessibility and intrinsic reactivities.

Shen *et al*. [82] reported the use of a k-nearest-neighbor QSAR model trained to predict the metabolic stability of 631 molecules in human hepatic S9 homogenate. The model was accurate for ~85% of molecules in both training and test datasets. A GRIND QSAR model was shown to be able to predict the stability of molecules incubated with human CYP3A4 75–85% accuracy on test datasets [83]. A Bayesian regularized neural network using electrotopological descriptors was used to predict the K_m values of CYP3A4 substrates [84].

Until recently, structure-based analyses of CYP450 metabolizing enzymes were limited to homology model studies due to the lack of crystal structures of human CYP450s [85–88]. In the last few years, multiple crystal structures of human CYP4503A4 have been solved [89–91]. Very interesting results were reported by Ekroos and Sjoegren [91]. They found that CYP3A4 is much more

flexible than previously reported, and that the active site can enlarge by greater than 80% upon binding to ketoconazole, a potent CYP450 inhibitor. In fact, the crystal structure showed two molecules of ketoconazole were bound within the active site. A CYP3A4–erythromycin complex suggested multiple binding modes. These results suggest further experimental studies will be needed to improve modeling results for CYP3A4.

Cruciani *et al*. [92] have developed the program Metasite for the prediction of the site of oxidative metabolism by CYP450 enzymes. Metasite uses GRID molecular interaction fields to fingerprint both structures of CYP450s (from homology models or crystal structures) and test substrates and then matches the fields. Zhou *et al*. [93] showed that Metasite was able to correctly predict the site(s) of metabolism 78% of the time for 227 CYP3A4 substrates. Caron *et al*. [94] used Metasite to predict the oxidative metabolism of seven statins.

7. CONCLUSION

Many advances have been made in computational ADME modeling. For many ADME properties, models now exist which provide reasonably good predictive quality and can be deployed to aid medicinal chemists in drug discovery projects.

The usefulness of computational ADME models depends on many factors, including the quality and breadth of data used to build them, how well the model approximates the physiological or physicochemical mechanism of interest, how the model is made available to chemists, and how well the chemist understands and uses the model. Ideally, ADME models are made available on the desktop, are easy to use, and are fast enough to help a chemist to better evaluate and prioritize a variety of molecular designs or even libraries each day. ADME models can also play a crucial role in helping the interpretation of experimental data by directly highlighting structural features the model associates with a particular ADME property, or at least allowing a chemist to quickly sketch different analogues and remove portions of a molecule to observe how the model's predictions change. A number of companies are reporting ADME/cheminformatics systems designed to aid in these efforts [95–98].

Two major issues for ADME modeling are data availability and optimization. The lack of larger data sets has hampered development of ADME models and reduced their potential quality; however, articles reviewed here show that this situation is improving. More human and animal ADME data would provide significant benefits. The fact that many ADME properties interact means that we must optimize a molecule's ADME properties simultaneously, or much work will be wasted traversing chemical space fixing one poor property but inadvertently causing a second to worsen [99–101]. This requires more work to develop systems with multiple ADME models having scoring functions for overall molecular (and series) quality based on both model predictions and experimental data as it becomes available, such as reported by Segall *et al*. [98].

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CHAPTER 30

Prediction of Human Volume of Distribution Using *in vivo*, *in vitro*, and *in silico* Approaches

R. Scott Obach

Contents

1. INTRODUCTION

It has become increasingly accepted that the pharmacokinetic behavior of new drugs represents an important attribute, along with efficacy and safety. The frequency with which a drug must be taken is a function of several factors: the half-life, the span between minimally efficacious concentrations and concentrations that cause side-effects, and the pharmacokinetic-pharmacodynamic relationship. Typically medicinal chemists optimize the predicted pharmacokinetics of

Department of Pharmacokinetics, Pharmacodynamics, and Drug Metabolism, Pfizer Global Research and Development, Groton Labs, Groton, CT, USA

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compounds in humans and the potency and other compound attributes simultaneously. Predicted human half-lives can be lengthened by decreasing the predicted clearance, and it is now commonplace in drug research to screen the newly synthesized compounds for *in vitro* metabolic lability in assays using human-derived reagents (e.g., hepatic microsomes).

The half-life ($t_{1/2}$) of a drug is a function of two variables: clearance and volume of distribution. Half-life is directly related to volume of distribution (VD) and inversely related to clearance (CL):

$$t_{1/2} \propto \frac{\mathrm{VD}}{\mathrm{CL}} \tag{1}$$

Thus, while the half-life can be lengthened by reducing the clearance (as stated above), the half-life can also be lengthened by increasing the VD.

2. DEFINITIONS OF VOLUME OF DISTRIBUTION

2.1 Volume of distribution: a fundamental definition

To understand VD at its most basic element, begin by considering a vessel with no volume markings on it that contains an unknown volume of solvent. Into this volume of solvent is dissolved a known mass of solute. Then, a sample of the solution is removed and the concentration of the solute is measured. By knowing the mass of solute added and subsequently measuring the concentration of the solution, the volume of the solvent can be computed:

$$\text{volume of the solvent} = \frac{\text{mass of solute}}{\text{concentration of the solution}} \tag{2}$$

This is analogous to a pharmacokinetic volume of distribution. In the example, the mass is analogous to the dose of a drug and the concentration is analogous to the concentration of drug in the plasma. By knowing the dose, and measuring the concentration, the distribution volume can be calculated. In a simplistic sense, this is what is done to measure the volume of distribution of a drug in a pharmacokinetic study.

To add a bit of complexity, now imagine that an unknown portion of this solute sticks to the inside walls of the vessel, and that the extent of this is not known. When this occurs, the measured concentration of a sample of the solution will be lower. When this lower measured concentration is combined with the known mass of solute that was dissolved, the calculation of the volume of the solvent will yield an overestimate. This is analogous to a drug that leaves the plasma and enters tissues – the calculated volume will increase.

2.2 Volume of distribution: a conceptual definition

Unlike the simple example of the solution described above, a human body is a complex mixture of components (tissues, proteins, membranes, etc.). Yet, in human pharmacokinetic studies, with rare exceptions only the plasma

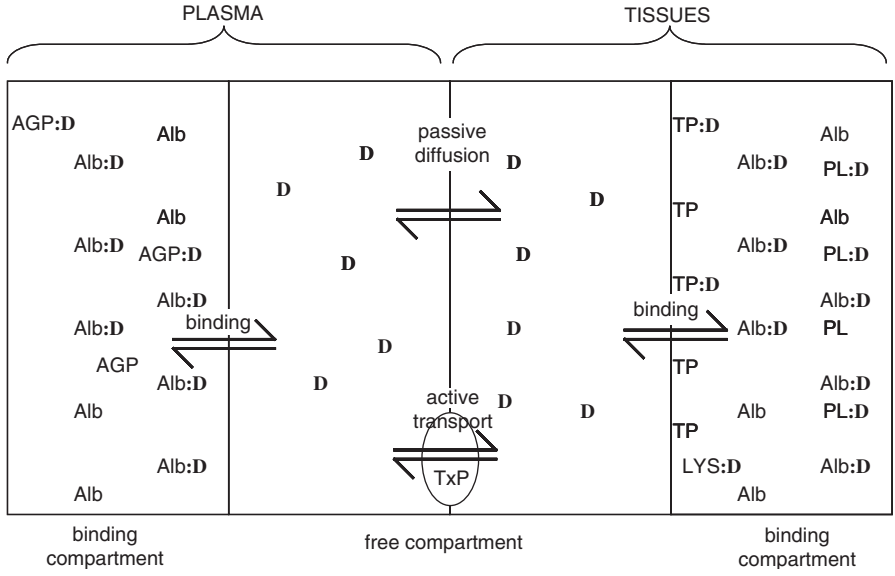

Figure 1 A schematic illustrating the concepts of drug distribution as a function of relative binding to plasma and tissue components. Abbreviations: D = drug, Alb = albumin, AGP = α_1-acid glycoprotein, TxP = drug transport protein, TP = tissue proteins, PL = phospholipid, LYS = lysosome. When the D is depicted as X:D, this refers to drug that is reversibly bound to the adjacent component, X. The plasma compartment, which is the one sampled during a pharmacokinetic study, is comprised of albumin and α_1-acid glycoprotein which to varying extents bind to the drug, as well as free drug (represented in this case by five D's). If the drug can readily penetrate membranes, then free drug in plasma is equal to free drug in tissue. In the case that the drug is a substrate for active transport proteins, this free plasma to free tissue concentration ratio can deviate from unity. (In this example the ratio is 5:6.) When in the tissue, the drug can bind to a variety of structures to varying extents, including soluble proteins like albumin in tissues, other soluble and insoluble proteins in tissues, phospholipid membranes, lipid vesicles, and be sequestered in lysosomes due to the pH differential in this organelle. The VD will be a function of the total drug in the plasma compartment (both free and bound; 13 D in this scheme) and the tissue compartment (both free and bound; 18 in this scheme).

concentration of drug is measured. The more that a drug leaves the plasma and enters the tissues, the greater its volume of distribution will be. A conceptualized simplification is shown in Figure 1. If a drug readily permeates the membranes that divide the body into its myriad tissues and compartments, then the volume of distribution will be a function of the extent to which the drug binds to plasma components ($f_{b(plasma)}$) versus the extent to which the drug binds non-specifically to tissue components ($f_{b(tissues)}$).

$$VD \propto \frac{f_{b(tissues)}}{f_{b(plasma)}} \tag{3}$$

For the purposes of this discussion, ''tissue components'' are being lumped into one huge mass; it is, however, clear that this actually represents a summation of a highly complex set of binding phenomena for each drug in a variety of tissue types. Non-specific binding sites in tissues to which drugs bind can be unique to various tissues and can also be common to several tissue types. For example, some drugs non-specifically partition into phospholipid membranes in tissues. Tissues that are relatively rich in phospholipids, such as brain tissue, can contribute a large share to the distribution volume of such drugs, even though there will be partitioning into all tissues that contain phospholipids. Binding to plasma is primarily driven by the extent to which drugs bind to the two major drug-binding proteins in plasma: albumin and α_1-acid glycoprotein, although other proteins such as globulins and lipoproteins are also present and can contribute to drug binding. Thus, drugs that are highly bound to plasma proteins tend to have smaller volumes of distribution. However, it should be kept in mind that VD is a function of the relative extents of binding to plasma and tissues. Thus, some drugs that are highly bound to plasma proteins also have high VD because they bind to tissues even greater (e.g., amiodarone).

Some physiological volumes are known or have been estimated. Over two decades ago, Oie and Tozer proposed a relationship between the volume of distribution of a drug and its extent of plasma and tissue binding, using various fixed values for plasma and extracellular fluid volumes [1]. This equation has been utilized in some methods used for prediction of steady-state VD, which will be discussed later:

$$\mathrm{VD_{ss}} = V_\mathrm{p}\left(1 + R_\mathrm{e/i}\right) + V_\mathrm{p}\left(\frac{1}{f_\mathrm{b(plasma)}}\right)\left(\frac{V_\mathrm{e}}{V_\mathrm{p}} - R_\mathrm{e/i}\right) + V_\mathrm{r}\left(\frac{f_\mathrm{b(tissues)}}{f_\mathrm{b(plasma)}}\right) \qquad (4)$$

The terms $f_\mathrm{b(plasma)}$ and $f_\mathrm{b(tissues)}$ represent fraction of the drug that is bound in plasma and tissues, respectively, the terms V_p, V_e, and V_r represent physiological constants of the volumes of plasma, extracellular fluid, and ''rest'' of the fluid in the body, respectively, and $R_\mathrm{e/i}$ is the ratio of concentrations of drug binding proteins (e.g., albumin) in the plasma versus extracellular fluids.

2.3 Volume of distribution: pharmacokinetic definitions of $\mathrm{VD_c}$, VD_β, and $\mathrm{VD_{ss}}$

In pharmacokinetic studies, several different types of volumes of distribution can be calculated from the data, and each of these volumes has a different meaning. Unfortunately, different investigators can utilize different terminologies for VD values in reports on pharmacokinetic data and this can be confusing. The key to understanding which VD is being discussed is to understand how the value was calculated. It is very important to note that true VD values can only be determined following direct administration into the plasma compartment, i.e., intravenous administration. VD values calculated following other routes of administration, such as oral, intramuscular, subcutaneous, etc., require that assumptions are made, and are of diminished value.

After intravenous dosing of a bolus of drug, plasma samples are obtained as quickly as possible after administration for measurement of drug concentrations. However, there is a practical limit to how fast the sample can be drawn and also there is a finite period for the blood to make a complete circuit through the body. Because of this, the plasma concentrations measured over the first few sampling times are back-extrapolated to time = 0 to afford an estimate of what the initial drug concentration would have been were it possible to deliver the drug to the entire plasma volume all at once (referred to as C_0). Drugs that can readily penetrate membranes will demonstrate some level of distribution even at these early sampling times. An estimate of VD_c can be obtained by relating the extrapolated concentration at $t = 0$ to the intravenous dose:

$$VD_c = \frac{\text{Dose}}{[C_0]} \tag{5}$$

Drugs that can distribute into tissues rapidly will have VD_c values that exceed the volume of plasma. The smallest possible value would be the volume of plasma (about 3 l in an average human).

A commonly reported VD value is the volume of distribution during the terminal elimination phase. This has been abbreviated with the terms VD_β, VD_z, or VD_{area}. (VD_β will be the nomenclature used below.) This volume is reflective of the partitioning of drugs into a ''deep'' tissue compartment, which refers to a tissue from which the drug slowly leeches out back into the plasma. This rate of return of drug from the deep tissue compartment is limiting and dictates the elimination rate of drug from the body. However, for determining dosing regimen, the terminal phase half-life may not be meaningful, VD_β can represent very small amounts of the total dose, and plasma concentrations can be substantially lower than those needed for efficacy. The terminal phase volume of distribution is calculated from the elimination rate constant (k_{el}) and the clearance (CL):

$$VD_\beta = \frac{CL}{k_{el}} = \frac{\text{Dose}}{AUC_{0-inf} \bullet k_{el}} \tag{6}$$

While this expression is written as the VD value being dependent upon CL, it is important to understand that VD and CL are the independent variables in this relationship and k_{el} (and hence $t_{1/2}$) is the dependent variable.

The most important VD value for dictating the dosing regimen is the steady-state volume of distribution (VD_{ss}). This volume represents the extent of distribution when the rate of transit to and from the tissues is equal. It is more representative of a time-averaged volume of distribution and its value will reside somewhere in between VD_c and VD_β. The steady-state VD is calculated from the mean-residence time (MRT).

$$VD_{ss} = \frac{\text{Dose} \bullet AUMC_{0-inf}}{(AUC_{0-inf})^2} = CL \bullet MRT \tag{7}$$

Experimentally, VD_{ss} is determined by calculating the area under the first moment of the plasma versus time curve (AUMC), which when combined with AUC will yield the mean residence time.

3. PREDICTING HUMAN VOLUME OF DISTRIBUTION FROM CHEMICAL, *IN VITRO*, AND *IN VIVO* DATA

3.1 General considerations

The accurate prediction of VD is clearly desired when making selections of new pharmacological agents for further development as drugs. Over the years, several types of approaches have been described that can be used for making such predictions. These approaches vary in the extent of effort needed to generate the data required for the prediction method. Obviously, methods requiring animal pharmacokinetic data are the most expensive and labor intensive, as they require synthesis of suitable quantities of test compound (e.g., 50–500 mg), administration to animals and collection of plasma samples, development of bioanalytical methods for measuring the test compound in plasma, and analysis of samples. *In vitro* approaches are somewhat less resource intensive but still require synthesis of test compound (typically 1–10 mg) and measurement of various *in vitro* parameters (e.g., plasma protein binding, etc.). Approaches that require only chemical data are more resource sparing and if computational approaches alone are used, then no test compound needs to be synthesized and no experimental measurements made.

These different types of approaches can be strategically placed in the drug research process so as to provide optimal value. For example, in the early phases when thousands of compounds are being considered in any given target pharmacology, it would be inappropriate to use VD prediction methods that rely upon animal pharmacokinetic data (from both a pragmatic/cost reason as well as to be consistent with efforts to reduce whole-animal experiments in scientific research). However, use of such an approach for a few selected compounds representative of different chemical series may be appropriate. Alternately, use of *in vitro* approaches (if automated) or *in silico* approaches to predict VD may be most appropriate when faced with thousands of test compounds. As each drug research project matures and the number of test compounds that continues to be considered for continued development is reduced, then more elaborate and resource-intensive prediction methods may be appropriate. Such a strategy is appropriate when these more resource intensive approaches yield predictions of VD that are considerably more accurate than those generated using more resource-sparing methods (i.e., *in silico* or *in vitro* methods). Whether simpler approaches to predict human VD are as accurate as methods that require animal data is debatable; the methods that use *in vitro* or *in silico* data have improved in recent years. In the three subsections that follow, different types of approaches to predict human VD are described.

3.2 Predicting VD from *in vivo* data (animal pharmacokinetic data)

Prediction methods based on animal pharmacokinetic data can be categorized into three types: (1) allometric scaling, (2) proportionality methods, and (3) correlative approaches. All three make a basic underlying assumption that the types

of molecular phenomena that cause drugs to bind to tissues are similar across species, and that the differences in VD between animals and humans is a function of differing tissue type compositions (i.e., masses of various drug binding tissues, such as muscle or adipose, per kg body weight).

3.2.1 Allometric scaling

Allometric scaling, as applied to pharmacokinetics, refers to the development of relationships between various pharmacokinetic parameters (e.g., clearance, volume of distribution, etc.) and physiologic constants. Despite being applied for decades, it remains somewhat controversial as its critics highlight its empirical nature and failure to account for interspecies differences in the biochemical processes (e.g., differing drug metabolizing enzymes) that drive the pharmacokinetics of drugs. Nevertheless, while the application of allometric scaling to predicting drug clearance may be subject to criticism, its application to predicting VD in humans from animal pharmacokinetic data remains a viable and effective method. For predicting VD, as for other pharmacokinetic parameters, the underlying concept of allometry is a description of the relationship between the VD and the body weight (W) of the species as a simple exponential function:

$$\mathrm{VD} = a \bullet W^{b} \tag{8}$$

in which a and b are referred to as the allometric coefficient and exponent, respectively. The VD for a given compound is measured in two or more animal species (typically, common laboratory animal species), and the values are plotted versus body weight on a logarithmic plot. The value for human is extrapolated from a standard body weight value of 70 kg.

There are numerous reports of the use of allometric scaling for predicting human pharmacokinetics for individual drugs, but the efforts summarized in this article describe only those that have attempted to broadly apply allometry for many drugs, as these have been the reports that have been most insightful on the topic. Efforts by Mahmood described in a series of articles, showed the ability of allometric scaling to predict human VD for several drugs [2–5]. Using a set of twelve structurally diverse drugs, the use of three species for prediction of VD in human was shown to offer no advantage over using just two [5]. The focus of these predictions has been for VD_c as opposed to VD_{ss}, and the value of this was claimed to be for using the predicted VD value to estimate appropriate first-dose-in-man levels in clinical trials. For a set of drugs, it was shown that VD_c was more accurately predicted than VD_{ss} [2]. Furthermore, allometric scaling of ndividual constants that describe the concentration versus time curve in a two-compartment model was done, and these constants scaled to human were used to estimate human VD_c, raising allometric scaling to another level of complexity [3]. However, this variation on the approach was not claimed to be significantly better than the aforementioned simpler direct scaling approach. In another report, allometric scaling was shown to be reliable at predicting VD_{ss}, but this was after the values were corrected for interspecies differences in plasma protein binding, i.e., free VD_{ss} was being predicted [6]. This makes intuitive sense: since VD is a function of both tissue binding (a gross composite of non-specific binding

phenomena) and plasma protein binding (a function mostly of the affinity for two main drug binding proteins in plasma, albumin, and α_1-acid glycoprotein). A cross-species difference in either of these plasma proteins with regard to its interaction with any given drug would confound an allometric relationship.

A recent variation on the prediction of human VD using allometric scaling involves the use of what has been termed ''fractal' volume of distribution (v_f) [7]. This refers to the VD value corrected to within the bounds of actual volumes within the body – in the case of human the upper and lower bounds would be 70 l and plasma volume, respectively. Thus, even if a compound were to have a VD_{ss} of 1000 l, its v_f would be 69.8 l. The authors of this approach have shown that v_f scales allometrically across species better than VD [8], with the explanation that body volume and body mass are exactly scaleable across species. Animal values for v_f are calculated from VD obtained from pharmacokinetic studies using the relationship:

$$\upsilon_f = \left(V_{total} - V_p\right) \bullet \frac{VD - V_p}{VD} + V_p \tag{9}$$

in which V_p and V_{total} refer to the plasma volume and total body volume, respectively, and VD can be any of the various VD terms (i.e., VD_c, VD_{ss}, VD_β). From the allometrically extrapolated human v_f, the VD is back calculated. The authors report a mean-fold error of prediction of 1.51 for VD_c when determined through allometric scaling of fractal volume of distribution compared to a value of 1.72 for the same set of compounds scaled using VD_c directly.

Some discussion of the allometric exponent (term ''*b*'' in equation 8) is worthwhile here. For allometric scaling of CL, exponents can vary somewhat from drug to drug, but it is common for them to reside near a value of 0.7. Interestingly, many physiologic and metabolic parameters demonstrate a similar exponent when scaled across species using body weight as the independent variable. This suggests that as animal species increase in size, their ''rates'' do not increase proportionately. However, for VD, allometric exponents are typically around unity, suggesting that simpler relationships between VD and body weight across species may exist, and this is important for some of the other VD prediction approaches described below. Overall, allometric scaling for the prediction of human VD is a generally reliable and accurate method, and when tested with large groups of compounds, typical predictions fall within 2-fold of the actual values.

3.2.2 Animal–human proportionality methods

Proportionality methods refer to those in which VD is measured in a single species, and the data are combined with a measurement of plasma protein binding in that species along with a plasma protein binding value for human. The underlying assumption is that the extents of tissue binding in the selected animal species and in human are similar – thus the unbound VD values are the same across species. This was demonstrated for a set of 10 basic drugs by Sawada *et al.*, over 20 years ago [9]. For individual drugs, linear relationships were shown to exist between VD and free fraction across species. And while a weak relationship

was shown to occur between VD in animals and VD in humans for this set of 10 drugs, the relationship between free VD in animals and free VD in humans was strong. For weakly basic drugs, the main driver for tissue binding is likely the extent of association between the cationic lipophilic drugs and the anionic phospholipid membranes in tissues, and this driver is likely quantitatively similar across species; hence the findings of Sawada *et al*. [9]. In another report, a proportionality approach was used to predict VD_{ss} in humans from VD_{ss} in dogs, correcting for any differences in plasma protein binding between humans and dogs [6]. Interestingly, this simple approach was shown to be more accurate than allometric scaling, despite requiring less input data. The overall mean-fold error of this method was considerably less than two. In the same paper [6], a method in which VD_{ss} is measured in multiple animal species along with plasma protein binding in those species plus human to predict human VD_{ss} was described and tested. In this method, equation 4 (above) is used to back-calculate tissue binding in each species, the values for tissue binding are averaged, and this value is used as an estimate of the value in human which is combined with the human plasma protein binding value to yield an estimate of human VD_{ss}. Interestingly, despite utilizing data from more species, this approach was not as accurate as the simpler one that utilized only data from the dog. It is possible that the dog represents a more similar species to human with regard to tissue binding properties of drugs, either through more similar body composition characteristics or more similarities in macromolecules that bind drugs non-specifically in tissues. However, this proposal remains speculative. It is also important to note that development of simple proportionality methods like the one described that uses dog pharmacokinetic data, but using rat or monkey data instead, did not yield methods that matched the dog method in overall accuracy (unpublished observations).

3.2.3 Correlative methods

Correlative methods represent some more recently described approaches driven from the development and analysis of pharmacokinetic databases. In these approaches, for a given set of drugs, VD data measured in human are plotted versus VD data measured in selected animal species, and linear relationships derived. Predictions for new compounds are made by extrapolating human VD values from measured animal VD values using these correlations. An analysis of a database of 103 drugs for which VD_{ss} data were available for human and laboratory animal species was conducted in which allometric scaling was examined as well as simple species versus species correlations [10]. Prediction methods were assessed using two different criteria: (a) evaluation of whether a method correctly classified human VD_{ss} values into low (<0.7 l/kg), mid, or high (>2.8 l/kg) ranges and (b) evaluation of the percentage of times that a method predicted human VD_{ss} within 2-fold of the actual value. Interestingly, a simple correlation between VD_{ss} data in monkey and human yielded the greatest performance, even when compared to the more elaborate multispecies allometric scaling method. It should be noted that for this dataset, when allometric scaling was performed, the allometric exponent was typically around 0.8 rather than unity as mentioned above. These investigators expanded on their work by

integrating computational chemistry parameters (e.g., molecular weight, rotatable bonds, lipophilicity, polar surface area, etc.) into estimates of likelihood of success of prediction using the correlative approach [11]. It should be noted that this is not an *in silico* method per se (like those described below); rather it is the use of computed physiochemical parameters for individual compounds to direct the investigator to the most successful VD prediction method. A set of physiochemical "rules" were developed to describe the appropriate instances for selection of the animal species that will provide the best prediction of human VD_{ss}. For example, if the monkey correlation approach yields a predicted human VD_{ss} between 0.7 and 3.5 l/kg, then those compounds possessing less than eight rotatable bonds and a polar surface area of less than 100 $Å^2$ are more likely to be successfully predicted. In this work, it was important to note that small VD_{ss} compounds were well predicted irrespective of the physiochemical properties examined.

While the research described above suggested that the monkey was the species that yielded the most predictive correlations from which to predict human VD, Caldwell *et al*. have conducted a similar analysis and showed that VD data obtained in the rat yielded a predictive correlation [12]. In their approach, simply by multiplying the measured rat VD_{ss} value by a factor of 188 yields a prediction for human (in units of volume that are not corrected for body weight) or by a factor of 0.67 when values are corrected for body weight. The approach yielded a mean-fold error of 1.85, which is a comparable level of error as other *in vivo* methods.

Wajima and coauthors offer an alternative approach to utilize animal VD data to predict human VD [13]. Several compound descriptors that included both chemical structural elements as well as animal VD_{ss} values were subject to multiple linear regression and partial least squares statistical analyses, with human VD_{ss} as the independent parameter to be predicted using a dataset of 64 drugs. Methods derived in this manner were compared to simple allometry for overall accuracy. Their analyses yielded the following regressions:

$$\log VD_{ss,man} = -0.03869 \bullet H_a + 0.009311 \bullet \log VD_{ss,rat} \bullet H_a + 0.1256 \bullet \log VD_{ss,dog} \bullet \log VD_{ss,dog} + 1.857 \quad (10)$$

and

$$\log VD_{ss,man} = 0.1859 \bullet \log VD_{ss,rat} \bullet \log VD_{ss,rat} - 0.3887 \bullet \log VD_{ss,rat} \bullet \log MW + 0.3089 \bullet \log VD_{ss,dog} \bullet \log MW + 0.003306 \bullet \log MW \bullet c\log P + 1.71 \quad (11)$$

In which the terms H_a refers to the number of free electron pairs, MW is the molecular weight, and *c*logP is the computed lipophilicity. While this method could be stated to be "partially *in silico*" because it utilizes some chemical descriptors, the need for *in vivo* animal data and their dominance in the individual terms really makes this approach more of an animal–human correlation than an *in silico* method. Finally, in the same report, the authors describe a regression based solely in animal data. Overall, the performance of these

methods was good, with over 70% of compounds yielding estimates of human VD_{ss} that were within 2-fold of actual values. Wajima and coworkers extended this work to use these values for predicted human VD_{ss} in the estimation of circulating drug concentration versus time courses [14].

3.3 Predicting VD from *in vitro* data

Utilizing *in vitro* data to predict human VD has been reported over several years. In these approaches, the underlying assumption is that an *in vitro* measurement adequately represents a surrogate of tissue binding *in vivo*. *In vitro* measurements made for use in predicting VD have included determination of binding of drugs to plasma and tissue homogenates, determination of propensity to bind membranes, and determinations of solvent partition coefficients under various conditions. The data have been used for simple estimates of partition coefficients through to more complex physiologically based pharmacokinetic modeling (PBPK).

Over the years, various tissues and tissue components have been discussed as compartments into which drugs can penetrate and bind. The ability of lipophilic drugs to partition into membranes has been known for many years, especially cationic drugs that can bind to anionic phospholipid membranes [15–20]. The phenomenon of lysosomal trapping has also been cited as a factor that contributes to the tissue binding and high VD values of weakly basic drugs [20–23].

For purposes of this discussion, *in vitro* methods for predicting VD are divided into two categories: (1) tissue binding approaches and (2) correlation to experimentally determined physio-chemical properties.

3.3.1 Tissue binding approaches

Demonstration that tissue partitioning can be determined *in vitro* was made by comparing tissue/plasma ratios generated using equilibrium dialysis to *ex vivo* tissue partitioning data in rats [24]. However, it was noted that this method tended to underestimate partitioning of bases into tissues that are rich in lysosomes, such as lung. Using rabbit muscle homogenates, Schuhmann *et al*. [25] demonstrated a strong correlation between free VD and total/free ratios in muscle homogenate for 9–11 drugs spanning a range of chemical properties. Muscle homogenate binding was determined using ultrafiltration, and the selection of this tissue type rather than others was made on the basis that muscle mass comprises the vast majority of body mass. There are some practical challenges in measuring binding to tissue homogenates including the fact that data need to be gathered in dilutions of homogenates followed by extrapolation to binding values that would be observed in undiluted material. Interestingly, the correlation between total/free ratios in rabbit muscle homogenate correlated even better to human free VD values than rabbit free VD values, reinforcing the notion that tissue binding is a non-specific phenomenon driven more by the physicochemical nature of the drug than any specific property of the tissue.

Hinderling reported linear correlations between red blood cell/buffer partition coefficients and free VD_{ss} in humans [26]. Separate correlation equations

were derived for acids:

$$\log VD_{ss,free} = 1.995 + 0.550 \bullet \log(\text{blood cell/buffer});\ r = 0.867 \quad (12)$$

and bases:

$$\log VD_{ss,free} = 1.945 + 1.669 \bullet \log(\text{blood cell/buffer});\ r = 0.924 \quad (13)$$

The conclusion was offered that binding into blood cells represents a reasonable surrogate of tissue binding, as blood cells would contain similar compositions to major tissue depots for drug binding.

However, others maintain that adipose tissue is an important contributor to VD. Bjorkman showed that adipose and muscle tissue partitioning are the two tissues that yield the best predictions of VD_{ss} and that such data obtained in other tissues did not offer more accuracy [27]. (Note that the tissue partitioning data used to predict human VD_{ss} were from rat or rabbit *ex vivo* measurements.) The emphasis on both adipose and muscle was also advocated by Poulin and Thiel in their prediction method that uses solvent/water partition coefficients [28] (see below).

Finally, among the work described in a comprehensive piece on predicting human pharmacokinetics using physiological-based pharmacokinetic modeling, predictions of human VD were included [29]. For most cases, these investigators utilized an *in vitro* approach described earlier by Poulin and Thiel [28] based on estimates of tissue/plasma partitioning that is described in greater detail below. However, in some instances that approach was deemed unsuitable when it did not provide a good estimate of VD in animals. In those cases, the authors either measured a total tissue partition coefficient in rat for use in predicting human VD, or even in a few cases the concentrations of drug in selected rat tissues were experimentally determined for making this prediction. The latter clearly represents a very resource-intensive approach to predicting human VD. It should be noted that in this PBPK approach, even the use of *in vitro* data to predict human VD was only made when such data adequately predicted VD in rats – thus necessitating the measurement of rat VD.

3.3.2 Correlations between volume of distribution and experimental physiochemical properties

Free VD values (i.e., VD/f_u) were shown to be highly correlated with $logD_{7.4}$ (octanol/water) for over 100 drugs [30]. Several linear regressions were derived correlating $logD_{7.4}$ with VD values – either VD_c, VD_{ss}, or VD_β, with r values in excess of 0.9 for most. The authors made a point of stating that VD_c was most highly correlated, however the correlations to other VD values were very close. The linear regression for VD_{ss} yielded the relationship:

$$VD_{ss} = 0.1139 \bullet \log D_{7.4} + 0.7762\ \ r = 0.942 \quad (14)$$

A weak correlation between octanol/water partition coefficient (logP) and $logVD_{ss,free}$ was shown for a set of 36 weak bases, while a similar relationship using VD data for 15 weak acids could not be demonstrated [26]. The correlation was binomial and likely would not be tight enough to enable prospective human

prediction of VD values from logP data alone, although such an analysis was not reported.

A comprehensive piece of work on predicting VD_{ss} from physiochemical data was reported by Poulin and Thiel for both rat and human [28]. The experimental input data required is the plasma protein binding, blood cell partitioning, octanol:water partition ratio, and olive oil:water partition ratio. The underlying relationship utilized was:

$$VD_{ss} = V_p + V_e \bullet B/P + \sum V_t \bullet P_{t:p} \quad (15)$$

In which V_p refers to the plasma volume, V_e is the volume of blood cells, B/P is the blood/plasma partition ratio, V_t is the volume of a tissue compartment, and $P_{t:p}$ is the partition coefficient for the drug between the tissue compartment and plasma. The authors make the claim that tissue compartments can be categorized as two parts: adipose and non-adipose tissue. Physiological constants were used for each of the volume terms. For the non-adipose tissue value for $P_{t:p}$, the octanol:water partition coefficient was combined with several fixed values for the volumes of neutral lipids, phospholipids, and water in tissues and plasma, as well as plasma free fraction and an estimate of tissue free fraction based on the ratio of drug binding proteins (e.g., albumin) in extracellular space versus plasma. For the adipose tissue value for $P_{t:p}$, a similar approach was taken, except that the olive oil:water partition coefficient was used, the rationale being that adipose tissue is better represented by a triglyceride than octanol, since it has a high composition of triglycerides. The authors divided the compounds into three groups: one for which the method predicted the actual VD_{ss} value within 2-fold, one for which the method failed to do this, and acids with very low VD_{ss} values, in order to discuss their findings. (Of course, when applying this approach in a prospective manner, one would not know whether a compound was predicted within 2-fold or not.) The group of compounds that failed in this approach was comprised of a large number of weak bases that were underpredicted, and the authors offer the possibility that this could be due to these compounds accumulating in lysosomes by charge-trapping. In the report, the average ratio of predicted to actual VD_{ss} values for the successful group was reported as 1.06, however in this calculation over- and underpredictions would compensate for each other. (Note: This author calculated a geometric mean-fold error using all of the data for human VD_{ss} prediction in the paper by Poulin and Thiel and determined a value of 1.73. This shows that this method possesses an acceptable ability to be used in pharmacokinetic predictions, and is comparable to many and better than some of the other methods described in this review.)

Lombardo and colleagues described a method wherein experimental logD (*e*logD) values determined using an HPLC method were combined with data on the cationic nature of molecules and measured values for free fraction in human plasma to predict human VD_{ss} for basic and neutral drugs [31,32]. The underlying premise of this approach, as with many of the approaches described in this review, is that tissue binding is related to lipophilicity and charge. Using the Oie–Tozer equation (equation 4), values for the fraction unbound in human

tissues (f_{ut}) for a training set of 64 drugs was calculated from VD_{ss} and f_u data. These calculated f_{ut} values were correlated with *e*logD, fraction cationic ($f_{i(7.4)}$, calculated from the pK_a), and plasma free fraction to obtain a linear regression:

$$\log f_{ut} = -0.0389 - 0.1739 \bullet e\log D_{7.4} - 0.8324 \bullet f_{i(7.4)} + 1.04 \bullet \log f_u \quad r^2 = 0.884 \tag{16}$$

From this regression, human logf_{ut} values were estimated for a test set of 14 compounds, from which human VD_{ss} predictions could be made. The overall mean-fold error for the test set was 2.20. Interestingly, when compounds exhibiting high protein binding ($f_u < 0.02$) were excluded from the analysis, the mean-fold error was considerably lower (1.62) providing a possible filter for the method (i.e., to not attempt it with highly bound drugs) and also possibly indicating that error in measuring very low plasma free fractions can confound the approach. In a later report, this method was extended to include larger training ($N = 120$) and test ($N = 18$ sets) and the coefficients of regression remained largely unchanged, indicating the overall robustness of the approach [32]:

$$\log f_{ut} = -0.0080 - 0.2294 \bullet e\log D_{7.4} - 0.9311 \bullet f_{i(7.4)} + 0.8885 \bullet \log f_u \quad r^2 = 0.867 \tag{17}$$

The mean-fold error improved slightly to 2.08.

A recent method was described in which human VD_{ss} can be predicted from HPLC measurements of albumin and phospholipid binding [33]. The underlying premise is that VD_{ss} is primarily driven by albumin binding in plasma and partitioning into phospholipid membranes in tissues. The investigators utilized specialized HPLC columns: one in which human serum albumin is immobilized and a second containing immobilized artificial membranes (i.e., resin bonded with phosphatidylcholine). These provide estimates of binding to albumin and phospholipid by calibrating them to known compounds assessed on these systems. The data obtained for 179 compounds yielded the following relationship:

$$\log VD_{ss} = 0.44 \bullet \log K_{IAM} - 0.22 \bullet \log K_{HSA} - 0.66 \tag{18}$$

In which K_{IAM} and K_{HSA} are derived from the retention times on the respective columns. Using a 30-drug subset as a test set, a mean-fold error using this approach of 2.09 was obtained, and some slight improvements could be obtained if parameters describing ionic character of the compounds were included.

3.4 Predicting human volume of distribution from *in silico* data

If the assertion that VD is driven by non-specific interactions between drugs and macromolecular structures in tissues, then it logically follows that VD would be correlated to physiochemical parameters. Since such parameters are amenable to computation from structure alone, the prediction of human VD from chemical structure is feasible. Such *in silico* approaches have only been described over the past few years, as computational chemistry tools have advanced.

In an early application of *in silico* approaches to predict human VD, Ritschel and coworkers described an approach using artificial neural networks (ANN), in this case for VD_β [34]. However, this was not a truly *in silico*-only approach as the ANN that yielded accurate predictions of human VD required animal pharmacokinetic data as input parameters, along with *in vitro* data (protein binding and logP).

The first report of a VD prediction method that utilized structural data alone was reported by Lobell and Sivarajah [35]. This is a fairly simple approach in which compounds were first divided into four groups based on charge: anionic, cationic, zwitterionic, and neutral. The VD data for a 204 compound training set and a 124 compound test set were obtained from the appendix of Goodman and Gilman's *Pharmacological Basis of Therapeutics*. (Note: a passage on VD databases is included below.) Among several computational descriptors examined, it was found that AlogP98 yielded correlations to human VD for some of these compound groups. For anionic compounds, it was concluded that no correlation existed between VD and AlogP98, however since all anionic compounds in the training and test sets possessed VD values proximal to 0.21/kg, the prediction method stated that this value was a suitable prediction for all anions. For cations, a correlation existed for compounds of AlogP98 between –2 and 5, and compounds outside those ranges had either very low or very high VD, respectively. Neutral compounds could be categorized into VD of 10 or 11/kg depending on whether the AlogP98 was greater or less than 5. Despite the promise of such a method, the overall predictive accuracy was only about 3-fold, which is not accurate enough for practical application in a drug research setting. This lack of accuracy is likely due to the simplicity of the approach.

Using a 70-drug set, Ghafourian *et al*., conducted a statistical analysis of computed chemical descriptors to predict human VD [36]. The descriptors found to provide a predictive model included logP, $\log D_1$, and μ_{MM} (a measure of dipole moment), in a linear regression:

$$\log \mathrm{VD} = -0.151 + 0.364 \bullet \log \mathrm{P} - 0.260 \bullet \log \mathrm{D}_1 - 0.086 \bullet \mu_{\mathrm{MM}} \qquad (19)$$

The overall accuracy of the predictions, assessed as the mean-fold error of prediction of the test set was 2.03, making this approach one that would possess suitable accuracy for use in drug design and human pharmacokinetic predictions. Similar methods developed separately for acids and bases showed an improvement in accuracy. This investigation also included a prediction of unbound VD, which should represent a simpler parameter to predict since it would be based only on tissue binding and not plasma protein binding. However, it is interesting to note that this approach was less accurate for this parameter, which would be unexpected.

The artificial neural network approach was applied to a training set of fifty compounds to develop models for predicting human VD_{ss}, as well as other pharmacokinetic parameters [37]. Eighty-five chemical descriptors were used to generate the ANN, after which these were pruned resulting in a model for VD_{ss} comprised by four descriptors, one specific for a functional group, one describing molecular connectivity, and two describing lipophilicity. The correlation between

predicted and actual VD_{ss} values for six remaining compounds used for the test set was very high (0.956). It would be interesting to determine how well this model stands up to use with a larger number of test compounds.

Recent *in silico* models for prediction of human VD_{ss} have included those generated using classification and regression trees (CART), Bayesian neural networks (BNN), and partial least squares (PLS) [38], stepwise regression [39] as well as random forest modeling [40]. In the CART, BNN, and PLS models, 75% of a dataset of 199 drugs was used as a training set to build the models for prediction of the remaining 25%. Statistical modeling yielded 23 descriptors upon which the models were built, and the models were judged by comparing regression statistics of the predicted versus actual values for the test set compounds. These were comparable among the three models, and the authors suggested using a consensus of the three models for even greater accuracy.

The model that utilized regression analysis was one that built upon previous work by the same authors [36,39]. In this case, the dataset was expanded to 125–129 drugs and the number of assessed descriptors increased to 210. Models for acidic and basic compounds were developed separately as well as a model using all compounds, and the advantages of analyzing acids and bases separately were minimal. Mean-fold errors were generally around 1.8. Descriptors that dominated the models included lipophilicity, fraction anionic or cationic, surface electrostatic potential, and parameters specific to aliphatic carbons and fluorine.

In the random forest approach, a large dataset of 384 human VD_{ss} values were used to build the model which employed 31 chemical descriptors that were statistically selected, using simulated annealing, out of over 1000 that were computed [40]. The descriptors selected for the model included expected ones such as *c*logP, fraction anionic, fraction cationic, as well as several structural fragments. Mean-fold error for a test set of 23 compounds yielded a value of 1.78, which to date is the highest accuracy reported for an *in silico* approach for predicting VD_{ss} and approaches the accuracy attained by methods that require experimental data as input. The large size of the training dataset in this model afforded an opportunity to conduct a leave-class-out analysis in which the model was recast after removing an entire structural class one at a time and VD values for the compounds in the removed structural class were predicted from the recast model. This permitted a ''real-world'' scenario in which the model would be used in predicting VD_{ss} for structurally novel sets of compounds in a drug research setting.

Finally, the utility of developing an *in silico* model from a more limited set of drugs of a given chemotype was demonstrated using different classes of antibiotics and a simulated annealing-nearest neighbor approach [41]. While such a model may not be able to be applied for molecules outside the structural type, it does demonstrate the potential for *in silico* modeling of VD to be applied to narrower compound sets, provided that an adequately large number of actual human VD values are available to use in model building.

Generating valid *in silico* models requires high quality databases for model training. True values of VD in human require that the parameters are calculated from pharmacokinetic data measured after intravenous administration. From equation 7 above, calculation of VD_{ss} requires that the dose that enters the bloodstream is known, which can only be guaranteed by intravenous

administration. Values for VD that are reported after oral administration are without value for understanding what the true distribution behavior of the drug is, unless the absolute bioavailability is known:

$$VD_{oral} = \frac{VD_{ss}}{F} \tag{20}$$

Volumes of distribution calculated after oral administration will therefore represent over-estimates of true VD and the values would represent a composite of VD as well as absorption and first-pass extraction. Many papers describing *in silico* models report the use of Goodman and Gilman's *The Pharmacological Basis of Therapeutics* as a source for human VD data, since a large table of human pharmacokinetic data is in one of the appendices of this textbook. While many values in this convenient resource are bona fide intravenous VD values, many others are actually VD/F values and should not be included in *in silico* models (either training or test sets). Nevertheless, these values tend to be included in some *in silico* models, and therefore may cloud the accuracy of predictions of intravenous VD values. Investigators who build these models, as well as those who use the models, should be aware of this possibility and should scrutinize the original source of the data used in model construction and testing.

4. CONCLUSIONS

It can be concluded that many methods exist which can provide predictions of human VD values for new chemical entities with acceptable accuracy in drug research efforts. In the search for new drugs, those with acceptable pharmacokinetic properties are sought because reasonable and convenient dosing regimens will provide a better chance for therapeutic success. Since VD plays a role in what the half-life of the drug will be, this parameter is important to predict. The determinants of VD (i.e., non-specific binding to tissues, plasma protein binding) appear to be driven by overall physiochemical properties of the compound and the gross physiological properties of the body. It is expected that the prediction of this parameter is more likely to be successful as compared to prediction of those parameters that are a function of specific biochemical processes (e.g., clearance).

Methods to predict human VD can be categorized by the type of data that are required as input, and these have been summarized in Table 1. Some methods utilize animal pharmacokinetic data, such as allometric scaling, and thus require the greatest effort in the generation of experimental data. Others require only *in vitro* data (biochemical data in some cases, physiochemical data in others), and while the conduct of *in vitro* experiments tends to be less labor intensive than *in vivo* studies (with some being amenable to high-throughput approaches), these still require synthesis of a small quantity of compound to test. *In silico* approaches offer the possibility of not requiring any experimental input data, and could thus be applied before compounds are even synthesized. The promise of *in silico* methods for prediction of human VD is high, and will be bolstered by an increased understanding of the chemical determinants that influence this parameter. Even now, overall accuracies of *in silico* VD prediction methods are close to or even exceed those achieved by methods that require experimental data.

Table 1 Summary of prediction methods for human VD

Approach/Method	Underlying assumptions	Input data required	References
In Vivo:			
Allometry	Determinants of VD (e.g., tissue and blood volumes; binding capacity) scale with body weight across species	VD_{ss} in two or more animal species	[2–5]
Allometry (unbound VD)	Determinants of tissue binding scale with body weight across species	VD_{ss} in two or more animal species, f_u in animals and human	[6]
Allometry (fractal VD)	Body volume and weight are related across species, tissue and plasma binding is similar across species	VD_{ss} in two or more animal species	[8]
Average animal tissue binding proportionality	Human tissue free fraction is represented by the average value in various animal species	VD_{ss} in two or more animal species, f_u in animals and human	[6]
Dog–human proportionality	Dog and human tissue binding capacities are similar	VD_{ss} in dog, f_u in dog and human	[6]
Cross-compound monkey/human correlation	Human VD is proportional to monkey VD across all drugs	VD_{ss} in monkey	[10]
Cross-compound rat/human correlation	Human VD is proportional to rat VD across all drugs	VD_{ss} in rat	[12]
Cross-compound correlation including chemical descriptors	Human VD is related to animal VD and chemical structure	VD_{ss} in rat and dog, computed chemical parameters	[13]

In Vitro:			
Rabbit muscle homogenate binding	Muscle tissue binding dominates all tissue binding and muscle binding in rabbit and human is similar	Fraction unbound in muscle homogenate by dialysis	[25]
Blood cell partitioning	Red blood cell binding is representative of tissue binding	Human blood cell/buffer partition ratio	[26]
Correlation to logD	VD is driven by lipophilicity	$logD_{7.4}$ (octanol:water)	[30]
Correlation to logD in octanol and olive oil	VD is a function of binding to adipose (triglycerides) and non-adipose tissues, and plasma free fraction	$logD_{7.4}$ (octanol:water), $logD_{7.4}$ (olive oil:water), protein binding in human, human blood cell partitioning	[28]
Correlation to *e*logD and fraction ionized	Tissue binding of bases and neutrals is primarily driven by lipophilicity and cationic character	*e*logD, pK_a, f_u in human	[32]
Correlation to HAS and IAM column retentivity	VD is a function of albumin binding and partitioning into phospholipid membranes, and these can be measured using affinity HPLC columns	Retentivity on human albumin and immobilized artificial membrane HPLC columns	[33]
In Silico:			
Correlation to AlogP98	VD is driven by lipophilicity	AlogP98	[35]
Correlation to logP, $logD_1$, and μ_{MM}	VD is a function of specific chemical attributes	logP, $logD_{pH=1}$, computed molecular connectivity	[36]
ANN	VD is a function of specific chemical attributes	Computed chemical descriptors	[37]
CART, BNN, and PLS	VD is a function of specific chemical attributes	Computed chemical descriptors	[38]
Regression	VD is a function of specific chemical attributes	Computed chemical descriptors	[39]
Random forest	VD is a function of specific chemical attributes	Computed chemical descriptors	[40]

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CHAPTER 31

Plasma Protein Binding and the Free Drug Principle: Recent Developments and Applications

George L. Trainor

Contents

1. INTRODUCTION

There has been increasing interest over the past few years in understanding the molecular processes that govern the behavior of drugs *in vivo*. The binding of drugs to plasma proteins such as serum albumin and α_1-acid glycoprotein is a long-known, well-understood phenomenon. The pharmacokinetic and drug–drug interaction implications of plasma protein binding are well described in the literature [1]. More recently, there has been increased appreciation of the role of plasma protein binding in limiting the effective activity of drugs at their site of action [2,3].

Research and Development, Bristol-Myers Squibb Co., Princeton, NJ 08543-4000, USA

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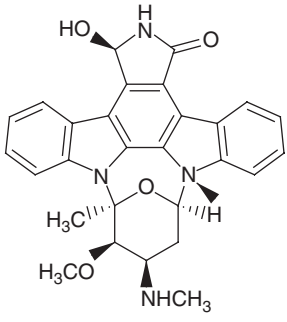
The fundamental premise of the so-called ''free drug principle'' is that the target occupancy and hence pharmacological activity is responsive to the free or unbound drug concentration (and not total drug concentration) at the site of action. An important corollary that derives from the same physical principles is that in the absence of energy-dependent phenomena (e.g. efflux or uptake transporters), the free drug concentration will be the same in all compartments that are in steady-state equilibrium. This chapter reports on literature, generally from 2006 to 2007, that supports or refines the understanding of the role of plasma protein binding on drug action *in vivo*. Reports from the medicinal chemistry literature where a deliberate attempt has been made to rationally modify plasma protein binding in a chemical series will be discussed. Advances in plasma protein binding determination will also be described.

2. REFINEMENTS IN UNDERSTANDING OF THE FREE DRUG PRINCIPLE

2.1 Preclinical studies

There have been a number of recent preclinical studies that highlight the importance of plasma protein binding when making cross-species comparisons, interpreting drug concentrations in various compartments, and determining effective drug concentrations at the site of action.

Although the degree of plasma protein binding is generally reasonably consistent across species, there are cases where free fraction varies significantly. An historical example is the case of the antitumor agent UCN-01 (**1**) which showed unexpectedly (based on extrapolations from preclinical studies) and dramatically low clearance in human clinical trials; this was attributed to exceptionally tight binding to human α_1-acid glycoprotein relative to mouse, rat, and dog (unbound fraction $<0.02\%$, 1.17%, 1.75%, and 0.49%, respectively) [4]. The opposite situation was recently encountered with the minor-groove-binding, antitumor agent SJG-136 (**2**) where the free fraction in mouse was very low compared to rat and human (unbound fraction $<1\%$, 16.2%, and 25%, respectively) [5]. The authors clearly stated their anticipation of possible species differences in pharmacokinetic behavior.



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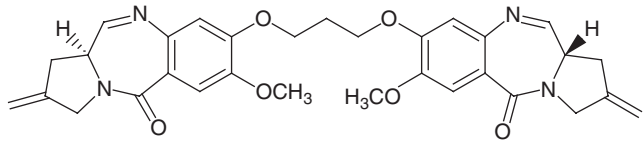

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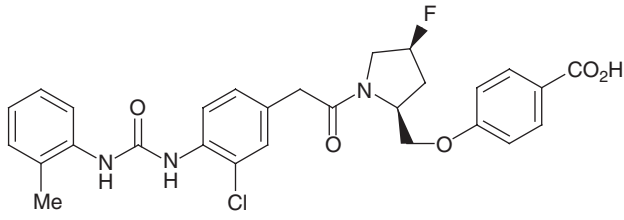
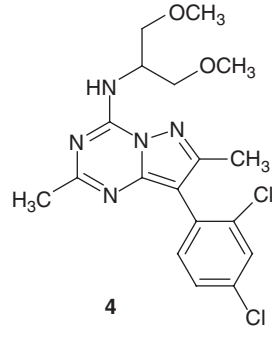
Significant differences in pharmacokinetics of drugs across strains of rats have been observed from time to time; for example in the case of diazepam the difference in pharmacokinetics has been shown to be attributable to polymorphisms in the enzymes responsible for enzymatic elimination [6,7]. In recent studies on the $\alpha_4\beta_1$ integrin antagonist D01-4582 (**3**) a roughly 20-fold difference in oral exposure (AUC) between SD and CD rats was attributed to a 17-fold difference in unbound fraction in plasma (0.26% and 0.015%, respectively) [8]. Additional rat strains were studied and differences in unbound fraction in plasma were found to be well correlated with genetic polymorphisms in two residues close to the binding site I in albumin. The authors note that the rare occurrence of genetic polymorphisms in human serum albumin suggests that there is a low probability of clinical risk.



3

In the past five years there has been a major advance in our understanding of the role played by plasma protein binding in controlling the distribution of drugs into deep compartments (e.g., the CNS), the interpretation of tissue to plasma drug level ratios, and the implications for drug action in those compartments. In a seminal paper, Kalvass and Maurer examined 18 diverse CNS compounds and established the concept of unbound fraction in the brain to link plasma concentration, plasma protein binding, and brain/plasma ratio [9]. Direct demonstration of the equivalence of plasma unbound drug and unbound drug in brain extracellular fluid using microdialysis following pharmacologic blockade of P-glycoprotein was reported for the HIV protease inhibitor amprenavir [10]. Studies with the corticotropin-releasing factor I antagonist DMP696 (4) relating *in vitro* receptor binding free plasma concentration, *in vivo* receptor occupancy and activity in a behavioral model were consistent with the concept of unbound drug at the site of action being pharmacologically relevant [11]. The Kalvass and Maurer finding was confirmed and expanded in subsequent papers based on nearly 100 compounds [3,12].



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These refinements in our knowledge of brain penetration and CNS activity of drugs feature prominently in a major medicinal review of the blood–brain barrier [14]. *In vivo* perfusion studies on the rate of brain uptake of several non-steroidal anti-inflammatory drugs in rats with increasing concentration of albumin in the perfusate clearly demonstrate the effect of plasma protein binding on the *rate* (in addition to the *extent* at steady-state) of brain uptake [15].

There have been several studies that underscore the importance of unbound concentration in cell-based studies of receptor function. In a model study of the effect of plasma protein binding on the renal transport of organic anions using the expression of various organic anion transporters (OATPs) in *Xenopus* oocytes, the transport of ochratoxin A, methotrexate, and estrone sulfate was found to be strongly inhibited by the addition of human serum albumin to the culture medium [16]. Similarly, the addition of α_1-acid glycoprotein was found to reverse the blockade of sodium-ion current by cocaine in a preparation of cardiac myocytes [17].

The free drug principle is finding application in toxicology studies with increased focus on free drug levels [18]. A study attempting to establish a predictive model for metabolism-based reactive-intermediate generation showed that *in vivo* covalent binding in the liver correlated best with free plasma AUC and *in vitro* binding [19]. Another study describing a predictive model for estimating the toxic serum concentration of compounds validated the use of shifts in cytotoxicity on addition of serum or albumin with extrapolation to 100% serum [20].

Although most plasma protein binding studies of drugs involve compounds bound to albumin and/or α_1-acid glycoprotein, there are occasional studies with other serum proteins. A recent study in mice with genetically knocked-out corticosteroid binding globulin showed the predicted increase in unbound fraction of endogenous corticosterone, but also highlighted the difficulty in predicting the overall effects in the face of both pharmacokinetic and pharmacodynamic changes [21].

2.2 Clinical studies

References to elements of the free drug principle are appearing with greater frequency in clinical publications where the target plasma levels of drug are discussed. The notion of adjusting plasma exposure/drug potency for plasma protein binding was first introduced in the HIV antiretroviral field and has been reviewed extensively [22]. A recent paper discussing the use of inhibitory quotients to optimize HIV therapy stresses the importance of adjusting potencies for plasma protein binding [23].

A number of recent publications indicate that the antibacterial field has adopted the concept of comparing free drug concentration at the site of action to *in vitro* drug potency reported as MIC [24–26]. A study of the antibacterial ertapenem in healthy volunteers was carried out to provide support for its use in skin and skin-structure infections [27]. Using microdialysis techniques, unbound drug concentrations in muscle and subcutaneous tissues were sampled at

multiple time-points and compared to total plasma concentrations. The concentrations versus time curves suggest that these three compartments are at steady-state equilibrium within 2 h after dosing. The unbound drug levels in the two tissues were similar and, relative to total plasma concentration, consistent with the reported free fraction for the drug (4–16%). The authors concluded that there was sufficient free drug present in tissue to support efficacy against a number of common pathogens.

In a similar microdialysis study in patients with the antibacterial agent linezolid, unbound concentrations in plasma, adipose tissue, and skeletal muscle were sampled to generate data to support dosing guidelines [28]. After reaching steady-state distribution, unbound drug concentrations were similar in the three compartments, though somewhat lower in adipose tissue. The penetration of linezolid into the cerebral spinal fluid (csf) was studied to support the use of this agent in neurosurgical patients [29]. The longer $T_{max,csf}$ and $t_{1/2,csf}$ relative to plasma and the csf/plasma ratio at $T_{max,csf}$ were fully consistent with slow movement into and out of the csf for the polar linezolid ($\log P = 0.55$) and a high free fraction (69%).

Plasma protein binding has also been taken into account in setting safety margins [30] and target concentrations [31] for antifungal agents.

The role of plasma protein binding in drug action has been of particular interest in the anticancer field where many agents are used within a narrow dose or concentration range defined by unacceptable toxicity at the high end and diminished efficacy at the low end. The significance of the moderately high affinity of the kinase inhibitor imatinib for α_1-acid glycoprotein has been subject of considerable study and debate [32–35]. Recent studies have indicated that much of the inter-individual variation in imatinib pharmacokinetics in patients can be attributed to variations in plasma α_1-acid glycoprotein concentration [36,37] and the presence of genetic variants of α_1-acid glycoprotein with altered affinity for imatinib [38]. Hematological toxicity was found to be better correlated with unbound AUC than total AUC [36].

The unbound fraction of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib has been extensively studied in cancer patients [39]. The drug was found to bind to serum albumin, α_1-acid glycoprotein, and red blood cells with a mean unbound fraction of 3.4% which was constant over 28 days of dosing. Unbound fraction ranged from 2.2% to 5.4% and was inversely correlated with pre-treatment levels of α_1-acid glycoprotein.

The effects of plasma protein binding and varying α_1-acid glycoprotein concentration on the clinical performance of docetaxel in cancer patients have been the subject of ongoing studies. Most recently, a logistic regression analysis established that unbound C_{max} and α_1-acid glycoprotein levels were major determinants of grade IV neutropenia [40].

Despite the challenges related to the extremely tight binding of the kinase inhibitor UCN-01 to human α_1-acid glycoprotein [4], this agent continues to be studied clinically. A phase I clinical trial of UCN-01 in combination with cisplatin showed that the drug could be safely administered at doses sufficient to achieve meaningful modulation of a pharmacodynamic biomarker for target inhibition [41].

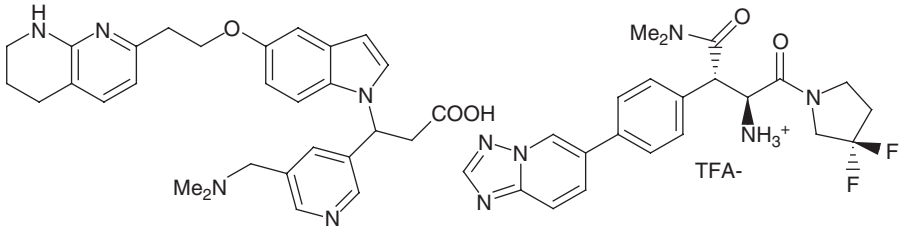
One interesting dataset emerging from this study has implications for the interpretation of saliva drug levels. It has been generally accepted that the total drug levels in saliva represent unbound plasma drug levels due to the very low levels of plasma proteins in saliva [42]. In the terminal phase of elimination, drug in plasma and in saliva were observed to eliminate in parallel, with a saliva/plasma ratio of 0.0005, which was consistent with extremely high plasma protein binding. However, the authors tested saliva from both patients and volunteers for α_1-acid glycoprotein levels in saliva using immunoblotting with a monoclonal antibody specific for human α_1-acid glycoprotein. They found that levels were very low (approximately 2% that of plasma) but sufficient to suggest significant binding of UCN-01 in saliva. Simple equilibrium considerations suggest that only drugs with very tight binding will be significantly bound to such saliva levels of α_1-acid glycoprotein.

3. APPLICATIONS FROM THE MEDICINAL CHEMISTRY LITERATURE

3.1 Extracellular targets

Application of the ''free drug principle'' is most straightforward for drugs which address extracellular targets since confounding factors such as cell penetration and active transport do not play a role. Examples from the recent medicinal chemistry literature attest to differing levels of engagement ranging from consideration of free fraction as a primary SAR variable to using plasma protein binding data to rationalize the *in vivo* activity of an advanced candidate.

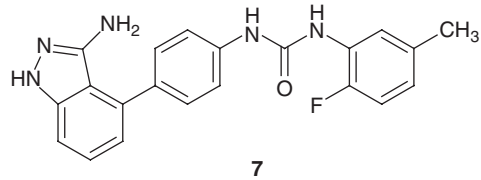
A paper describing the design of dual $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonists is a good example of an aggressive approach to managing plasma protein binding [43]. In a previous series, the authors had attributed poor *in vivo* activity for potent antagonists to high plasma protein binding. In the current work, the extent of plasma protein binding, as assessed by both binding via Biacore and serum shift, was presented along with integrin binding for all final compounds. In general, a strategy of introducing polar functionality in the series reduced binding to albumin, sometimes dramatically, but often potency versus target was ablated as well. At the end, one molecule **5** was identified which maintained high affinity for target and had insignificant binding to albumin (unbound fraction: 60%). Unfortunately, no *in vivo* data were presented to allow one to assess the overall success of this strategy.



5 **6**

A similar strategy was employed to identify a DPP-IV inhibitor (**6**) with good *in vivo* potency in a mouse model of diabetes [44]. Plasma protein binding, as assessed by shift assay (50% serum), was presented for all final compounds. The compound selected as having the best overall profile was active *in vivo* at 0.1 mg/kg. The activity at 1 h post-dose was consistent with the free drug principle – total plasma concentration: 269 nM; murine-free fraction: 4%; unbound plasma concentration: 11 nM; *in vitro* potency versus murine DPP-IV: 6 nM.

A paper detailing the properties of the multikinase inhibitor ABT-869 (**7**) did not indicate whether plasma protein binding data were used in the optimization leading to this highly protein-bound (mouse: 98.2%, human 99.0%) compound [45]. A dose which provided a 69% reduction in tumor growth and >50% inhibition of receptor phosphorylation and pharmacodynamic response afforded plasma concentration that remained above the cellular IC_{50} for receptor phosphorylation in the presence of plasma for 4 of 12 h in the bid dosing cycle.



7

Similarly, though plasma protein binding data were not ostensibly used in the identification of an oxytocin antagonist suitable for advancement to the clinic, such data were used to explain the differences between *in vivo* and *in vitro* potency [46].

As previously mentioned (*vide supra*), plasma protein binding remains a key factor in assessing the suitability of HIV antiretroviral drugs where disease models are not generally available. A paper describing the properties of the CCR5 antagonist TAK-220 notes the attractively low plasma protein binding (human: 53–57%) of this clinical agent [47].

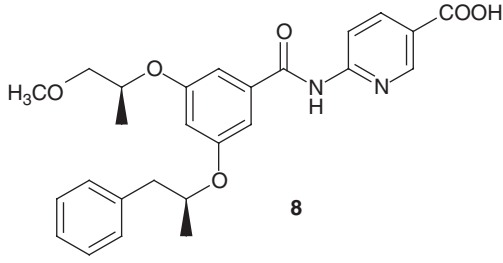
Although the plasma protein binding literature centers mainly on relatively lipophilic, ''small molecule'' drugs, other classes of therapeutic agents may exhibit high plasma protein binding as well. A report describing the optimization of series of antibacterial peptides clearly shows that the addition of serum strongly reduced the antibacterial activity of some peptides while others, including one peptide with demonstrated *in vivo* efficacy, retained activity in the presence of serum [48].

3.2 Intracellular targets

Intracellular targets can present a more complex situation with respect to the application of the free drug principle. The unavailability of reliable general methods for determining free drug concentrations inside the cell (as opposed to total drug associated with the cell, which can usually be measured), often renders

it difficult to apply the principle in a rigorous, quantitative fashion. Still, it is possible to use the serum shift approach to gauge the sensitivity of the cellular drug response to the presence of serum, though there are caveats and limitations with this approach [3].

Recent efforts to identify a glucokinase activator with good *in vivo* efficacy provide an excellent example of the challenges often encountered in balancing free fraction with target potency [49]. A series of carboxylic acids was found to be extensively bound in serum (presumably to albumin). In general, plasma protein binding and target affinity were found to be highly correlated. The optimal compound **8** (in terms of *in vivo* efficacy) was a clear outlier in the correlation. The paper also highlighted the need to simultaneously decrease intrinsic clearance as reducing plasma protein binding exposes more drug to clearance processes.



8

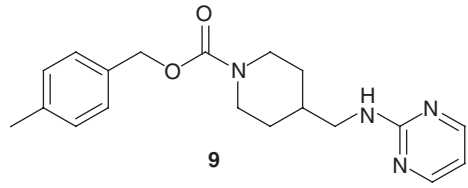
One industry group has used protein NMR spectroscopic analysis of lead compounds bound to domain III of human serum albumin to guide efforts to reduce plasma protein binding. This data, used in conjunction with protein crystallographic data on compound binding to target allowed for improvements in both target potency and free fraction for series of methionine aminopeptidase-2 inhibitors [50]. A similar approach was taken with a series of extremely high plasma protein-bound antagonists of bcl-2 family proteins [51]. The approach resulted again in improvements in both target affinity and free fraction, leading to a compound with *in vivo* efficacy.

Improving free fraction continues to be a goal in the anti-infective area, particularly in areas where animal efficacy models are not readily available. The importance of adequate free fraction was noted in publications describing a series of HIV integrase inhibitors [52], the HCV protease inhibitor VX-950 [53], a series of cyclosporine derivatives active against HCV [54], and a series of antibacterial MurB inhibitors [55].

3.3 CNS targets

There has been a major advance in our understanding of drug exposure in the CNS as detailed in the seminal paper of Kalvass and Maurer and in follow-up publications (*vide supra*) [9,12,13]. This work emphasizes the importance of unbound drug in the brain, and its relationship to unbound drug in the plasma and brain/plasma ratio. One expects that this work should have a major impact on how CNS drugs are optimized and evaluated. There appears to be little

evidence of this in the medicinal literature, but this is likely due to the well-known time lag between what is going on in the laboratory and what is being submitted for publication.



9

A recent report on a NR2B selective NMDA receptor antagonist (**9**) supports the findings of Kalvass and Maurer [56]. Rapid equilibration between plasma and CNS coupled with the lack of Pgp substrate activity led the authors to assume that plasma-free and brain-free drug concentrations were equivalent. An *ex vivo* receptor binding assay showed 50% occupancy at a total plasma concentration of 230 nM. Given a rat-free fraction of 15.3%, the authors concluded that 50% brain occupancy occurred at 35 nM unbound brain concentration, which was in reasonable agreement with the measured K_i of 3.4 nM versus the human receptor.

4. COMPUTATIONAL STUDIES

There have been a number of recent studies attempting to predict the plasma protein binding of compounds. Earlier work pointed out the importance of being able to discriminate highly plasma protein-bound compounds (i.e., submicromolar to nanomolar affinity for plasma proteins) [57]. This work also demonstrated that, in spite of the well-accepted dogma that increasing polarity decreases plasma protein binding, lipophilicity alone was a poor descriptor for plasma protein binding across chemical series. There have been a number of papers detailing various QSAR approaches to the prediction of plasma protein binding [58–62].

One novel predictive approach utilized a comparison of the change in plasma protein binding for a large number of pairs of molecules related by a single, specific structural change [63]. Some changes had counterintuitive effects: monomethylation of a primary amide reduced plasma protein binding despite increasing lipophilicity. The results of this study may be useful in guiding medicinal chemists when more comprehensive modeling approaches are not available.

There have been several reports where plasma protein binding data was used in the prediction of *in vivo* properties of compounds. Two papers noted that the ability to predict *in vivo* clearance from *in vitro* microsome data was greatly improved when a plasma protein binding term was included [64,65]. In another study, binding to phospholipids and human serum albumin was assessed by HPLC retention times (on IAM and HAS columns, respectively) and used to predict volume of distribution [66].

5. EXCEPTIONS TO THE FREE DRUG PRINCIPLE

Exceptions to the free drug principle refer to situations where the unbound drug levels in a pharmacologically relevant compartment cannot be easily rationalized or target occupancy does not appear to be driven by unbound drug levels. In most such cases, the discrepancies relate to non-steady-state behavior or reflect the action of an energy-driven transport phenomenon. A number of such real or apparent exceptions have been cataloged in a recent review [3].

A microdialysis study was carried out to examine transport of oxycodone into the brain of rats [67]. Oxycodone was administered by i.v. infusion, and unbound drug concentrations were monitored in both vena jugularis and striatum. Steady-state equilibrium was reached rapidly and drug levels in the two compartments declined in parallel at the end of the infusion. An unbound brain to unbound plasma ratio of 3.0 was measured which is 3- to 10-fold higher than for other opioids, and explains the similar *in vivo* potency of oxycodone in spite of lower receptor affinity. The authors interpret these data as *de facto* evidence of the existence of an as-yet unidentified transporter that carries oxycodone across the blood–brain barrier.

A study of the potency of the antibiotic daptomycin cited plasma protein binding of 92%, but it claimed only a 2-fold shift in potency in serum (expected: 12-fold) [68]. This type of discrepancy is relatively common and can often reflect substantial binding to components in the ''serum-free'' media. In the cases of HIV-directed non-nucleotide reverse transcriptase inhibitors, this has been dealt with by measuring the unbound drug concentration in the ''serum-free'' medium and using that data to calculate the intrinsic, serum-free potency [69].

A report on the binding of the anesthetic propofol to human serum albumin and to plasma presents a dataset that challenges simple notions of equilibria [70]. The unbound fraction of propofol was found to *increase* sharply at low drug concentrations. The authors appear to have carefully eliminated possible artifacts. Explanations based on cooperative binding modes are discussed though no clear explanation emerges.

Examination of apparent exceptions to the free drug principle is important because such studies can lead to the identification of new processes that may be important for understanding drug action in certain circumstances.

6. ADVANCES IN METHODOLOGY FOR MEASURING PLASMA PROTEIN BINDING

The ''gold standard'' methods for measuring plasma protein binding are equilibrium dialysis, ultrafiltration, and ultracentrifugation [71]. Although these methods are highly reliable, they are relatively resource-intensive and are not well suited to high throughput application. These characteristics have, in many cases, been limiting to the medicinal chemist who would like to track plasma protein binding SAR. In the past 12–18 months, there has been considerable literature from the analytical community detailing attempts to refine current

methodology, to adapt current methods for high throughput, and to identify new methods.

A system based on microdialysis coupled with flow-injection chemiluminescence detection allows for direct sampling of unbound drug without extractive sample preparation [72]. A similar approach based on continuous ultrafiltration has also been reported [73]. Modifications designed to overcome challenges of low solubility and high-non-specific binding in the ultrafiltration approach have also been described [74].

Several methodologies with potential to provide higher throughput have been offered. A system using ultrafiltration in a 96-well format followed by automated sampling and analysis of filtrate by LC/ESI-MS/MS has been reported [75]. In an alternate approach, conventional equilibrium dialysis was carried out on several dozen compounds followed by pooling of extracts to facilitate LC-MS analysis [76].

Several methods for measuring drug binding to human serum albumin involving the determination of retention times on HPLC columns with bound albumin have been reported [77,78]. Solid-phase microextraction [79,80], capillary electrophoresis [81], and displacement of near-infrared fluorescent labels [82] have all been studied.

There have also been advances in serial monitoring of unbound drug concentration *in vivo*. Methodology involving peritoneal microdialysis in freely moving rodents for the measurement of unbound drug concentrations allows for sampling every 10–20 min and affords useful pharmacokinetic profiles and parameters after appropriate scaling [83].

Finally, work that may facilitate understanding the role of α_1-acid glycoprotein variants in inter-individual variations in plasma protein binding, pharmacokinetic behavior, and drug action has been described. A capillary zone electrophoresis method that allows for the determination of 11 intact forms (i.e., isoforms, glycoforms) of α_1-acid glycoprotein has been described [84].

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CHAPTER 32

To Market, To Market – 2006

Shridhar Hegde and **Michelle Schmidt**

The pharmaceutical market in 2006 saw the launch of 27 new molecular entities (NMEs) for therapeutic use, including five first-in-class drugs [1–5]. Anticancer and anti-infective therapies were by far the most prolific areas of new product introductions; together they accounted for almost half of the total NMEs. The United States continued to be the most active market with the launch of 15 new products, followed by the European and the Japanese markets with 8 and 2 entries, respectively. In addition to the NMEs, the year also saw the market entry of eight new vaccines, including the first-ever preventive therapy for cervical cancer. Furthermore, as in previous years, there was a continued focus on innovative combinations of the existing drugs to provide enhanced patient benefit. Of the major drug companies, Pfizer had a very productive year with the launch of three NMEs and the first-ever inhaled insulin formulation, whereas Merck had the distinction of introducing two first-in-class small molecule drugs and three highly significant vaccines. BMS was accredited with three new product introductions while Amgen, Genentech, GSK, J&J, Novartis, Sanofi-Aventis, and Schering-Plough had a marketing or co-marketing role in one each. Keeping with the trend of recent years, the number of new combinations, new formulations, and new indications of existing drugs continued to grow rapidly. While these line extensions as well as the new vaccines of the year are not elaborated in this review of NMEs, they comprised a substantial portion of the new products in 2006.

The new anticancer drugs of the year included eight NMEs and the vaccine for cervical cancer. Merck's Gardasil®, arguably the highest profile new product of 2006, is a vaccine against the Human Papillomavirus (HPV). It prevents infection against four different strains of HPV, including the two types that cause

Pfizer, Inc, St. Louis, MO 63017, USA

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most cervical cancers and the two types that cause the most genital warts. HPV is estimated to be one of the most common sexually transmitted infections in the US. The oncolytic NMEs were highlighted by two new small molecule drugs that inhibit multiple receptor tyrosine kinases (RTKs) and two new biological agents that target the epidermal growth factor receptor (EGFR). Sutent® (sunitinib), launched by Pfizer, inhibits multiple RTKs, including platelet-derived growth factor receptors (PDGFR) and vascular endothelial growth factor receptors (VEGFR). It is an oral treatment for gastrointestinal stromal tumors (GIST) and metastatic renal-cell carcinoma (RCC). In GIST, Sutent® is indicated as a second-line agent in disease refractory to imatinib or patients with intolerance to imatinib. Sprycel™ (dasatinib), marketed by BMS, is also an oral inhibitor of multiple RTKs, including ABL kinase and Src family of kinases. It is indicated for use following resistance or intolerance to prior therapy, such as Gleevec®, for treatment of adults with chronic myeloid leukemia. Sprycel™ also has orphan drug status for the treatment of Philadelphia-chromosome positive acute lymphoblastic leukemia. Panitumumab (Vectibix™) and nimotuzumab (Biomab EGFR) are the two anti-EGFR monoclonal antibodies introduced last year for treating metastatic colorectal cancer, and head and neck cancer, respectively. Another significant highlight of last year's anti-cancer NMEs was the introduction of Zolinza™ (vorinostat), a first-in-class drug, by Merck. Vorinostat is the first example of a histone deacetylase inhibitor to reach the market, and it received orphan drug status in the US for the treatment for advanced cutaneous T-cell lymphoma. Inhibiting the hypoacetylation of nucleosomal histones represents a new type of epigenetics-based therapy, distinct from the well-explored class of DNA methyltransferase inhibitors. Rounding out the category of cancer-related drugs are Revlimid® (lenalidomide), an oral TNF-α inhibitor from the thalidomide class, and Dacogen™ (decitabine), an injectable DNA hypomethylating agent. Both products are indicated for the treatment of myelodysplastic syndromes, a family of bone marrow disorders that can progress to leukemia.

The anti-infective domain had four NMEs, a new combination product, and six new vaccines introduced in 2006. Eraxis™ (anidulafungin), an injectable product for the treatment of invasive *Candida* infections, was launched in the US. Anidulafungin is an echinocandin, a class of antifungal drugs that inhibits the biosynthesis of 1,3-β-D-glucan, an essential component of fungal cell walls. It is the third echinocandin to reach the market behind caspofungin and micafungin, which were launched in 2001 and 2002, respectively. Also reaching the market was Schering-Plough's Noxafil™ (posaconazole), the newest member of the azole class of antifungals, for the oral treatment of invasive *Aspergillus* and *Candida* infections, as well as oropharyngeal candidiasis. A new HIV protease inhibitor Prezista™ (darunavir) was introduced by Tibotec and J&J. It is the tenth protease inhibitor on the market to date, and it is indicated for co-administration with ritonavir and with other antiretroviral agents for the treatment of HIV infection in treatment-experienced adults, such as those with strains resistant to more than one protease inhibitor. Sebivo® (telbivudine), a new member of the L-nucleoside class of antiviral agents, was introduced for the treatment of chronic hepatitis B virus (HBV) infection. This agent is a highly specific and selective

inhibitor of HBV DNA polymerase without inhibiting human cellular polymerases. It offers clinicians a more potent alternative to lamivudine and adefovir in the treatment of HBV infection. As in previous years, the trend of combining two or more HIV drugs to simplify the dosing regimens continued in 2006 with the launch of Atripla™, the first-ever once-daily single tablet regimen intended as a stand-alone therapy or in combination with other antiretrovirals. The product is a combination of three different reverse transcriptase inhibitors commonly prescribed for HIV infection: efavirenz, emtricitabine, and tenofovir. In addition to the NMEs and the combination product, the 2006 portfolio of anti-infectives included a battery of new vaccines: Rotarix®, an oral rotavirus vaccine for the prevention of gastroenteritis; Zostavax® for the prevention of herpes shingles; HepaGam B™ for the treatment of acute exposure to blood containing hepatitis B surface antigen; Supervax, an adjuvanted hepatitis B vaccine; Rabirix™ for the prevention and treatment of human rabies infection; and Quinvaxem™, a pentavalent vaccine that combines antigens for protection against five important childhood diseases: diphtheria, tetanus, pertussis, hepatitis B, and *Haemophilus influenzae* type B.

In the area of endocrine and metabolic diseases, two landmark products were introduced for the treatment of diabetes. Exubera®, the first ever inhaled insulin, and the dipeptidyl peptidase-4 (DPP-4) inhibitor Januvia™ (sitagliptin), a first-in-class oral drug, were both launched last year. Exubera is a fast-acting, dry powder formulation of recombinant human insulin that is inhaled into the lungs via the mouth before meals using a simple-to-use, handheld device. It represents the first new insulin delivery option introduced since the discovery of insulin in the 1920s, and it is currently licensed for use by both type 1 and type 2 diabetics over the age of 18. Sitagliptin is a once-daily oral drug for the treatment of type 2 diabetes. It acts by increasing the levels of active incretins, which are hormones released from the small intestine that increase insulin secretion and decrease glucagon secretion in response to elevated blood glucose. Since the action of incretins are only triggered when the blood glucose is elevated, sitagliptin therapy is expected to have a low risk of hypoglycemic side effect. Also entering the diabetes market were two new combination drugs: Avandaryl™ (rosiglitazone maleate and glimepiride), and Duetact™ (pioglitazone hydrochloride and glimepiride). Both of these drugs combine a PPARγ agonist and a sulfonylurea in a single tablet formulation in order to simplify the treatment regimen of diabetic patients who are often prescribed the two drugs in combination due to their complementary mechanisms of action. A high profile entry into the metabolic disease market last year was Acomplia® (rimonabant), a first-in-class oral drug for obesity. Rimonabant is a selective antagonist of cannabinoid type-1 (CB1) receptor. It acts by reducing the overactivity of the endocannabinoid system, thereby regulating food intake and energy balance and improving lipid and glucose metabolism. Additional drugs entering this market last year were two enzyme-replacement therapies that are the first-ever treatments for two rare but serious metabolic diseases. Myozyme® (alglucosidase alfa) was launched for the treatment of Pompe's disease, which is an inherited neuromuscular disorder characterized by insufficient metabolism of glycogen and its accumulation in

heart and muscle cells, often leading to fatality in infants. In addition, Elaprase™ (idursulfase) was introduced for the treatment of mucopolysaccharidosis II, also known as Hunter's syndrome, which is a rare lysosomal storage disorder that involves a variety of physical and neurologic problems, including abnormal bone and joint growth, respiratory problems, learning disabilities, and hearing loss.

In the cardiovascular sector, two new oral drugs were introduced for treating chronic stable angina (CSA): Procorolan™ (ivabradine) and Ranexa™ (ranolazine). Ivabradine acts by reducing the heart rate by a mechanism different from β-blockers and calcium channel blockers, two most commonly prescribed anti-anginal drugs. It selectively inhibits the I_f channel in the sino-atrial node that controls the diastolic depolarization and regulates heart rate. Ivabradine does not have the bradycardia or respiratory side effects commonly seen with β-blockers, and it is specifically indicated for the treatment of CSA patients with normal sinus rhythm who have a contraindication or intolerance for β-blockers. Ranolazine is a late-stage sodium channel blocker, and its anti-anginal effects do not depend on reductions in heart rate or blood pressure. Ranolazine is indicated as a second-line CSA treatment in combination with amlodipine, β-blockers, or nitrates. In addition to the new angina therapies, the cardiovascular area also saw the launch of Thelin® (sitaxsentan) for the treatment of pulmonary arterial hypertension (PAH). Sitaxsentan is a selective endothelin A receptor antagonist, and it is the first once-daily oral treatment available for patients with PAH.

The CNS area was represented by the entry of two new drugs: Neupro® (rotigotine) for the treatment of Parkinson's disease and Chantix™ (varenicline) as an aid to smoking-cessation treatment. Neupro® is a nonergolinic dopamine D2/D3 receptor agonist that is formulated as a transdermal patch for once-daily application. Varenicline, a partial agonist of the α4β2 nicotinic receptor, is a first-in-class drug launched last year. It exhibits dual action by decreasing craving and withdrawal symptoms and by decreasing the reinforcement associated with smoking. Varenicline is the first new prescription for smoking cessation approved in over 10 years.

Three new urologic drugs were introduced last year. Vaprisol® (conivaptan) and Physuline (mozavaptan) are the two new vasopressin antagonists launched last year for the treatment of hyponatremia, a condition that occurs when the blood sodium level falls significantly below normal. Blocking the activity of arginine vasopressin receptors restores proper fluid balance by causing increased urine output without loss of sodium. This differs from the commonly used diuretics, which stimulate loss of both water and electrolytes. The third new drug in this market was Urief (silodosin), an α_{1A} adrenoceptor (α_{1A}-AR) antagonist selective for prostatic receptors. It entered the Japanese market last year as an oral treatment for dysuria associated with benign prostatic hypertrophy (BPH). Unlike the previously marketed AR antagonists for treating BPH, silodosin has an improved cardiovascular safety profile as a result of its higher selectivity for α_{1A}- over α_{1B}-adrenoreceptors.

The field of gastrointestinal drugs saw the entry of Amitiza™ (lubiprostone), the first selective and locally acting CIC-2 chloride channel activator, for the

treatment of chronic idiopathic constipation in adults. By targeting ClC-2 chloride channels on the gastrointestinal epithelial cells, lubiprostone treatment produces a chloride-rich intestinal fluid without significantly affecting the serum electrolyte levels. Increased intestinal fluid production promotes gastrointestinal motility and spontaneous bowel movements.

The rheumatoid arthritis (RA) market and the ophthalmic market each had a new biological agent added to the portfolio last year with the launch of Orencia® (abatacept) and Lucentis™ (ranibizumab), respectively. Abatacept is a fully human fusion protein that works by selectively modulating a co-stimulatory signal which is required for full T-cell activation. It is indicated for reducing the signs and symptoms of RA, slowing the progression of structural damage, and improving physical function in RA patients who have had an inadequate response to methotrexate or anti-TNF therapy. Ranibizumab is a monoclonal antibody fragment (Fab) that inhibits angiogenesis by neutralizing vascular endothelial growth factor A (VEGF-A). Ranibizumab is marketed by Genentech as an intravitreal injection for the treatment of age-related macular degeneration (AMD). The corresponding full-length antibody, bevacizumab, had been launched previously by Genentech for treating colorectal cancer. The ophthalmic sector also had two combination drugs launched for glaucoma treatment: Ganfort® (bimatoprost/timolol maleate) and DuoTrav™ (travoprost/timolol maleate). Both of these products combine a prostaglandin analog and a β_2-adrenoreceptor antagonist in a single eyedrop formulation in order to simplify the treatment regimen.

Finally, two new diagnostic agents were introduced last year: Vasovist™ (gadofosveset trisodium), a gadolinium-based contrast agent designed to provide improved imaging of the vascular system using magnetic resonance angiography (MRA), and Sonazoid® (perflubutane), an ultrasound contrast agent to facilitate the detection and characterization of lesions associated with hepatic tumors. Although these diagnostic agents are not considered drug entities for therapeutic use and are not covered in this review, they add to the rapidly growing portfolio of innovative products in the field.

Abatacept	(Rheumatoid arthritis)	[6–13]	
Country of origin:	US	Class:	CTLA-4-Ig
Originator:	Bristol–Myers Squibb		fusion protein
First introduction:	US	Type:	CD28 antagonist
Introduced by:	Bristol–Myers Squibb	Molecular weight:	92 kDa
Trade name:	Orencia	Expression system:	Mammalian cell
CAS registry no:	332348-12-6	Manufacturer:	BMS

Rheumatoid arthritis (RA) is a debilitating autoimmune disease causing joint destruction and pain via the infiltration of inflammatory mediators. While some patients find relief using traditional disease-modifying antirheumatic drugs (DMARDs), such as methotrexate or tumor necrosis factor-blocking agents, a large proportion of individuals fail to respond adequately to existing therapy. For those that may initially benefit, issues with side effects may lead to the termination of treatment. Abatacept attempts to address the unmet need as a new

class of DMARD that targets T-cell function. With the recognition that T cells play a central role in the pathogenesis of RA, abatacept has been developed as a novel, rational approach to interfere with the upstream effector of the inflammation. As a recombinant protein consisting of a fusion between the extracellular domain of human cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and the modified heavy chain constant segment of human immunoglobulin G1, abatacept acts as a costimulatory modulator of the CD80/86:CD28 pathway. In addition to the antigen-specific signal elicited by recognition of an immunogenic peptide bound to the major histocompatibility complex on an antigen-presenting cell, T cells also require the engagement of CD28 on T cells with CD80/86 on antigen-presenting cells for full activation. Abatacept acts as a downregulator by binding to CD80/86 with higher affinity than CD28, thereby, mimicking endogenous CTLA-4 in its inhibition of T cell costimulation. The immunoglobulin portion of the protein serves as a handle to facilitate purification of the protein that is produced by recombinant DNA technology in a mammalian cell expression system. It also enhances the solubility and serum half-life of the fusion protein. The dosing regimen involves a 30-min infusion every four weeks after the initial protocol (initial dose followed by a dose at two weeks and four weeks) and may be used as monotherapy or in conjunction with DMARDs other than TNF antagonists. From phase I/II studies, the optimal efficacious dose was determined to be 10 mg/kg. Linear pharmacokinetic properties were observed with little variability among patients, and comparable results were obtained for both RA and healthy subjects. The serum half-life of 14.7 days was independent of dose, and the rate of elimination remained constant supporting the lack of anti-drug antibody generation. After multiple 10 mg/kg doses in RA patients, the average C_{max} was 295 μg/mL, clearance was 0.22 mL/h/kg, and volume of distribution was 0.07 L/kg. The route of metabolism and excretion is currently unknown. A phase I study sought to determine both safety and possibly efficacy by enrolling RA patients who failed at least one DMARD. The primary endpoint was a 20% improvement in American College of Rheumatology response criteria (ACR 20). At day 85 of the trial, 53% of patients achieved an ACR 20 response at the highest dose (10 mg/kg). In a double-blind, placebo-controlled, phase II trial, 339 patients with active RA were randomized to receive placebo plus methotrexate, abatacept (2 mg/kg) plus methotrexate, or abatacept (10 mg/kg). The 10 mg/kg abatacept dose resulted in significant improvement in ACR 20 response at six months compared to placebo plus methotrexate (60% vs. 35%). Furthermore, patients in the abatacept group demonstrated improvements in quality of life measurements, such as overall physical health, pain, vitality, and social function. Along with the clinical improvements, reductions in inflammatory biomarkers were also observed, including C-reactive protein, rheumatoid factor, soluble IL-2 receptor, IL-6, and E-selectin. Regarding phase III, two major studies were conducted to evaluate the efficacy of abatacept in patients who were refractory to two traditional methods of treatment. The Abatacept in Inadequate Responders to Methotrexate (AIM) trial was a one-year, double-blind, placebo-controlled study with ACR 20 response and structural damage progression as primary end

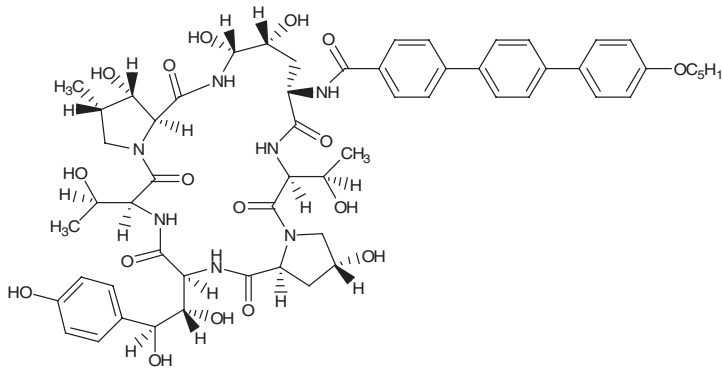
points. After one year, 73% of the abatacept group achieved ACR 20 responses compared to 40% of the placebo group although it should be noted that background therapy could be adjusted after six months. As the first study to include radiological outcome, inhibition of structural damage progression was confirmed. The Abatacept Trial in Treatment of Anti-TNF Inadequate Responders (ATTAIN) also utilized ACR 20 responses. The 391 patients with refractory RA were permitted to remain on at least one background DMARD, but all anti-TNF therapy was previously discontinued. At six months, 50% of patients in the abatacept group achieved ACR 20 responses compared to 20% in the placebo group. The drug-treated group also had higher remission rates (10% vs. 1%). Throughout the clinical studies, it was demonstrated that abatacept was well-tolerated and safe. The most common adverse affects were headache, upper respiratory tract infection, nasopharyngitis, and nausea while serious infections and malignancies were similar to placebo. Infusion-related events included dizziness, hypertension, and headaches. Abatacept is contraindicated in patients with a known hypersensitivity to any of the components of the drug. Furthermore, abatacept should not be used concomitantly with TNF antagonists since the combination increases the risk of serious infection. Since abatacept modulates the immune system, patients with an existing infection or a history of recurring infection should be monitored closely and possibly discontinue use. Patients with chronic pulmonary obstructive disorder (COPD) should also proceed with caution since abatacept may exacerbate COPD symptoms. During abatacept treatment, live vaccinations should be avoided because the drug may diminish the effectiveness of some immunizations. Finally, formal drug interaction evaluations have not been conducted.

Alglucosidase Alfa	(Pompe Disease)	[14–16]	
Country of origin:	US	Class:	Recombinant human protein
Originator:	Duke University		
First introduction:	US	Type:	Glucosidase
Introduced by:	Genzyme General	Molecular weight:	110 kDa Precursor
Trade name:	Myozyme	Expression system:	CHO cell
CAS registry no:	420784-05-0	Manufacturer:	Genzyme

Pompe disease is a lysosomal storage disorder that is characterized by a deficiency in the acid alpha-glucosidase enzyme that is responsible for the breakdown of glycogen to glucose. The lack of degradation results in the accumulation of glycogen in lysosomes, predominantly affecting cardiac and skeletal muscles. In cases of complete enzyme deficit, such as observed in the infantile manifestation, cardiomyopathy, and skeletal muscle myopathy occur with fatal consequences. As with other lysosomal storage disorders, enzyme replacement therapy (ERT) is the patient's only hope. Alglucosidase alfa has been developed

and launched as the ERT for Pompe disease. As a recombinant human enzyme, it is produced by transfected CHO cells as a 110-kDa precursor that targets lysosomes via the mannose-6-phosphate (M6P) receptor. Following endocytosis, the enzyme is transformed to its mature 76-kDa form that restores glycogen processing and reverses accumulation. The dosing regimen of alglucosidase alfa is 20 mg/kg infused over a period of 4 h every two weeks. The pharmacokinetic properties are dose-proportional between 20 and 40 mg/kg. Following a single infusion of 20 mg/kg, a C_{max} of 162 ± 31 μg/mL, a clearance of 25 ± 4 mL/h/kg, a volume of distribution of 96 ± 16 L, and a half-life of 2.3 ± 0.4 h are observed. Two separate clinical trials evaluated the safety and efficacy of alglucosidase alfa. The first study restricted inclusion to patients less than seven months of age with demonstrated cardiac hypertrophy but no ventilatory support at first infusion. Efficacy was determined by decreased mortality and prevention of invasive ventilatory support. There were no deaths within the first 12 months of treatment; however, 3 of 18 treated patients required ventilatory support. By the 20-month follow-up with continued treatment, two of the ventilator-dependent patients had died, but 16 patients survived. Other endpoints included motor function assessment by the Alberta Infant Motor Scale (AIMS) and changes in baseline of the left ventricular mass index (LVMI). Gains in AIMS-assessed motor function were initially observed in 13 patients although two patients eventually regressed despite continual treatment. Regarding LVMI, for the 15 patients with baseline echocardiography, all patients experienced a decrease from baseline ranging from 45 to 193 g/m^2. In summary, improvement in cardiac function appeared to be more dramatic than the response in motor function. A greater preponderance of M6P receptors in cardiac tissue compared to skeletal muscle may allow more efficient uptake of the recombinant enzyme in the heart; however, skeletal muscle biopsy has indicated that enzyme levels within the normal range are attainable. In cases where initial improvements in motor function were followed by regression with continued treatment, antibodies to the recombinant enzyme were detected. Interestingly, while the presence of antibodies was deleterious to muscle strength and pulmonary function, cardiac function was uncompromised. As with other types of ERT, infusion reactions and antibody formation were the most common side effects. Other adverse events included fever, rash, diarrhea, vomiting, cough, pneumonia, upper respiratory tract infection, gastroenteritis, otitis media, and decreased oxygen saturation. At this time, no formal drug interaction studies have been conducted.

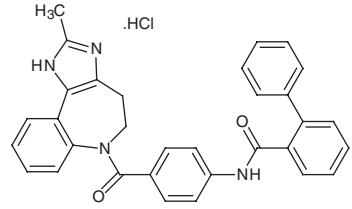
Anidulafungin	(Antifungal)	[17–19]
Country of origin:	US	
Originator:	Eli Lilly	
First introduction:	US	
Introduced by:	Pfizer	
Trade name:	Eraxis	
CAS registry no:	166663-25-8	
Molecular weight:	1140.3	



Anidulafungin, a semi-synthetic derivative of echinocandin B, has been developed and launched as an intravenous treatment for serious fungal infections, such as candidemia, Candida-derived peritonitis, intra-abdominal abscesses, and esophageal candidiasis. As a non-competitive inhibitor of 1,3-β-D-glucan synthase, which is responsible for the formation of glucan polymers, anidulafungin interferes with the cell wall synthesis of most pathogenic fungi. This mode of action is characteristic of the echinocandin class of antifungals. While the first member of this class, cilofungin, was withdrawn due to toxicity associated with the formulation vehicle, anidulafungin follows the successful introduction of caspofungin and micafungin. Compared to the other echinocandins, anidulafungin appears to be more potent (MIC_{90} of $\leq 0.25\,\mu g/mL$ for *C. albicans*, $0.5\,\mu g/mL$ for *C. glabrata*, $1\,\mu g/mL$ for *C. krusel* and *C. tropicalis*, $2\,\mu g/mL$ for *C. lusitaniae*, and $2\,\mu g/mL$ for *Aspergillus* spp) and is devoid of significant drug interactions since it is neither an inhibitor nor substrate of the cytochrome P450 isoenzymes. The emergence of the echinocandins circumvents the concern regarding the rising resistance to the azole and amphotericin B antifungals; no cross-resistance is expected because the echinocandins work at the cell wall rather than the cell membrane. The convergent synthesis of anidulafungin involves the acylation of the cyclic peptide core, prepared by the enzymatic deacylation of echinocandin B, with the side chain 1-carbomethoxy-4''-(pentyloxy) terphenyl generated by coupling the boronic acid of 4-bromo-4′-(pentyloxy) biphenyl with methyl 4-iodobenzoate. Following intravenous infusion, systemic exposures are dose-proportional. With a loading dose of 70 mg on day 1 and subsequent daily maintenance doses of 35 mg, a C_{max} of 3.55 mg/L, a clearance of 0.84 L/h, and a terminal elimination half-life of 43 h are achieved. The volume of distribution of 30–50 L is comparable to total body fluid volume. At physiologic temperature and pH, the drug is slowly degraded to an inactive ring-opened metabolite that is further processed prior to elimination. Using radiolabeled anidulafungin, it has been determined that less than 10% of intact drug is eliminated in the feces. In a phase 2 clinical study against invasive candidiasis, an almost 90% clinical response rate was realized at currently approved doses. Evaluation of anidulafungin for esophageal candidiasis was the subject of one of the phase III trials. Although end-of-therapy responses were high (97% by

endoscopic, 87% by mycological, and 99% by clinical determination), the 2-week follow-up endoscopic response dropped to 64%. Maintenance of positive response was better in the invasive candidiasis phase III study; the end of therapy response was 76% with continued success of 65% of patients at the 2-week follow-up. The most common adverse events included headache, nausea, vomiting, neutropenia, phlebitis, and hypokalemia.

Conivaptan	(Hyponatremia)	[20–22]
Country of origin:	Japan	
Originator:	Yamanouchi	
First introduction:	US	
Introduced by:	Astellas	
Trade name:	Vaprisol	
CAS registry no:	168626-94-6	
Molecular weight:	535.04	

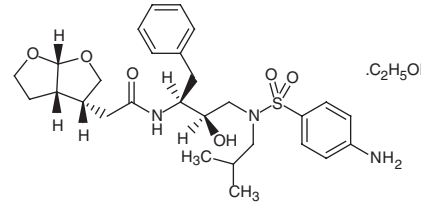


Arginine vasopressin is intimately involved in volume homeostasis, and elevated levels of arginine vasopressin are responsible for the pathogenesis and progression of diseases with an imbalance of sodium and water, particularly congestive heart failure. To restore homeostasis, antagonism of vasopressin receptors is a practical solution. As such, conivaptan has been developed and launched as a dual V_{1a} and V_2 vasopressin receptor antagonist. As a competitive, reversible inhibitor of both subtypes, conivaptan can modulate systemic vascular resistance through the V_{1a} receptor ($K_i = 0.48$ nM) distributed in vascular smooth muscle cells, cardiomyocytes, hepatocytes, and platelets and blocks the renal V_2 receptor ($K_i = 3.04$ nM) resulting in enhanced diuresis, thereby increasing serum sodium concentration and reducing total body volume. Currently, the drug is approved for the management of refractory hyponatremia and potentially life-threatening sodium and water imbalance, but it has shown promise as a potential treatment option for other diseases, such as congestive heart failure, syndrome of antidiuretic hormone, diabetes insipidus, and liver cirrhosis. The large-scale production of conivaptan incorporates the biphenyl acid moiety as the last step to minimize the waste of this costly intermediate. Initially, the 1,2,3,4-tetrahydrobenzo[b]azepin-5-one core is acylated with *p*-nitrobenzoyl chloride followed by alpha-bromination of the ketone. The alpha-bromo ketone is then transformed to the imidazole ring by reaction with acetamidine. Subsequent reduction of the nitro group by catalytic hydrogenation affords the amine handle for coupling with the biphenyl acid. Selective acylation is achieved without protection of the imidazole. The drug may be intravenously or orally administered. For intravenous applications, a loading dose of 20 mg is given over 30 min followed by 20–40 mg/day by continuous infusion. The oral dose is typically twice the intravenous amount, and for a continual 24-h effect, it is divided into at least a twice- to three-times-daily dose. Despite inter-subject variability, pharmacokinetic parameters were determined following a 60-mg oral dose in healthy

patients. The oral bioavailability was 44% with peak drug effect (diuresis) occurring after 2 h and no residual activity after 12 h. Peak plasma concentrations, however, were achieved in 1.1 h. In this study, the half-life ranged from 1.4 to 2.2 h, but the range has also been reported from 3.1 to 7.8 h. The volume of distribution was approximately 34 L, and the protein binding was 98.5%. The mean total body clearance was 26 L/h. The drug is predominantly metabolized by CYP3A4 to generate four inactive metabolites that are excreted in the feces. In addition to being metabolized by this liver enzyme, conivaptan acts as an inhibitor of hepatic enzyme activity, thereby increasing the effects of concomitant drugs metabolized by the cytochrome P450 isoenzymes; increased plasma concentrations of statins, benzodiazepines, and amlodipine have been observed with co-administration of conivaptan. In a placebo-controlled, double-blind, randomized trial of 74 patients with euvolemic hyponatremia, conivaptan was administered intravenously as a 20-mg loading dose followed by 40 or 80 mg/day as a continuous infusion for four days. A slightly better response was achieved with the 80 mg/day maintenance dose; 82% of patients demonstrated an increase in serum sodium concentration (>135 mEq/L) compared to 71% of patients receiving the 40 mg/day maintenance dose and 48% of patients on placebo. Furthermore, the 80 mg/day dose required on average 12.1 h to increase sodium serum >4 mEq/L above baseline while the 40 mg/day dose required 27.5 h and placebo registered 71.7 h. In another study of six normotensive subjects, conivaptan increased urine output seven-fold and also considerably reduced urine osmolality (600 to 100 mOsm/L) following a 60-mg single oral dose and a 50-mg single intravenous dose at intervals of one week. The most common adverse events included infusion site complications, such as local inflammation, phlebitis, and pain at the infusion site. Other side effects included headache, hypokalemia, thirst, vomiting, pollakiuria, peripheral edema, diarrhea, and orthostatic hypotension. With increasing doses, these adverse events increased. Conivaptan is contraindicated in patients with hypovolemia or total body fluid depletion due to the potential for renal failure, ischemic organ damage, and shock. While conivaptan corrects sodium imbalance, monitoring of serum sodium concentration is essential to avoid a rapid change that could precipitate neurological symptoms. Dose adjustment may be necessary in patients with renal and hepatic impairment. Finally, concomitant use of conivaptan with other inhibitors of hepatic enzymes is contraindicated.

Darunavir (HIV) [23–26]

Country of origin:	US
Originator:	Tibotec/J&J
First introduction:	US
Introduced by:	Tibotec/J&J
Trade name:	Prezista
CAS registry no:	206361-99-1
Molecular weight:	593.73

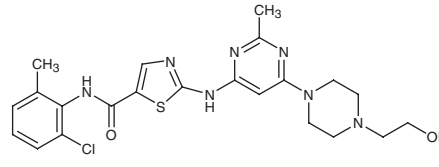


Darunavir is the latest weapon in the arsenal of agents to combat human immunodeficiency virus type 1(HIV-1). As an HIV-1 protease inhibitor, its mechanism of action involves blocking the cleavage of the *gag* and *gag–pol* polyproteins into functional proteins essential for the production of infectious progeny virus; the result is the production of immature, noninfectious viral particles. Compared to predecessor HIV protease inhibitors, darunavir retains activity against resistant stains, a critical factor with the continual emergence of multidrug-resistant (MDR) mutants. Despite experiencing a 13-fold reduction in binding to MDR HIV-1 protease, this binding is 1.5 orders of magnitude tighter than the first-generation protease inhibitors. Furthermore, darunavir exhibits less than a 10-fold decrease in susceptibility in cell culture against 90% of 3309 clinical isolates resistant to amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir. In contrast, darunavir-resistant viruses display limited susceptibility to only tipranavir, suggesting limited cross-resistance between these two protease inhibitors. To avoid the issues of the peptide-based protease inhibitors, darunavir has evolved from a structure-based design effort to minimize peptidic features and reduce molecular weight and complexity. As expected from modeling, the strong interaction between the bis-tetrahydrofuranyl urethane moiety and Asp29 and Asp30 contribute to its tight binding to wild-type HIV-1 protease ($K_d = 4.5$ pM). In addition, darunavir possesses potent activity against a broad spectrum of laboratory strains and clinical isolates of HIV-1 with EC_{50} values ranging from 1.2 to 8.5 nM. In the presence of human serum, the EC_{50} values increase by a median factor of 5.4. While darunavir has been prepared by several procedures, one interesting route involves the manipulation of three epoxide intermediates. 2-Vinyloxirane is treated with phenylmagnesium bromide in the presence of copper cyanide to provide *trans*-4-phenyl-2-buten-1-ol, which is enantioselectively epoxidized with Ti(O-*i*-Pr)$_4$ and diethyl D-tartrate. Subsequent reaction of the (*2R,3R*) epoxide with trimethylsilyl azide and Ti(O-*i*-Pr)$_4$ yields a chiral azidodiol that in turn is converted into another chiral epoxide by treatment with 2-acetoxyisobutyryl chloride and sodium methoxide. This final epoxide is opened with isobutylamine generating a secondary amine that is sulfonylated with 4-aminophenylsulfonyl chloride. The azido moiety is then reduced with hydrogen over palladium/carbon catalyst. The primary amine is ultimately condensed with the essential bis-tetrahydrofuran via its mixed carbonate ester, prepared separately in five steps. Darunavir is supplied in 300-mg, orange, film-coated tablets for oral administration. To increase its bioavailability, darunavir must be taken with ritonavir; the oral bioavailability of a single 600-mg dose is 37% while co-administration with 100 mg of ritonavir twice daily results in a boost to 82%. The effectiveness of concomitant ritonavir stems from its inhibition of CYP3A4, the primary hepatic enzyme responsible for the metabolism of darunavir. The outcome is an increase in the plasma levels of darunavir. Following this standard, approved regimen, a T_{max} of approximately 2.5–4 h is observed with an AUC (0–24 h) of 60.4 $\mu g \cdot h/mL$. The plasma protein binding of darunavir is 95% with alpha 1-acid protein as the major contributor. Excretion occurs primarily in the feces (79.5%) with 41% identified as parent compound. The terminal elimination half-life is 15 h. In a randomized, open-label

trial, 50 experienced patients with HIV not responding to their current retroviral regimen were randomized to receive either 300 or 600 mg of darunavir twice daily or 900 mg once daily. Each dose was accompanied with 100 mg of ritonavir while the control group consisted of individuals continuing with their current protease inhibitor. At the end of 14 days, the HIV viral load was reduced by a minimum of 1 $\log_{10}$ copies/mL in 76% of patients in the combined darunavir group. Similar results were achieved by only 17% of patients in the control group. In another clinical study where patients also received other active antiretrovirals, including at least two nucleoside reverse transcriptase inhibitors, the 48-week analysis of the darunavir 600 mg/ritonavir 100 mg twice daily dose demonstrated a reduction of HIV viral load of at least 1 $\log_{10}$ copies/mL in 61% of 110 patients compared to 15% of 120 patients in the control group. Adverse effects following darunavir treatment were similar to other protease inhibitors and included nausea, headache, diarrhea, and increased aminotransferase activity and serum lipid concentrations. As with other antiretroviral treatments, redistribution of body fat has been experienced by patients, and the mechanism and long-term consequences of these events are currently unknown. While other protease inhibitors have reported the worsening of or onset of diabetes, this has not been experienced with darunavir. Since darunavir and ritonavir both modulate CYP3A4 activity, increased plasma levels of concomitant drugs metabolized by this hepatic cytochrome P450 may result in prolonged therapeutic effects and adverse events. Specific drugs that are contraindicated for co-administration with darunavir include anticonvulsants (carbamazepine, phenobarbital, and phenytoin), antihistamines (astemizole and terfenadine), antimycobacterial (rifampin), ergot derivatives (dihydroergotamine, ergonovine, ergotamine, and methylergonovine), motility agent (cisapride), herbal supplement (St. John's wort), HMG-CoA reductase inhibitors (lovastatin and simvastatin), neuroleptic (pimozide), and sedative/hypnotics (midazolam and triazolam). In these cases, serious or life-threatening side effects may occur.

Dasatinib (Anticancer) [27–33]

Country of origin: US
Originator: Bristol–Myers Squibb
First introduction: US
Introduced by: Bristol–Myers Squibb
Trade name: Sprycel
CAS registry no: 302962-49-8
Molecular weight: 480.01

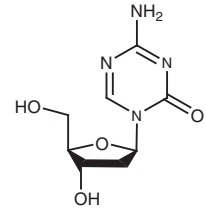


Chronic myeloid leukemia (CML), a hematological stem-cell disorder, is definitively diagnosed by the detection of the Philadelphia chromosome, a truncated version of chromosome 22 resulting from the reciprocal translocation of chromosomes 9 and 22 induced by a single mutagenic event. The consequence is the juxtaposition of two genes creating a fusion gene BCR-ABL. This gene translates a fusion protein with increased tyrosine kinase activity that contributes to

the pathogenesis of CML. Targeting the BCR-ABL protein has led to the successful intervention of the disease. Now established as first-line therapy for CML, imatinib is the first selective tyrosine kinase inhibitor of BCR-ABL. Since imatinib only binds to an inactive conformation of the ABL kinase portion, the conformational restrictions contribute to its selectivity. While imatinib has produced clinical remissions in most patients, encounters with subsequent drug resistance are increasing. The culprit is the emergence of mutations in the kinase domain of BCR-ABL that interfere with drug binding. As a second-generation tyrosine kinase inhibitor, dasatinib has been designed to possess less stringent conformational constraints for ABL binding. In practice, dasatinib has demonstrated activity against all imatinib-resistant mutants except the T315I mutant. With the relaxation of conformational restrictions, selectivity is also diminished; dasatinib is a potent inhibitor of several kinases, including BCR-ABL ($K_i = 30 \pm 22$ pM), SRC ($K_i = 16 \pm 1.0$ pM), SRC family members, c-KIT, and PDGFR-β. In addition, it is greater than 300-fold more potent than imatinib ($IC_{50} = 0.6$ vs. 280 nmol/L). While dasatinib can be constructed by several routes, one process involves the amination of 4,6-dichloro-2-methylpyrimidine with 1-(2-hydroxyethyl)piperazine. The remaining chloro handle of this intermediate is displaced with the left-hand 2-aminothiazole derivative utilizing a palladium-catalyzed reaction ($Pd(OAc)_2$, BINAP, and potassium carbonate in toluene at 100°C). Over a dose range of 15 mg to 240 mg/day, dasatinib displays a dose-proportional increase in AUC. Within 0.5 and 6 h, maximum plasma concentrations are achieved. The oral bioavailability ranges from 14% to 34%. Its terminal half-life is 3–5 h, and the plasma protein binding is 96%. With a volume of distribution of 2505 L, the drug is extensively distributed in the extravascular space. Dasatinib is primarily metabolized by CYP3A4 although FMO-3 and UGT enzymes are also involved. An active metabolite is equipotent to dasatinib and attributes to 5% of the AUC. Elimination of the parent and its metabolites occurs via the feces. The efficacy and safety of dasatinib were evaluated in four multicenter single-arm studies in patients with all phases (chronic, accelerated, and blast) of CML and Philadelphia chromosome-positive acute lymphoblastic leukemia (PH+ALL) who were resistant or intolerant of imatinib therapy. The starting dose was 70 mg orally twice daily; however, the dose could be adjusted in 20-mg increments (+ or −) to compensate for inadequate response or toxicity, and this has been retained as the standard dosing regimen. A complete hematologic response was achieved in 90% of patients ($N = 186$) in the chronic phase while 52% had a major cytogenetic response. In the accelerated phase group of 107 patients, 33% experienced a complete hematologic response and 22% realized a complete cytogenetic response. Even in the most advanced form of the disease, 26% of blast phase patients ($N = 74$) achieved a complete hematologic response, and 27% managed a complete cytogenetic response. In the PH+ALL study comprising 36 patients, a complete hematologic response was seen in 31% and a major cytogenetic response in 58%. It should be noted that all studies included patients without and with BCR-ABL mutations that confer resistance to imatinib treatment. Since dasatinib is metabolized by CYP3A4 and is a time-dependent inhibitor of CYP3A4, concomitant use of drugs that induce or inhibit CYP3A4 should be

avoided. Also, because the solubility of dasatinib is pH-dependent, concurrent use of H_2 blockers or proton pump inhibitors is not recommended. Antacids may be used 2 h before or after dasatinib dosing. Diarrhea, nausea, vomiting, abdominal pain, and bleeding were the most frequent adverse events. Although difficult to assess in patients with cytopenias, myelosuppression is common. QT prolongation can occur, so cardiac function should be monitored. In comparison to imatinib, major thrombocytopenia (54% vs. 14%) and neutropenia (58% vs. 28%) as well as pleural effusion were more common; however, fluid retention was more of an issue with imatinib (43% vs. 25%).

Decitabine	(Myelodysplastic syndromes)	[34–37]
Country of origin:	Netherlands	
Originator:	Pharmachemie	
First introduction:	US	
Introduced by:	SuperGen, MGI Pharma	
Trade name:	Dacogen	
CAS registry no:	2353-33-5	
Molecular weight:	228.21	



Decitabine, 5-aza-2′-deoxycytidine, has been launched for the treatment of myelodysplastic syndromes (MDS). MDS are a set of hematologic disorders affecting the bone marrow that result in ineffective formation and development of blood cells. Furthermore, patients with MDS have a high risk of progressing to acute myeloid leukemia (AML). Traditional treatments include blood transfusions, hematopoietic growth factors, and prophylactic antibiotics, but these measures merely improve the quality of life with questionable effects on disease modification. While stem-cell transplantation is an aggressive, potentially curative approach, the advanced age or the other complicating health issues of most patients preclude them from considering this option. Recent advances in the underlying etiology of MDS, however, have led to the development of a new class of compounds known as ''demethylating agents''. Decitabine follows the successful introduction of the first DNA methyltransferase inhibitor azacitidine. Whereas azacitidine may be incorporated into RNA and DNA, decitabine, without a 2′ hydroxyl, is preferentially taken up by DNA. While both agents are cytotoxic at high doses, low doses promote hypomethylation of DNA, upon analog phosphorylation and incorporation, by inhibition of DNA methyltransferase. Since aberrant DNA hypermethylation in the promoter regions of tumor suppressor genes and their subsequent silencing is characteristic of MDS, decitabine restores the normal gene control of cellular differentiation and proliferation, thereby resulting in the reversal of pathogenesis. The original synthesis of decitabine was conducted in 1964. The reaction of 2-methylisourea with 3,5-di-*O*-*p*-toluyl-2′-deoxy-D-ribofuranosyl isocyanate affords an intermediate that is condensed with ethyl orthoformate to construct the triazine ring. Prolonged treatment of the triazine derivative with methanolic ammonia at room temperature provides the desired 5-aza-2′-deoxycytidine as the major product with the

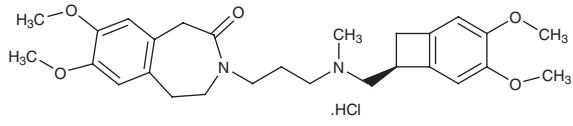
alpha-anomer as the minor component. The recommended dose of decitabine is 15 mg/m^2 administered by continuous intravenous infusion over 3 h repeated every 8 h for 3 consecutive days. With this dosing regimen, a C_{max} of 79 ng/mL and an AUC (0 – infinity) of 170 ng · h/mL were achieved. The steady-state volume of distribution was 148 mL/kg, and the elimination half-life was 35 min. Negligible plasma protein binding (<1%) was encountered. Decitabine is metabolized by nucleoside activation and degradation enzymes, particularly cytidine deaminase found in the liver, granulocytes, intestinal epithelium, and whole blood. In a randomized, open-label, controlled phase III trial involving 170 patients with MDS classified by International Prognostic Scoring System (High Risk, Intermediate-2, and Intermediate-1), patients were randomized to receive decitabine in addition to supportive treatment (blood and blood product transfusions, prophylactic antibiotics, and hemopoietic growth factors) or supportive care alone. Patients with AML were not intended for inclusion. Decitabine was administered at the recommended dose described above with this cycle being repeated every 6 weeks, depending on the patient's clinical response and toxicity. The overall response rate (complete or partial response) and time to AML or death were co-primary endpoints of the study. In the decitabine group, 17% of patients achieved a response (8 complete and 7 partial) while 0% of patients registered a response in the supportive care population. While the median duration of response for decitabine responders was 288 days, treatment did not substantially change the median time to AML or death compared to supportive therapy. The most frequent side effect is myelosuppression, including neutropenia, thrombocytopenia, and anemia. Although this side effect is serious, supplemental growth factor therapy may minimize the complications associated with myelosuppression. Other common adverse events include tiredness, fever, nausea, vomiting, cough, constipation, diarrhea, high sugar, petechiae, arthralgia, headache, and insomnia. Decitabine is contraindicated in women considering imminent pregnancy since it may have teratogenic consequences. Furthermore, men should not father a child during treatment and should wait for two months after completion. Caution is also recommended in patients with liver and renal insufficiency. Finally, formal drug–drug interaction studies have not been conducted; however, decitabine is neither a substrate nor an inhibitor of the cytochrome P450 enzymes; no interactions are, therefore, anticipated.

Idursulfase	(Mucopolysaccharidosis II (Hunter Syndrome))		[38–39]
Country of origin:	UK	Class:	Recombinant human protein
Originator:	Shire Transkaryotic therapies		
First introduction:	US	Type:	Sulfatase
Introduced by:	Shire	Molecular weight:	76 kDa Precursor
Trade name:	Elaprase	Expression system:	HT-1080 cells
CAS registry no:	50936-59-9	Manufacturer:	Shire

Mucopolysaccharidosis II, also known as Hunter Syndrome, is a lysosomal storage disorder characterized by a deficiency in iduronate-2-sulfatase, an enzyme responsible for the hydrolysis of the terminal 2-sulfate esters from the glycosaminoglycans dermatan sulfate and heparin sulfate in the lysosomes of various cells. This enzyme deficiency causes an accumulation of glycosaminoglycans (GAGs) in tissue. The clinical manifestations of this deficiency are short stature, joint stiffness, harsh facial features, hepatosplenomegaly, and progressive mental retardation. As with other lysosomal storage disorders, the patient's only recourse is enzyme replacement therapy (ERT). Idursulfase is a recombinant human enzyme that has been developed and launched as the ERT for Hunter syndrome. Unlike most recombinant enzymes, it cannot be produced in prokaryotic cells. For proper post-translational attachment of *N*-linked oligosaccharides and the crucial mannose-6-phosphate groups as the targeting passport into lysosomes, idursulfase is produced from HT-1080 cells. In addition to being fully glycosylated with eight mannose-6-phosphate groups, the enzyme possesses sialylated moieties that improve its stability in circulation. For full activity, Cys[59] must undergo modification to formylglycine. With one unit being defined as the amount required for hydrolysis of one micromole of heparin disaccharide substrate per hour, idursulfase has an activity of 41–77 units/mg of protein. The recommended dose of idursulfase is 0.5 mg/kg of body weight administered via a weekly intravenous infusion. As the dose was increased from 0.15 to 1.5 mg/kg following a single 1-h infusion, the AUC increased in greater than a dose-proportional manner. In 10 patients ranging in age from 7.7 to 27 years, the pharmacokinetic parameters were evaluated at Week 1 and Week 27 and found to be comparable at both time points. At Week 1, the data were as follows: AUC $= 206 \pm 87\,\text{min} \cdot \mu\text{g/mL}$, $C_{\max} = 1.5 \pm 0.6\,\mu\text{g/mL}$, $t_{1/2} = 44 \pm 19\,\text{min}$, Cl $= 3.0 \pm 1.2\,\text{mL/min/kg}$, and V_{ss} (% BW) $= 21 \pm 8$. In a randomized, double-blind, placebo-controlled clinical study evaluating the safety and efficacy of idursulfase, patients were included who had a documented deficiency in iduronate-2-sulfatase enzyme activity. If patients could not perform the required pulmonary function testing or could not follow protocol instructions, they were excluded from the study. The duration of the study was 53 weeks, and patients were randomized to receive 0.5 mg/kg of idursulfase every week, 0.5 mg/kg of idursulfase every other week, or placebo. A two-component composite score, based on the sum of the ranks of the change from baseline to Week 53 in distance walked during a six-minute walk test (6-MWT) and the ranks of the change in percent-predicted forced vital capacity (FVC), was the primary efficacy outcome assessment. While the changes in percent-predicted FVC were not statistically significant between drug and placebo groups, the weekly idursulfase-treated group achieved a 35 m greater mean increase in the 6-MWT than placebo. Urinary GAG levels and changes in liver and spleen size were further measures of bioactivity. At baseline, urinary GAG levels were elevated in all patients. After 53 weeks, patients receiving weekly infusions of idursulfase experienced markedly reduced urinary GAG levels although half of the patients were still above the upper limit of normal. In addition, sustained reductions in both liver and spleen volumes were also attained. In the placebo group, urinary GAG levels

remained elevated, and there were no measurable changes in liver and spleen volumes. As with other ERT, antibody development is an issue. In the weekly infusion group, 53% of patients developed anti-idursulfase IgG as assessed by ELISA or conformation specific antibody assay. While the full effect of circulating antibodies to idursulfase is unknown, patients with demonstrated anti-idursulfase antibodies experienced less of a reduction in urinary GAG excretion and were more likely to have increased incidence of infusion hypersensitivity. Regardless of antibody production, the most common adverse events were infusion-related, and specific warnings about the seriousness of hypersensitivity reactions are listed on the label. Since reactions have included respiratory distress, hypoxia, hypotension, angioedema, and seizure, appropriate medical support should be readily available upon idursulfase administration. In addition to the infusion-related reactions, pyrexia, headache, and arthralgia were observed most frequently. As for drug–drug interactions, no formal studies have been conducted at this time.

Ivabradine (Angina) [40–46]

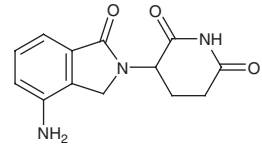


Country of origin:	France
Originator:	Servier
First introduction:	UK
Introduced by:	Servier
Trade name:	Procoralan
CAS registry no:	148849-67-6
Molecular weight:	505.05

In an effort to develop angina agents without the unwanted negative inotropic and hypotensive effects associated with β-adrenergic blockers and calcium channel blockers, a new class of heart-rate reducing compounds that act specifically on the sinoatrial (SA) node has been explored. These bradycardic agents interact directly with the pacemaking cell of the SA node and the hyperpolarization-activated I_f , the primary pacemaking current. Ivabradine has evolved as a specific inhibitor of I_f current through its contact with f-channels on the intracellular side of the plasma membrane. As a consequence, ivabradine reduces the speed of diastolic depolarization and decreases heart rate. It has been approved for the treatment of chronic stable angina and provides a viable alternative to patients with a contraindication or intolerance of β-blockers. Evaluation is also underway for the potential treatment of ischemic heart disease. Using a patch-clamp technique on rabbit sinoatrial node cells, inhibition of I_f current ranged from 6% (0.03 μM) – 80% (10 μM). The preparation of ivabradine involves the convergence of the left and right-hand pieces. Regarding the benzocyclobutane portion, 4,5-dimethoxybenzocyclobutane-1-carbonitrile is reduced with borane in tetrahydrofuran to afford an amine that is acylated with ethyl chloroformate. The resulting carbamate is treated with lithium aluminum hydride providing racemic *N*-(4,5-dimethoxybenzocyclobutan-1-yl-*N*-methylamine. The desired (*S*)-enantiomer is subsequently obtained by optical resolution with camphorsulfonic acid. As for the benzazepinone piece, the known 3-(3-chloropropyl)-7,8-dimethoxy-2,3-dihydro-1*H*-3-benzazepin-2-one is converted into its

corresponding iodide by reaction with sodium iodide. The chiral, right-hand amine is then condensed with the terminal iodide of the left-hand component. The double bond of the final intermediate is reduced with hydrogen over $Pd(OH)_2$ in acetic acid to generate ivabradine. The active drug component is isolated as its hydrochloride salt. Following a single, 10-mg dose, ivabradine was rapidly absorbed with an oral bioavailability of 40%. A C_{max} of 45.0 ± 36.6 ng/mL, t_{max} of 0.96 ± 0.41 h, and an AUC(0 – infinity) of 128 ± 87 ng · h/mL were achieved. The plasma protein binding was approximately 70%, and the volume of distribution at steady state was nearly 100 L. The half-life was 1.92 ± 0.39 h with a total clearance of about 400 mL/min. The primary route of metabolism is in the liver via CYP3A4; only 4% of intact drug is found excreted in the urine; however, metabolites are distributed equally in the urine and feces. The *N*-desmethylated derivative is the major metabolite, and its metabolism also involves CYP3A4. While ivabradine does not inhibit or induce CYP3A4, concomitant use of the potent CYP3A4 inhibitor ketoconazole results in a three-to-four-fold increase in C_{max} and $t_{1/2.}$ In a placebo-controlled, phase II study consisting of 360 patients with stable angina, patients were randomized to receive placebo or one of three oral doses of ivabradine (2.5, 5, or 10 mg b.i.d.) for two weeks. Efficacy was determined by a standardized, bicycle exercise tolerance test (ETT). Ivabradine, at the trough of drug activity, induced dose-dependent reductions versus placebo in heart rate at rest and during exercise of 15 and 14 bpm, respectively, with the 10-mg twice daily dose. Not only did these reductions in exercise heart rate result in significant anti-ischemic and anti-anginal efficacy, but patients also reported reductions in the frequency of angina attacks during routine activities with ivabradine treatment. The most common adverse events were visual disturbances. Inhibition of I_h current in retinal hyperpolarization-activated, cyclic nucleotide-gated channels is the culprit. Other general disorders included headache and dizziness. In addition to being contraindicated for concomitant use with moderate-to-strong CYP3A4 inhibitors, ivabradine should be avoided in patients with heart rate below 60 bpm, cardiogenic shock, acute myocardial infarction, severe hypotension, severe hepatic insufficiency, sick sinus syndrome, sinoatrial block, heart failure, pacemaker dependency, unstable angina, and AV-block of 3rd degree. Since animal reproduction studies have demonstrated embryotoxic and teratogenic effects, ivabradine is also contraindicated during pregnancy and breast-feeding.

Lenalidomide (Myelodysplastic syndromes, multiple myeloma) [47–53]



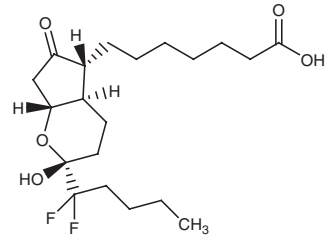
Country of origin:	US
Originator:	Celgene
First introduction:	US
Introduced by:	Celgene
Trade name:	Revlimid
CAS registry no:	191732-72-6
Molecular weight:	259.26

Lenalidomide is a derivative of thalidomide differing in the presence of an amino moiety in the 4-position and removal of one of the carbonyls of the phthaloyl ring. This derivative evolved from a structural-based effort to eliminate the adverse effects (somnolence, neuropathy, and teratogenicity) of thalidomide while maintaining or enhancing the appealing attributes. While the mechanism of action remains to be fully identified, lenalidomide inhibits TNF-α production in stimulated monocytes. In addition to inhibiting TNF-α secretion, the production of other pro-inflammatory cytokines is blocked. Complimentary to inhibition of pro-inflammatory cytokines, lenalidomide also increases the secretion of anti-inflammatory cytokines, such as IL-10. Furthermore, lenalidomide inhibits secretion of angiogenic cytokines, VEGF and bFGF. Due to its immunomodulatory and antiangiogenic properties, lenalidomide has the potential for a wide spectrum of therapeutic applications. While other indications are under evaluation, lenalidomide has initially been launched for the treatment of myelodysplastic syndromes (MDS), a set of hematologic disorders that affect the bone marrow and result in a deficiency of mature blood cells. There are various types of MDS, and lenalidomide is approved for the type associated with a truncation of chromosome 5 known as deletion 5q MDS. In combination with dexamethasone, it has also been designated for the second-line treatment of multiple myeloma, a B cell malignancy characterized by excess monotypic plasma cells in the bone marrow. Lenalidomide is a racemic drug with its *S*-enantiomer possessing more pharmacological activity; the *S*-enantiomer has an IC_{50} for TNF-α inhibition of 3.9 nM compared to 94 nM for the *R*-enantiomer. Since lenalidomide is significantly more potent than thalidomide, it can achieve responses at lower doses to minimize undesired side effects. Lenalidomide may be prepared starting with a Cbz-protected glutamine derivative that is transformed into its corresponding methyl ester. Liberation of the amine followed by condensation with methyl 2-(bromomethyl)-3-nitrobenzoate affords methyl 5-amino-2-(4-nitro-1-oxoisoindolin-2-yl)-5-oxopentanoate. The nitro group may then be reduced with hydrogen over 10% Pd/C prior or subsequent to base-catalyzed cyclization (potassium *t*-butoxide in tetrahydrofuran) to generate the racemic lenalidomide. In a phase I clinical study, it was determined that the pharmacokinetic parameters of lenalidomide increase linearly with dose. Following 5-mg, 10-mg, and 20-mg doses, respectively, the following data were observed: AUC = 305, 540, and 1879 ng · h/mL; C_{max} = 80, 107, and 350 ng/mL; T_{max} = 0.8, 1.5, and 1.2 h; $t_{1/2}$ = 2.1, 3.4, and 5.1 h; Cl/F = 278, 409, 342 mL/min; and V/F = 52, 104, and 1161. The plasma protein binding is approximately 30%. While the full metabolic profile in humans has not been evaluated, two-thirds of lenalidomide is eliminated unchanged through urinary excretion. In a clinical study evaluating the safety and efficacy of lenalidomide for MDS with a deletion 5q cytogenetic abnormality, 148 patients who had RBC transfusion-dependent anemia received either 10-mg once daily on a continuous basis or 10-mg once daily for 21 days of a 28-day cycle. To compensate for toxicity, reductions in dose to 5-mg daily or every other day, as well as dose delays, were permitted. For patients who developed neutropenia or fever associated with neutropenia, granulocyte

colony-stimulating factor was also introduced. A modification of the International Working Group response criteria for MDS was utilized to document the frequency of RBC-transfusion independence. In 67% of patients, transfusion independence was observed with a median duration of 44 weeks. Ninety percent of the patients who received a transfusion benefit did so by the completion of three months into the study. Due to adverse events, the dose of lenalidomide was reduced at least once in nearly 80% of patients while 33% required a second dose adjustment with a median interval of 51 days between the two dose modifications. Lenalidomide was also investigated in a multiple myeloma clinical study comparing its efficacy in combination with high-dose dexamethasone therapy to dexamethasone alone. In the lenalidomide/dexamethasone group, patients received 25 mg of lenalidomide on days 1–21 and placebo on days 22–28 of each 28-day cycle while patients in the placebo/dexamethasone group received placebo on each day of the cycle. Patients in both groups observed a dosing regimen of 40 mg of dexamethasone on days 1–4, 9–12, and 17–20 of each 28-day cycle for the first four cycles. On the fifth cycle, the dexamethasone dose was reduced to only days 1–4 of the 28-day cycle. The primary end point of the study was time to progression (TTP). A statistically significant improvement was seen in the lenalidomide/dexamethasone arm. With the study lasting 18 months, the median TTP was 13.3 months for the combination treatment compared to 5.1 months for dexamethasone alone. While thromboembolic events occurred with nearly twice the frequency in the combination therapy group (8.5% vs. 4.5%), the remaining safety profile was comparable to mono-therapy with dexamethasone. The recommended starting dose of lenalidomide is 10 mg daily, but the drug is supplied in 5-, 10-, 15-, and 25-mg capsules for facile dose adjustment. In addition to the common side effects of diarrhea, itching, rash, and tiredness, the most serious adverse events include myelosuppression and deep vein thrombosis, but both may be managed by dose interruption or prophylactic drug supplements. While mutagenicity and fertility tests revealed no toxicity issues, due to its structural similarity to the known teratogen thalidomide, lenalidomide is contraindicated in pregnant women or women and their male partners who are contemplating pregnancy. Since *in vitro* studies indicated that lenalidomide is not an inhibitor, substrate, or inducer of the cytochrome P450 enzymes, it is unlikely to cause drug interactions in humans.

Lubiprostone (Chronic idiopathic constipation) [54–58]

Country of origin: US
Originator: Sucampo
First introduction: US
Introduced by: Sucampo/Takeda
Trade name: Amitiza
CAS registry no: 333963-40-9
Molecular weight: 390.46

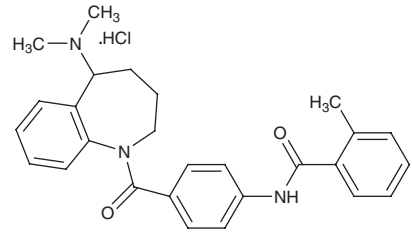


Chronic constipation is an affliction affecting 4–5 million Americans alone. When no specific cause is identified, it is classified as idiopathic. Dietary and lifestyle modifications are the first-line conventional approaches followed by the administration of laxatives. Unfortunately, chronic idiopathic constipation is frequently refractory to traditional therapy; thus, the need for novel agents exists. Lubiprostone is a bicyclic fatty acid with a novel mechanism of action. Without affecting sodium and potassium ion concentrations, lubiprostone activates intestinal chloride ion channels, thereby, increasing intestinal water secretion and intestinal fluid chloride ion concentration. In basolateral membrane-permeabilized T84 gastrointestinal epithelial cells under chloride gradient conditions, lubiprostone concentration-dependently increased short-circuit current with an EC_{50} of approximately 20 nM. Lubiprostone may be prepared in 10 steps starting from the commercially available Corey's lactone. Desilylation with tetrabutylammonium fluoride liberates the primary alcohol that is subsequently oxidized with oxalyl chloride to afford the aldehyde. The fluorinated side chain is installed via a titanium ethoxide-mediated condensation of the aldehyde with the phosphonate of the fluorinated chain. The unsaturated difluoroketone is subsequently hydrogenated over palladium/carbon before reducing the ketone with sodium borohydride. Diisobutylaluminum hydride is then utilized to reduce the carbonyl of the lactone to provide the lactol for condensation with 4-carboxybutyl triphenylphosphonium bromide. The resulting prostaglandin $F_{2\alpha}$ intermediate is esterified with benzyl bromide. At this point, both alcohol moieties are oxidized with chromium trioxide and pyridine followed by removal of the THP protecting group with acetic acid. The final step involves the concomitant cleavage of the benzyl ester and reduction of the double bond with hydrogen over palladium/carbon. Lubiprostone is supplied in 24-μg gelatin capsules with the recommended daily dose being 24 μg twice daily with food. Following oral administration, lubiprostone is quickly metabolized making an accurate assessment of its pharmacokinetic parameters impossible. Data, however, has been obtained for the major active metabolite M3 following the standard 24-μg dose. The C_{max} was 41.9 pg/mL with peak plasma levels occurring in 1.4 h. The mean AUC of M3 was 59.1 pg.h/mL, and its elimination half-life ranges from 0.9 to 1.4 h. Using radiolabeled lubiprostone, 60% of the radioactivity was recovered in the urine within 24 h while 30% was excreted in the feces within 168 h. Lubiprostone is not metabolized by the cytochrome P450 enzymes; microsomal carbonyl reductase is involved in the biotransformation of lubiprostone to M3; thus, there is a low probability of drug–drug interactions. Following a two-week, drug-free period, the safety and efficacy of lubiprostone were evaluated in 242 patients in a multi-center, randomized, placebo-controlled phase III trial for 4-week duration. Acceptance into the study was based on the criteria of having fewer than three spontaneous bowel movements (SBMs) a week and a 6-month history of constipation. Patients were randomized to receive 24-μg of lubiprostone twice daily or placebo. Lubiprostone was well tolerated; the most common adverse events were nausea, diarrhea, and headache. The lubiprostone-treated group experienced a significant increase in SBMs in all weeks of the study (5.1–5.7 versus 2.8–3.5). In addition, more patients in the drug-treated group had

a BM within 24 h of the first dose (57% vs. 37%). Lubiprostone is contraindicated in patients with a history of mechanical gastrointestinal obstruction. Since studies in guinea pigs have demonstrated the potential for fetal loss, in contrast to other animal species, lubiprostone should only be used during pregnancy if the potential benefit justifies the possible risk to the fetus.

Mozavaptan (Hyponatremia) [59–63]

Country of origin: Japan
Originator: Otsuka
First introduction: Japan
Introduced by: Otsuka
Trade name: Physuline
CAS registry no: 137975-06-5
Molecular weight: 464

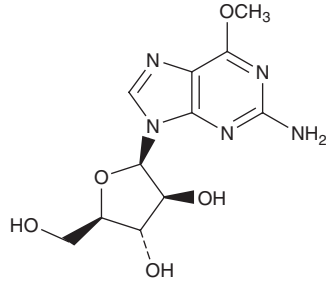


Mozavaptan is an oral vasopressin V_2 antagonist that has been launched in Japan for inappropriate antidiuretic hormone secretion syndrome (IADHS), an affliction manifesting as hyponatremia. It joins another nonpeptidic benzazepine, conivaptan, which corrects sodium and water imbalance by blocking the renal V_2 receptor resulting in enhanced diuresis, thereby effectively increasing serum sodium concentration. While conivaptan inhibits both V_1 and V_2 receptors, mozavaptan is significantly more selective for V_2 (IC_{50} of 14 nM vs. 1.2 μM for V_1). Mozavaptan may be prepared starting from the tosylated benzazepin-5-one. Its dimethylamino moiety is installed by two consecutive reductive aminations (methylamine and sodium borohydride followed by formaldehyde and sodium cyanoborohydride). Deprotection of the tosyl group is accomplished with polyphosphoric acid at 150°C, and the liberated amine is acylated with *p*-nitrobenzoyl chloride. Subsequent reduction of the nitro functionality with hydrogen over palladium/carbon affords an amine handle for coupling with 2-methylbenzoyl chloride to provide mozavaptan. The drug is formulated as its hydrochloride salt and distributed in 30-mg tablets. The SAR suggests that a simple methyl confers selectivity for V_2 while elaboration of the 2-position by introduction of a phenyl or a 4-substituted phenyl increases affinity for both subtypes. While the pharmacokinetic properties of mozavaptan were unavailable, the pharmacodynamics were examined in six healthy, hydrated men in six dose steps (3, 15, 30, 60, 100, and 200 mg). The drug was well tolerated at all doses. Measuring the 6-h hypotonic urine volume, increases were dose-dependent; a 30-mg dose raised the urine volume to two times, 100 mg to three times, and 200 mg to four times (1828.0 ± 130.2 mL/6 h) relative to that observed with the placebo (470.4 ± 52.1 mL/6 h). At all doses, mozavaptan increased urine flow maximally between 1 and 1.5 h. In addition, mozavaptan lowered urine osmolality for four hours, particularly between 60 and 90 min (72.3 ± 2.3 and 62.3 ± 5.1 mOsm/kg at 100 and 200 mg, respectively). At the highest dose (200 mg), mean free-water clearance of

the 6-h urine reached 2.82 ± 0.21 mL/min, the culmination of a dose-proportional increase into a positive range. The plasma osmolality also increased dose-dependently before reaching a maximum of 300 ± 1 mOsm/g. Furthermore, plasma sodium and chloride increased maximally at 6 h (142.8 ± 0.3–148.5 ± 0.3 mEq/L for sodium and 109.7 ± 0.4 to 112.5 ± 0.6 mEq/L for chloride) without altering plasma potassium. In a phase II trial, mozavaptan (single dose of 0.25 and 0.5 mg/kg i.v.) was administered to 11 patients with IADHS. An increase in urine volume with concomitant decrease in urine osmolality was observed after 4 h while plasma sodium increased 3 mEq/L. The noted side effects included dry mouth and thirst. While the metabolites of mozavaptan have been characterized (*N*-dealkylation, *N*-oxide formation, benzazepine hydroxylation, etc.), the route of metabolism has not been fully disclosed. No formal drug–drug interaction studies have been conducted.

Nelarabine (Anticancer) [64–68]

Country of origin:	US
Originator:	Glaxo Wellcome
First introduction:	US
Introduced by:	GSK
Trade name:	Arranon
CAS registry no:	121032-29-9
Molecular weight:	297.27



Nelarabine is a new member of the purine nucleoside antimetabolite class of drugs. It was launched last year as an intravenous infusion for treating relapsed or refractory T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) after at least two prior chemotherapy regimens. Nelarabine is a pro-drug of 9-β-D-arabinofuranosylguanine (ara-G), a deoxyguanosine derivative with a high level of T-cell selective cytotoxicity. Although Ara-G has been known since the 1960s, it has not been used in clinical studies due to its poor solubility. Nelarabine is the *O*-methyl derivative of ara-G with approximately 10 times greater aqueous solubility. It is demethylated *in vivo* by adenosine deaminase to produce ara-G, which is subsequently converted to the active 5′-triphosphate, ara-GTP. Accumulation of ara-GTP in leukemic blasts allows for incorporation into DNA, leading to inhibition of DNA synthesis and cell death. The selective cytotoxicity of ara-G in T-cells is postulated to be due to the higher accumulation and slower elimination of ara-GTP in these cells as compared to B-cells. The chemical synthesis of nelarabine involves a biocatalysis step wherein 2-amino-6-methoxypurine is subjected to a trans-glycosidation reaction with arabinosyl uracil by means of purine nucleoside phosphorylase and uridine phosphorylase. *In vitro*, nelarabine exhibits similar growth-inhibitory

activity to ara-G in human T-cell leukemia cell lines (CEM $IC_{50} = 0.3–0.4\,\mu M$; U-937 $IC_{50} = 1.0–1.5\,\mu M$; Monomac-6 $IC_{50} = 0.8\,\mu M$), whereas it is ineffective against B-cell leukemia IM-9 cells ($IC_{50} > 100\,\mu M$). Pharmacokinetic studies in patients with refractory leukemia or lymphoma have demonstrated that nelarabine and ara-G are rapidly eliminated from plasma with a half-life of approximately 30 min and 3 h, respectively. Plasma ara-G C_{max} values generally occur at the end of the nelarabine infusion and are generally higher than nelarabine C_{max} values, suggesting rapid and extensive conversion of the pro-drug to ara-G. The principal route of metabolism for ara-G is its hydrolysis to form guanine, which is subsequently metabolized to xanthine, uric acid, and allantoin. Both nelarabine and ara-G exhibit extensive distribution and very low protein binding (<25% *in vitro*). They are partially eliminated by the kidneys. The recommended dose of nelarabine in pediatric patients is $650\,mg/m^2$ administered intravenously over 1 h daily for 5 consecutive days, and the regimen repeated every 21 days. The recommended dose in adults is $1500\,mg/m^2$ administered intravenously over 2 h on days 1, 3, and 5 repeated every 21 days. The safety and efficacy of nelarabine were demonstrated in two clinical studies, one conducted in children and the other in adults. Both studies enrolled patients with relapsed or refractory T-ALL/T-LBL. In both studies, complete response (CR) was defined as bone marrow blast counts $\leq 5\%$, no other evidence of disease, and full recovery of peripheral blood counts. Complete response without full hematological recovery (CR*) was also assessed. In the pediatric study, of the 39 patients who had received at least two prior induction regimens, 23% achieved either CR or CR*. In the study with adult subjects, of the 28 patients who received ≥ 2 prior inductions, 21% achieved either CR or CR*. The dose-limiting toxicity of nelarabine is neurotoxicity, and the label contains a black-box warning strongly recommending close monitoring for neurological events. Common side effects reported with nelarabine treatment are fatigue, nausea, vomiting, and diarrhea. Nelarabine has been granted orphan drug status in the US.

Nimotuzumab	(Anticancer)		[69–72]
Country of origin:	Cuba	Class:	Humanized murine IgG2a monoclonal antibody R3
Originator:	Center for Molecular Immunology	Type:	EGFR antagonist
		Molecular weight:	~150 kDa
First introduction:	India	Expression System:	NSO cells
Introduced by:	YM Biosciences Biocon Pharmaceuticals	Manufacturer:	Center for Molecular Immunology
Trade name:	BioMab EFGR, Theraloc		
CAS registry no:	828933-51-3		

Overexpression of epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase, is prevalent in malignant tumors of epithelial origin and is especially common in breast, head and neck, colon, and lung cancer. With EGFR overexpression, prognosis is poor due to associated tumor invasion, metastasis, enhanced angiogenesis, and resistance to chemotherapy; thus, modulation of EGFR-mediated signaling is an attractive target for intervention. Small-molecule kinase inhibitors (gefitinib and erlotinib) and a monoclonal antibody (cetuximab) specific for EGFR are in clinical evaluation, but treatment cessation due to development of severe acne-like rash is common with these EGFR antagonists. Notably, nimotuzumab, a humanized form of the murine IgG2a monoclonal antibody that has been launched in India for treatment of head and neck cancers overexpressing EGFR, demonstrates clinical efficacy devoid of the rash toxicity. Presumably, humanization of the antibody sequence eliminates hypersensitivity reactions and human anti-mouse antibody (HAMA) responses, as well as, improves pharmacokinetic and effector functions in patients. While beyond the scope of this review, a technetium-labeled (^{99m}Tc) nimotuzumab has been developed for diagnostic purposes while its ^{188}Re-labeled counterpart emits β-particles for targeted radioimmunotherapy. The pharmacokinetic parameters and tissue localization, however, were determined with [^{99m}Tc]nimotuzumab. Following a 27mCi dose (3 or 6 mg i.v.), the radiopharmaceutical was cleared rapidly from the blood with a normal tissue distribution half-life of 10.8 ± 3.8 min.The volume of distribution and clearance were 180 ± 37 mL/kg and 14 ± 37 mL/kg/min, respectively. The liver, spleen, and kidneys demonstrated the largest radioactivity uptake, and it was determined that 19–24% of the agent was excreted in the urine. From repeated weekly intravenous dosing of nimotuzumab to patients with squamous cell carcinoma of the head and neck (SCCHN), the minimum and maximum steady-state concentrations increased linearly with dose. For the 200-mg dose, the range was 19–76 mg/mL compared to 39–147 μg/mL for the 400-μg dose. Nimotuzumab is prepared by genetic engineering of the murine IgG2a monoclonal antibody R3, secreted by hybridoma obtained from the fusion of murine myeloma cells with splenocytes from BALB/c mice immunized with partially purified human placental EGFR. The humanized h-R3 IgG1 antibody (nimotuzumab) is obtained by grafting the complementarity-determining regions of R3 onto the human frameworks, the light and heavy chains of REI and EU chosen for their high homology with the corresponding sequences of R3. Since this original approach led to a dramatic reduction in the binding of the antibody, evaluation of selective mutation back to a few original murine amino acids concluded that Ser^{75}, Thr^{76}, and Thr^{93} should be retained. By interacting with one epitope in the extracellular domain of EGFR, nimotuzumab blocks ligand binding and subsequent EGF-dependent receptor phosphorylation. In addition, nimotuzumab displays a similar affinity to that of EGF (~1 nM). The efficacy of nimotuzumab was evaluated in patients with advanced (unresectable) SCCHN with demonstrable overexpression of EGFR. For a duration of 6 weeks in combination with radiotherapy (60–66 Gy in 30 fractions), a total of 24 patients were administered weekly intravenous infusions of nimotuzumab (50, 100, 200, or 400 mg). Seven patients demonstrated a partial or complete response while one

patient receiving the 200-mg dose was disease-free after resection of the residual tumor. The overall survival was significantly increased at the two higher doses (median of 44.3 months) compared to the lower doses (median of 8.6 months). Furthermore, three-year survival was superior (66.7%) in patients receiving the higher doses of 200 or 400 mg compared with the survival rate for patients at the lower doses (16.7%). These results were confirmed by a parallel study in Canada where 70% of SCCHN patients achieved complete responses after doses of 100 mg and 200 mg of nimotuzumab in combination with radiation therapy. Equivalent response rates to those achieved with chemoradiation were observed without the toxicities associated with chemotherapy. The adverse events associated with nimotuzumab treatment included mild-to-moderate fever, hypotension, hypertension, vomiting, nausea, dry mouth, and tremors; however, no anaphylactic or skin reactions, prevalent with anti-EGFR agents, were observed.

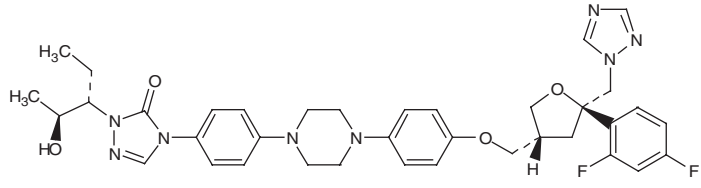
Panitumumab	(Anticancer)	[73–77]	
Country of origin:	US	Class:	Humanized IgG2
Originator:	Abgenix		monoclonal antibody
First introduction:	US	Type:	anti-EGFR
Introduced by:	Amgen	Molecular weight:	147 kDa
Trade name:	Vectibix	Expression system:	CHO cell line
CAS registry no:	339177-26-3	Manufacturer:	Amgen

Panitumumab is a recombinant, fully human IgG2 kappa monoclonal antibody that is highly selective for the epidermal growth factor receptor (EGFR), and it is indicated for the treatment of EGFR-expressing, metastatic colorectal carcinoma (mCRC) with disease progression on or following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens. Overexpression of EGFR is detected in many human cancers, including those of the colon and rectum. Panitumumab blocks the binding of ligands such as epidermal growth factor (EGF) and transforming growth factor (TGF-α) to various EGFR-expressing cell lines, which results in inhibition of EGF-dependent tumor cell activation, induction of apoptosis, and decreased pro-inflammatory cytokine and vascular growth factor production. *In vitro*, panitumumab binds EGFR with high affinity ($K_D = 50$ pM; $IC_{50} = 3$ nM) in human cervical epidermal cell line A-431. Panitumumab is the second anti-EGFR antibody to reach the market. Its predecessor, cetuximab (Erbitux®), launched in 2003, is also indicated for the treatment of mCRC. The key difference between these two drugs is the fact that cetuximab is a human/mouse chimeric monoclonal antibody with ~30% murine protein, whereas panitumumab is fully human. Chimeric antibodies are potentially immunogenic and treatment can lead to infusion reactions. In addition, the potential for development of human anti-chimeric antibodies can limit their efficacy. Fully human antibodies can be expected to produce minimal, if any,

immunogenic response, and may be more suitable for the chronic treatment of cancer patients. Panitumumab is derived from the use of Xenomouse^{TM} technology, wherein human immunoglobulin genes are introduced into transgenic mice that lack functional mouse immunoglobulin genes. The recommended dose regimen of panitumumab is 6 mg/kg once every 2 weeks as a 1-h intravenous infusion. The steady-state plasma concentrations of panitumumab are reached by the third infusion, and the elimination half-life is approximately 7.5 days. Panitumumab exhibits nonlinear pharmacokinetics that are similar across multiple patient populations. The clearance of panitumumab is mediated by two separate pathways. The EGFR itself acts as a sink, with clearance decreasing in a dose-dependent manner. Once the receptor becomes saturated, the reticular-endothelial system assumes a greater role and provides clearance similar to that of endogenous immunoglobulin. Serum levels are quite predictable with little interpatient variation. The safety and efficacy of panitumumab were studied in an open-label, randomized, controlled trial involving 463 patients with EGFR-expressing metastatic carcinoma of the colon or rectum. Subjects were randomized to receive panitumumab at a dose of 6 mg/kg given once every 2 weeks plus best supportive care (BSC) ($n = 231$) or BSC alone ($n = 232$) until disease progression. Statistical significance was seen in prolongation of progression free survival for the subjects treated with panitumumab versus those treated with BSC alone, with a mean of 96 days vs. 60 days, respectively. Adverse events associated with the use of panitumumab included dermatological toxicities, ocular toxicities, hypomagnesemia, fatigue, abdominal pain, nausea, diarrhea, and constipation.

Posaconazole (Antifungal) [78–82]

Country of origin:	US
Originator:	Schering-Plough
First introduction:	UK
Introduced by:	Schering-Plough
Trade name:	Noxafil
CAS registry no:	171228-49-2
Molecular weight:	700.78



Posaconazole, launched last year in the UK, is the newest member of the azole class of antifungal agents to reach the market. It is indicated for the treatment and prophylaxis of a range of invasive fungal infections, including aspergillosis,

fusariosis, chromoblastomycosis, mycetoma, and coccidiomycosis in patients who are refractory to, or intolerant of, standard therapy with amphotericin B and/or itraconazole. In the US, it is approved for the prophylaxis of invasive *Aspergillus* and *Candida* infections in patients ≥13 years of age who are at high risk of developing these infections due to being severely immunocompromised. Additionally, it is approved for the treatment of oropharyngeal candidiasis. Posaconazole has an expanded spectrum of activity over other members of the azole antifungals. In addition to potent activity against refractory cases of aspergillosis and fluconazole-resistant *Candida*, it demonstrates activity against Zygomycetes. As with other azole antifungals, posaconazole inhibits fungal ergosterol synthesis through inhibition of lanosterol 14-α demethylase and is highly selective for fungal cytochrome P450 systems. *In vitro*, posaconazole has similar potency as voriconazole against *Candida* and *Aspergillus*, with MIC_{90} ranges of 0.063–2.0 μg/ml and 0.25–0.5 μg/ml, respectively, and it is generally more potent than itraconazole against both species. Against *Candida*, posaconazole is typically 16- to 32-fold more potent than fluconazole. In addition, posaconazole has activity against many fluconazole-resistant strains of *Candida*. Posaconazole is the only member of the azole class that shows significant activity against the Zygomycetes ($MIC_{90} \leq 4$ μg/mL); however, it is generally 2-fold less active than amphotericin B against the 86 isolates tested. Posaconazole is administered as an oral suspension. Absorption of posaconazole is enhanced when co-administered with food. Maximum plasma concentrations are observed approximately 5.8 to 8.8 h following a single oral dose, and steady state levels are reached in approximately 10 days of twice-daily dosing. Posaconazole has high protein binding (98–99%), a large volume of distribution (343–1,341 L), and a long half-life (~25 h). In contrast to other azole antifungals, posaconazole is not extensively metabolized by CYP450 enzymes. Approximately 77% of an administered dose is excreted in the feces as the parent compound, and ~14% is excreted in the urine as multiple glucuronidated derivatives. The efficacy of posaconazole against a range of fungi was demonstrated in a phase III clinical study of 609 patients with invasive fungal infections who were refractory to, or intolerant of, other antifungal therapy. In this study, 330 patients received posaconazole (800 mg/day) and 279 patients served as external controls. In aspergillosis, the global response success rate at the end-of-therapy visit (primary endpoint) was significantly higher in posaconazole recipients than in external controls (42% vs. 26%). Posaconazole was also associated with overall success rates of 54% in zygomycosis, 46% in fusariosis, 43% in *Pseudallescheria* infection, 80% in phaeohyphomycosis and 100% in histoplasmosis. Success rates were 48% in refractory candidiasis, 69% in refractory coccidioidomycosis, 48% in refractory cryptococcal infection, and 82% in refractory chromoblastomycosis or mycetoma. Oral posaconazole suspension was generally well tolerated in patients with invasive fungal infections, including patients who received treatment for ≥1 year. The most commonly reported adverse events associated with posaconazole therapy are fever, headache, rigors, fatigue, hypertension, diarrhea, nausea, vomiting, coughing, and dyspnea.

Posaconazole has a high degree of structural similarity to itraconazole. It can be synthesized starting from a chiral oxazolidinone derivative of 2,4-difluoro-γ-methylene-benzenebutanoic acid via hydroxymethylation of the titanium enolate, followed by iodocyclization to produce an iodomethyltetrahydrofuran intermediate in high diastereoselectivity. Subsequent conversion into posaconazole is accomplished by a sequence of synthetic steps involving reductive cleavage of the oxazolidinone chiral auxiliary with lithium borohydride, displacement of the iodine with sodium-triazole, formation of a tosylate derivative of the hydroxyl group, and displacement with a 4-piperazinylphenol intermediate.

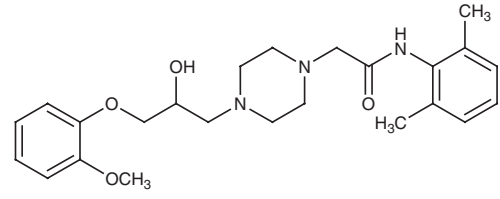
Ranibizumab	(Age-related macular degeneration)		[83–86]
Country of origin:	US	Class:	Humanized IgG1 monoclonal Fab
Originator:	Genentech		
First introduction:	US	Type:	anti-VEGF
Introduced by:	Genentech/ Novartis	Molecular weight:	48 kDa
Trade name:	Lucentis	Expression system:	*E. coli*
CAS registry no:	347396-82-1	Manufacturer:	Genentech

Ranibizumab is a recombinant, humanized, IgG1 monoclonal antibody fragment (Fab) that neutralizes all active forms of vascular endothelial growth factor A (VEGF-A), and it is indicated for the treatment of neovascular age-related macular degeneration (AMD). It consists of a nonbinding human sequence and a high-affinity binding epitope (Fab fragment) derived from the mouse. The full-length RhuMab VEGF (bevacizumab) was launched previously by Genentech for the treatment of colorectal cancer. Both the antibody fragment and the full-length antibody bind to and inhibit all active forms of VEGF-A and are derived from the same mouse monoclonal antibody. However, ranibizumab has been genetically engineered through a process of selective mutation to increase its affinity for binding and inhibiting the growth factor. The Fab domain of ranibizumab differs from the Fab domain of bevacizumab by six amino acids, five on the heavy chain (four of which are in the binding site) and one on the light chain. In addition, the smaller size of ranibizumab (~48 kDa) is expected to facilitate retinal penetration, and hence is more suitable for intraocular use. The binding of ranibizumab to VEGF-A prevents the interaction with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells, thereby reducing endothelial cell proliferation, vascular leakage, and new blood vessel formation. The recommended dosing regimen of ranibizumab is 0.5 mg once a month, administered by intravitreal injection. Pharmacokinetic studies in animal models indicate that systemic exposure of ranibizumab is more than 2000-fold lower than in the vitreous. In AMD patients, peak serum concentrations of 0.3–2.36 ng/mL

are achieved in approximately 24 h after intravitreal administration of ranibizumab 0.5 mg.These serum levels are well below the concentration of 11–27 ng/mL thought to be necessary to inhibit the biological activity of VEGF-A by 50%, as measured in an *in vitro* cellular proliferation assay. Average vitreous half-life is estimated at 9 days. The clinical efficacy and safety of ranibizumab were evaluated in three randomized, double-blinded, sham- or active-controlled studies in 1323 patients representative of the population usually affected with AMD. The primary efficacy end point in these studies was the proportion of patients who maintained vision, defined as losing fewer than 15 letters of visual acuity at 12 months compared with baseline. Nearly 95 percent of the participants who received a monthly intravitreal injection of 0.3- or 0.5-mg ranibizumab maintained their vision at 12 months compared to approximately 60 percent of patients who received the control treatment. Additionally, up to 40% demonstrated an improvement in vision of at least 15 letters. The most commonly reported adverse events associated with ranibizumab treatment include conjunctival hemorrhage, eye pain, vitreous floaters, increased intraocular pressure, and intraocular inflammation. If monthly injections are not tolerable, the regimen may be reduced to one injection every three months after the first three monthly injections. However, in one year clinical studies, dosing once every three months after three once-monthly doses was linked to a loss of approximately 5 letters in visual acuity for the following 9 months compared with continued monthly dosing.

Ranolazine (Angina) [87–91]

Country of origin: US
Originator: Roche Bioscience
First introduction: US
Introduced by: CV Therapeutics
Trade name: Ranexa
CAS registry no: 95635-55-5
Molecular weight: 427.54



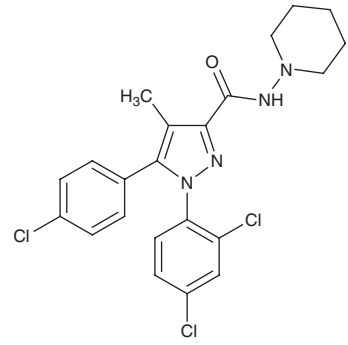
Ranolazine is an orally available, extended release drug for the treatment of chronic angina in patients who have failed to respond to prior angina therapy. Chronic stable angina (CSA) is a common symptom of coronary artery disease wherein plaques in the coronary vasculature restrict blood flow to the heart, which in turn leads to insufficient oxygenation of the heart, typically during physical exertion or emotional stress. A vast majority of the existing anti-anginal and anti-ischemic therapies aim to correct the imbalance between myocardial oxygen demand and supply through mechanisms that produce reductions in heart rate or blood pressure. For example, the nitrates act by dilating the blood vessels, thereby allowing more blood to flow to the heart while reducing its

workload. Likewise, the calcium channel blockers such as amlodipine dilate blood vessels and lower blood pressure, and the beta-blockers reduce heart rate and lower blood pressure. Although these are effective drugs, it is often not possible to increase their doses to the levels necessary to fully control chronic stable angina, owing to intolerable effects on blood pressure or heart rate. The anti-anginal and anti-ischemic effects of ranolazine do not depend on reductions in heart rate or blood pressure, thus representing a new mechanism of action. However, the mechanisms underlying the pharmacology of ranolazine are not clear. Recently, investigations of the electrophysiological effects of ranolazine have found that it inhibits pathologic increases in late Na^+ current induced during myocardial ischemia. Because of Na^+/Ca^{2+} coupling, this would be expected to reduce ischemia-induced calcium overload, thereby improving related diastolic function (i.e. more normal diastolic relaxation and decreased wall tension). Improved diastolic function decreases oxygen demand and increases coronary blood supply. Ranolazine is marketed as a racemic mixture. The chemical synthesis of ranolazine entails the reaction of 2-chloro-*N*-(2,6-dimethylphenyl) acetamide with excess piperazine to produce the monoalkylated piperazine intermediate, which is subsequently condensed with racemic [(2-methoxyphenoxy)methyl]oxirane. The pharmacokinetics of the (+)*R* and (−)*S*-enantiomers of ranolazine are similar in healthy volunteers. After oral administration, peak plasma concentrations of ranolazine are reached in about 2–5 h. It is 62% bound to plasma proteins. Ranolazine is extensively metabolized by CYP3A4. Following a single oral dose, approximately 75% of the dose is excreted in urine and 25% in feces, primarily as metabolites. Less than 5% is excreted unchanged in the urine and stool. Absorption of ranolazine is variable. The half-life of ranolazine is approximately 7 h, and the average oral bioavailability is 55%. The recommended dosing regimen of ranolazine is 500 mg b.i.d orally and can be increased to 1000 mg b.i.d. as needed. The efficacy and safety of ranolazine has been evaluated in several large randomized, placebo-controlled trials involving patients with chronic angina who remained symptomatic despite treatment with another anti-anginal agent. One clinical trial with 565 patients compared ranolazine 1000 mg twice daily (b.i.d.) to placebo over 6 weeks. All patients were also on amlodipine and about half were on long-acting nitrates. In this trial, ranolazine significantly decreased the frequency of angina attacks (mean 3.3 attacks per week vs. 4.3 for placebo; $p = 0.028$) and need for intervention treatment with nitroglycerin (mean 2.7 doses per week vs. 3.6 for placebo; $p = 0.014$). Another study containing 823 patients compared ranolazine at 750 mg b.i.d and 1000 mg b.i.d. with placebo over 12 weeks. In this trial, ranolazine treatment gave a significant increase in modified Bruce treadmill exercise tolerance ($p < 0.05$) and time to angina onset ($p < 0.05$) at both peak (4 h post-dose) and trough (12 h post-dose) drug plasma concentrations. Both doses significantly reduced angina frequency (750 mg: 2.5 attacks/week and 1000 mg: 2.1 attacks/week; vs. 3.3 attacks/week for placebo; $p = 0.006$ and $p < 0.001$, respectively) and nitroglycerin intervention (750 mg: 2.1 doses/week and 1000 mg: 1.8 doses/week; vs. 3.1 doses/week for placebo; $p = 0.016$ and $p < 0.001$). Ranolazine was less effective in women than in men. Anginal attacks were reduced by 1.3 per week in men, but

only 0.3 per week in women. The most frequent adverse events associated with ranolazine treatment were dizziness, headache, constipation, and nausea. Ranolazine prolongs the QT interval and should not be used with other drugs that prolong the QT interval (e.g. fluoroquinolones, ziprasidone, sotalol, and dofetilide). This effect is dose-related; therefore 1000 mg b.i.d. is the maximum allowed dose. An electrocardiogram should be performed before ranolazine is started and periodically thereafter.

Rimonabant (Anti-obesity) [92–96]

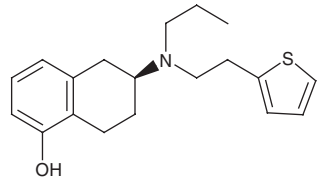
Country of origin: France
Originator: Sanofi-Synthelabo
First introduction: UK
Introduced by: Sanofi-Aventis
Trade name: Acomplia
CAS registry no: 168273-06-1
Molecular weight: 463.79



Rimonabant is a first-in-class drug launched last year as an oral treatment for obesity, and its mechanism of action involves the selective antagonism of cannabinoid type 1 (CB1) receptor. It is specifically indicated as an adjunct to diet and exercise for the treatment of obese patients (body mass index [BMI] $\geq 30\,kg/m^2$), or overweight patients (BMI $> 27\,kg/m^2$) with associated risk factors such as type 2 diabetes or dyslipidemia. Additionally, rimonabant is currently under development as a treatment for nicotine dependence. The CB1 and CB2 receptors, along with their endogenous ligands, constitute the endocannabinoid system. The CB1 receptor is expressed in the brain, adipose tissue, and several peripheral organs; the CB2 receptor is predominantly expressed in immune cells. Activation of the CB1 receptor in the CNS is associated with appetite stimulation and the modulation of brain reward mechanism, whereas activation in the periphery favors metabolic processes that lead to hepatic lipogenesis and impaired glucose homeostasis. Rimonabant acts by selectively blocking the action of central and peripheral CB1 receptors, thereby reducing food intake and improving lipid and glucose metabolism. It is a potent antagonist of the CB1 receptor, with high binding selectivity for CB1 over CB2 (CB1 $K_i = 2\,nM$; CB2 $K_i = > 1{,}000\,nM$). Rimonabant is chemically synthesized starting from Claisen condensation of 4′-chloropropiophenone with diethyl oxalate and subsequent cyclization with 2,4-dichlorophenylhydrazine to construct the pyrazole carboxylate ester intermediate, which is hydrolyzed to the corresponding acid and coupled with 1-aminopiperidine. The recommended dosage of oral rimonabant is 20 mg once daily before breakfast. Rimonabant therapy should be

undertaken in conjunction with a mildly hypocaloric diet. The pharmacokinetic parameters of rimonabant are relatively dose-proportional up to 20 mg, beyond which the increases in AUC values are less in proportion to dose. *In vitro*, rimonabant exhibits high human plasma protein binding (>99.9%). Following multiple, once-daily 20-mg doses in healthy subjects, maximum plasma concentrations (C_{max}) of rimonabant are achieved in approximately 2 h, and steady state plasma levels are achieved within 13 days. Peripheral volume of distribution is related to body weight, with obese patients having higher volume of distribution and taking longer to reach steady state. The elimination half-life is also longer in obese patients (16 days) than in non-obese patients (9 days). Rimonabant is predominantly eliminated through biliary excretion (~86%) as unchanged drug and metabolites. It is metabolized by CYP3A4 and amidohydrolase pathways, and the circulating metabolites do not contribute to its pharmacological activity. The efficacy of rimonabant has been demonstrated in multiple clinical trials, which included two studies in obese patients ($n = 1507$ and 3045), one study in obese patients with dyslipidemia ($n = 1036$), and one study in obese patients with type 2 diabetes ($n = 1047$). The mean BMI ranged from 34 to 38 kg/m^2 in the studies. All patients were required to adhere to a mildly hypocaloric diet wherein the normal diet was reduced by 600 kcal per day. In all four trials, the mean weight change from baseline to the end of one year was significantly greater in patients treated with rimonabant (5 mg or 20 mg) than with placebo ($p < 0.002$). The mean weight loss with 20 mg once-daily rimonabant ranged from 5.3 to 7.4 kg, compared with 1.4–1.9 kg with placebo. Additional endpoints that were significantly improved from baseline were waist circumference (mean net difference −3.3 to −4.7 cm), plasma HDL-cholesterol (+7 to +8%), and triglycerides (−12 to −16%). In overweight diabetic patients, fasting glucose concentrations and HbA1c also showed greater improvements than placebo by −0.97 mmol/L and −0.7%, respectively. Continuation of treatment with rimonabant 20 mg for a second year ($n = 333$) in one study showed maintenance of weight loss, but those patients who changed to placebo ($n = 327$) regained most of their previous loss. Rimonabant was generally well tolerated, with most adverse events considered mild to moderate in severity. The most common side effects associated with rimonabant treatment included nausea and upper respiratory tract infection (≥10% of patients in pooled clinical trials).

Rotigotine (Parkinson's disease) [97–105]



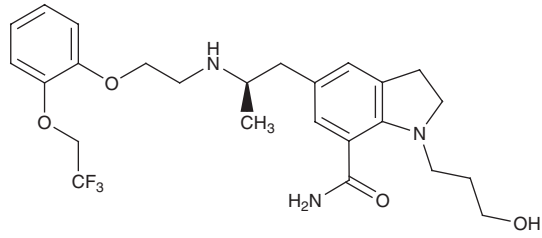
Country of origin:	US
Originator:	Aderis
First introduction:	Germany
Introduced by:	Schwarz Pharma
Trade name:	Neupro
CAS registry no:	99755-59-6
Molecular weight:	315.47

While levodopa is still considered the cornerstone of treatment of Parkinson's disease, many patients begin to experience treatment-related problems, such as a wearing-off phenomenon and the development of dyskinesias as the disease progresses. Continuous dopaminergic stimulation by means of a dopamine agonist has been recognized as being associated with a lower incidence of dyskinesias. Using a selective dopamine agonist as monotherapy in early disease may delay the onset of levodopa therapy, or at a minimum, lower its dose in adjunctive situations to minimize the adverse neurotoxic effects of levodopa. Rotigotine is a nonergolinic dopamine $D_3/D_2/D_1$ receptor agonist, and it is the first dopamine agonist to be launched as a transdermal patch. Transdermal delivery avoids potential interactions with food, bypasses hepatic first-pass metabolism before reaching the drug site, and improves patient compliance. Furthermore, by ensuring stable plasma levels with 24-h application, fluctuations in symptom control may be avoided. Unlike other dopamine agonists, such as cabergoline, pergolide, pramipexole, and ropinrole, rotigotine has considerable affinity for D_1, an attribute that results in the synergistic enhancement of the effect mediated by the D_2 receptor. While the propyl branch of the amine confers dopamine receptor affinity in general, the thiophene ring enhances D_2 selectivity and contributes to the lipophilicity essential for transdermal delivery. Using recombinant rat D_1, D_2, and D_3 receptors expressed in CHO cell, affinity constants of 364, 11, and 0.94 nM were obtained, respectively. Rotigotine is prepared starting from 5-methoxy-2-tetralone. Reductive amination with propylamine provides the 2-aminotetraline. The thienylethyl substituent may be installed by either direct alkylation with the corresponding halide or by acylation followed by reduction with lithium aluminum hydride. Demethylation with boron tribromide affords the racemic product. Since the *R*-enantiomer is significantly less potent, and possibly antagonistic, the enantiomers are separated by chiral chromatography (Chiralcel OD) to isolate rotigotine, the *S*-enantiomer. A silicone-based, adhesive-matrix is used for the formulation of rotigotine for transdermal delivery. Following application of a 10 cm^2 patch with a drug content of 4.5 mg, a median C_{max} of 0.215 ng/mL was reached in 16 h. The AUC(0 – tz) was 3.94 ng · h/mL, and after patch removal at 24 h, the median terminal half-life was 6.82 h. The mean steady-state plasma concentration increased dose-proportionally with titration from the 10 cm^2 to the 40 cm^2 patch. While plasma levels decreased slightly after application of a new patch with a lag phase of about 3 h, stable concentrations were maintained over a 24-h period. The metabolism of rotigotine is extensive with both the parent and metabolites (*N*-dealkylation) undergoing 5-*O*-glucuronidation and sulfation. Less than 0.1% of the absorbed dose is eliminated as the parent compound in the urine. After intravenous administration of radiolabeled rotigotine, 75% of the radioactivity was recovered in the urine and 25% in the feces. It was determined from *in vitro* studies that rotigotine metabolism is not prevented with any selective CYP isoform inhibitor. In addition, co-administration of various CYP substrates is not anticipated to elicit drug interactions. In a randomized, double-blind, placebo-controlled trial of 242 patients with untreated, early Parkinson's disease, the efficacy of rotigotine as

monotherapy was evaluated. Patients were randomized to receive placebo, 2.0, 4.0, 6.0, or 8.0 mg of transdermal rotigotine. Using the Unified Parkinson's Disease Rating Scale (UPDRS), there was a significant dose-related improvement in motor activities between baseline and week 11 for the 6.0 (–5.07) and 8.0 mg (–5.30) doses compared to placebo (–0.3). Rotigotine was also investigated as adjunctive treatment in patients with advanced disease that continued to receive a stable dosage of levodopa during the clinical trial. The primary efficacy end point was the change from baseline in off-time per day. The 40 cm^2 and 60 cm^2 patches produced a reduction in off-time of approximately 2.7 and 2.2 h, respectively, compared to 1 h for placebo. It was also noted that the decrease in off-time was associated with an increase in "on without troublesome dyskinesia" time. Rotigotine was well tolerated with the most common adverse events including application-site reactions, nausea, somnolence, and dizziness. While rotigotine is administered once daily for 24 h, no recommended dosage is available. For early Parkinson's disease, patch sizes of 10, 20, 30, and 40 cm^2 with drug content of 4.5, 9, 13.5, and 18 mg, respectively, were employed. The patch sizes for advanced disease were 20, 40, and 60 cm^2 (27 mg drug content). Lastly, rotigotine is also under evaluation for the treatment of restless leg syndrome.

Silodosin (Dysuria) [106–108]

Country of origin: Japan
Originator: Kissei
First introduction: Japan
Introduced by: Kissei/Daiichi
Trade name: Urief
CAS registry no: 160970-54-7
Molecular weight: 495.53

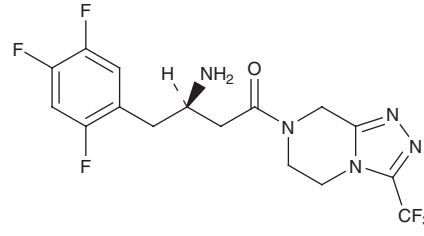


Silodosin, an α_{1A} adrenoceptor (α_{1A}-AR) antagonist selective for prostatic receptors, was launched last year as an oral treatment for dysuria associated with benign prostatic hypertrophy (BPH). The regulation of smooth muscle tone in the bladder neck and prostate is thought to be primarily mediated by α_{1A}-AR. Blockade of these receptors can cause smooth muscle relaxation in these areas, resulting in improved symptoms and urinary flow rates. Conversely, α_{1B}-AR are largely located on vascular smooth muscle, and antagonism of these receptors can cause tissue relaxation and potentially decrease cardiac compensation mechanisms involved in regulating blood pressure. Of the α_1-AR antagonists currently in clinical use for the treatment of BPH, alfuzosin, doxazosin, prazosin and terazosin are non-selective, and tamsulosin is modestly selective for α_{1A} and α_{1D} (7–38 fold) over α_{1B}-AR. Silodosin exhibits significantly improved selectivity for human α_{1A}-AR (α_{1A} $K_i = 0.036$ nM, α_{1B} $K_i = 21$ nM, α_{1D} $K_i = 2$ nM). In rat and dog models of BPH, comparative studies with tamsulosin and prazosin have shown that silodosin produces favorable uroselectivity, as determined by the ratio

between the dose required to inhibit intraurethral pressure and that to decrease blood pressure. The recommended dosing regimen for oral silodosin is 4 mg twice-daily. Following a single oral dose in healthy volunteers, the peak plasma concentrations (C_{max}) are reached within about 2 h, indicating rapid absorption of the drug. The absolute bioavailability of silodosin is about 32% and the elimination half-life is approximately 6 h. The *in vitro* human plasma protein binding of silodosin is 95.6%. Silodosin is a dual substrate for CYP3A4 and *p*-glycoprotein. Its metabolism occurs primarily via oxidation and glucuronidation mechanisms, and it is predominantly excreted via the feces. The clinical efficacy of silodosin was evaluated in 457 patients with BPH, wherein the subjects were randomized to treatment with silodosin 4 mg twice daily, tamsulosin 0.2 mg once daily, or placebo for a total of 12 weeks. The patient pool consisted of men aged ≥50 years with an International Prostate Symptom Score (IPSS) of ≥8 and a quality-of-life (QoL) score of ≥3. The primary endpoint was the change in IPSS from baseline. In the silodosin group, the IPSS fell by 8.3, compared to a drop of 6.8 in the tamsulosin group and 5.3 in the placebo group. In the subgroup of patients with severe symptoms (IPSS≥20), silodosin gave a significantly better improvement than placebo (–12.4 vs. –8.7). In addition, silodosin showed a significant improvement in the QoL score than placebo. The most common adverse event in the silodosin group was abnormal ejaculation, which occurred more often in the silodosin than in the tamsulosin group (22.3% vs. 1.6%). Silodosin is chemically synthesized starting from 1-acetyl-5-propionylindole in 15 synthetic steps. The key step in the sequence involves the preparation of a (2,2,2-trifluoroethoxy)phenoxy]-ethylamino]propylindoline intermediate in racemic form, which is optically resolved via its salt with (+)-mandelic acid.

Sitagliptin (Antidiabetic) [109–114]

Country of origin:	US
Originator:	Merck
First introduction:	Mexico
Introduced by:	Merck
Trade name:	Januvia
CAS registry no:	486460-32-6
Molecular weight:	407.31



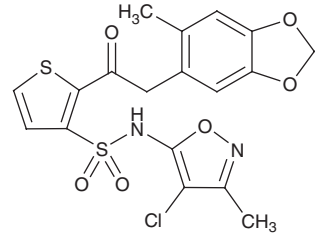
Sitagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, is a first-in-class oral drug launched last year for the treatment of type 2 diabetes. It acts by slowing the inactivation of incretins, which are endogenous peptides involved in the physiologic regulation of glucose homeostasis. Incretin hormones, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are released by the intestine throughout the day, and levels are increased in response to a meal. When blood glucose concentrations are normal or elevated,

GLP-1 and GIP increase the synthesis and release of insulin from pancreatic β cells via intracellular signaling pathways involving cAMP. GLP-1 also lowers glucagon secretion from pancreatic α cells, which leads to reduced hepatic glucose production. However, although GLP-1 and GIP effectively lower blood glucose, they are short-lived as a result of rapid inactivation by the ubiquitous serine protease DPP-4. By inhibiting DPP-4, sitagliptin increases the concentration and duration of active incretin levels, which in turn results in increased insulin release and decreased glucagon levels in a glucose-dependent manner. Sitagliptin is a potent, competitive, reversible inhibitor of DPP-4 ($IC_{50} = 18$ nM). The (*S*)-enantiomer of sitagliptin is considerably less potent than the (*R*)-enantiomer, exhibiting an IC_{50} of 440 nM. In addition, sitagliptin is highly selective for DPP-4 versus other proline specific proteases with DPP-4-like activity such as aminopeptidase P, prolidase, and QPP ($IC_{50} > 10$ μM), and the more closely related enzymes in the DPP-4 gene family such as fibroblast activation protein-α (FAPα, $IC_{50} > 100$ μM), DPP-8 ($IC_{50} = 48$ μM) and DPP-9 ($IC_{50} > 100$ μM). The recommended regimen of sitagliptin for all approved indications is 100 mg once daily with or without food. After oral administration of a single 100-mg dose, sitagliptin exhibits an absolute bioavailability of approximately 87%, with peak plasma concentrations occurring 1–4 h postdose. Sitagliptin has low protein binding (38%), a volume of distribution at steady state of approximately 198 L, and a mean half-life of 12.4 h. It is minimally metabolized by CYP3A4 and CYP2C8 (~16%), with 79% of the drug excreted unchanged in the urine. The recommended dose of sitagliptin is 100 mg once daily as monotherapy, or as combination therapy with metformin or a PPARγ agonist (e.g. thiazolidinediones). The efficacy of sitagliptin was assessed in four randomized, double-blind, placebo-controlled trials involving 2,316 patients with type 2 diabetes. In pooled data from these studies, sitagliptin reduced hemoglobin A_{1C} (HbA_{1C}), fasting plasma glucose, and 2-h post-prandial glucose compared to placebo. Monotherapy with sitagliptin 100 mg daily was associated with a –0.8% change in HbA_{1C}, a –17 mg/dL change in fasting plasma glucose, and a −47 mg/dL change in 2-h post-prandial glucose relative to placebo. In combination therapy with metformin, sitagliptin 100 mg daily was associated with a −0.7% change in HbA_{1C} relative to metformin alone. In combination therapy with pioglitazone, sitagliptin 100 mg daily was associated with a −0.7% change in HbA_{1C} relative to pioglitazone alone. Treatment was not linked to weight gain or loss from baseline, and the incidence of hypoglycemia was similar to that of placebo (1.2% vs. 0.9%). The most frequent adverse events associated with sitagliptin treatment included stuffy or runny nose and sore throat, upper respiratory tract infection, and headache. Gastrointestinal effects were also noted, including abdominal pain, nausea, and diarrhea. The chemical synthesis of sitagliptin involves a peptide-coupling reaction of a β-amino acid intermediate, 3(*R*)-(benzyloxyamino)-4-(2,4,5-trifluorophenyl)butyric acid, with a heterocyclic amine intermediate, 3-trifluoromethyl-5,6,7,8-tetrahydro-[1,2,4]-triazolo[4,3-a]pyrazine, and subsequent cleavage of the benzyloxy group by hydrogenolysis. The β-amino acid intermediate is derived in five synthetic steps starting from 3-oxo-4-(2,4,5-trifluorophenyl)butyric acid methyl ester, by first reducing it enantioselectively to

3(*S*)-hydroxy-4-(2,4,5-trifluorophenyl)butyric acid methyl ester using hydrogen over (*S*)-BINAP-$RuCl_2$ catalyst, followed by sequential hydrolysis to the corresponding acid, amidation with *O*-benzylhydroxylamine, cyclization to an azetidinone intermediate, and cleavage of the azetidinone with lithium hydroxide. The heterocyclic amine component is made in three steps from 2-hydrazinopyrazine by acylation with trifluoroacetic anhydride, followed by polyphosphoric acid mediated cyclization to the triazolopyrazine system, and subsequent hydrogenation.

Sitaxsentan (Pulmonary hypertension) [115–119]

Country of origin: US
Originator: Encysive
First introduction: UK
Introduced by: Encysive
Trade name: Thelin
CAS registry no: 184036-34-8
Molecular weight: 454.90

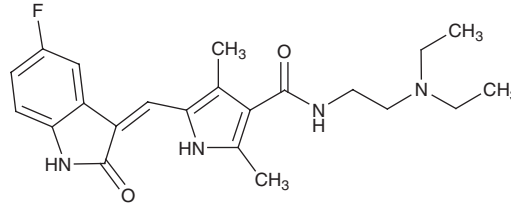


Sitaxsentan is a selective endothelin-A (ET_A) receptor antagonist launched last year for the treatment of patients with pulmonary arterial hypertension (PAH), to improve exercise capacity. It is the second ET receptor antagonist to be marketed for this indication behind bosentan. PAH encompasses a heterogeneous group of disorders characterized by inappropriate overactivation of the endothelin system and is characterized by a progressive increase in pulmonary vascular resistance resulting from vascular remodeling, vasoconstriction, and cellular proliferation. Endothelin-1 (ET-1), a potent vasoconstrictor and smooth muscle mitogen, is a key contributor to the acceleration of the disease, and its effects are mediated through activation of ET_A and ET_B receptors. ET_A receptors are found primarily on smooth muscle cells and, when activated, induce vasoconstriction and cellular proliferation. ET_B receptors are expressed in both pulmonary vascular endothelial cells and smooth muscle cells, and their activation is associated with the renal and pulmonary clearance of ET. Sitaxsentan binds to human ET_A receptors with high potency ($K_i = 0.43$ nM) and high selectivity (ET_A $IC_{50} = 1.4$ nM, ET_B $IC_{50} = 9800$ nM). By comparison, bosentan is significantly less selective (ET_A $IC_{50} = 4.7$ nM, ET_B $IC_{50} = 95$ nM). Although both sitaxsentan and bosentan are efficacious in the treatment of PAH, it is hypothesized that the ET_A receptor selectivity of sitaxsentan may confer a greater clinical benefit by inhibiting the deleterious ET_A-mediated vasoconstriction while preserving the beneficial vasodilator and clearance functions of ET_B receptors. Sitaxsentan is chemically derived starting from condensation of 3-(chlorosulfonyl)-thiophene-2-carboxylic acid methyl ester with 4-chloro-3-methylisoxazol-5-amine. The resulting sulfonamide intermediate is subjected to a sequence of ester hydrolysis, amidation of the carboxylic acid group with *N*,*O*-dimethylhydroxylamine, and Grignard

reaction with 6-methyl-1,3-benzodioxol-5-ylmethyl magnesium chloride. Sitaxsentan is rapidly absorbed after oral administration, reaching maximum plasma concentrations in PAH patients within 1–4 h. The oral bioavailability of sitaxsentan is high (70–100%), and is unaffected by food. The terminal elimination half-life is 10 h. Steady-state drug levels are reached within about 6 days. Sitaxsentan is >99% bound to plasma proteins and does not appear in erythrocytes or cerebrospinal fluid. It is extensively metabolized by CYP2C9 and CYP3A4 enzymes. The most common metabolites are >20-fold less potent as ET_A receptor antagonists than sitaxsentan in *in vitro* assays. Approximately 50–60% of an oral dose of sitaxsentan is excreted in the urine, with the remainder being eliminated in the feces; <1% of the dose is excreted as unchanged drug. The clinical efficacy of sitaxsentan has been evaluated in four separate studies. An uncontrolled open-label study and a randomized placebo-controlled study showed sitaxsentan to improve exercise tolerance in patients with PAH, as evidenced by significant increases in the distance walked in 6 min. However, significant hepatotoxicity developed in patients receiving sitaxsentan at 300 mg.The benefits of sitaxsentan with respect to exercise tolerance and hemodynamics were sustained in a one-year extension of the placebo-controlled study. The results of a multicenter, randomized, placebo-controlled trial of 50 mg once-daily and 100 mg once-daily doses of sitaxsentan with an open-label bosentan arm suggested that only the 100 mg dose provided superior benefit in exercise tolerance and improvement in WHO functional class. Treatment-related adverse effects included headache, insomnia, peripheral edema, nausea, nasal congestion and dizziness.

Sunitinib (Anticancer) [120–123]

Country of origin:	US
Originator:	Sugen
First introduction:	US
Introduced by:	Pfizer
Trade name:	Sutent
CAS registry no:	557795-19-4
Molecular weight:	398.47



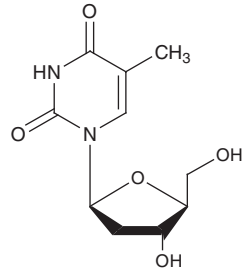
Sunitinib is an inhibitor of multiple receptor tyrosine kinases (RTKs) involved in tumor proliferation and angiogenesis, including platelet-derived growth factor receptors (PDGFR), vascular endothelial growth factor receptors (VEGFR), and stem cell factor receptor (KIT). It was launched last year as an oral treatment for gastrointestinal stromal tumors (GIST) and advanced renal-cell carcinoma (RCC). *In vitro*, sunitib inhibits VEGFR2, PDGFRα, PDGFRβ, KIT, and FLT3 receptors with IC_{50} values in the 4–14 nM range, and the ligand-dependent autophosphorylation of VEGFR2 and PDGFRβ with IC_{50}s of approximately 10 nM. In addition, it inhibits the growth of tumor cells expressing dysregulated target

RTKs *in vitro* and inhibits PDGFRβ- and VEGFR2-dependent tumor angiogenesis *in vivo*. Sunitinib exhibits broad and potent antitumor activity, causing regression in murine models of human epidermal (A431), colon (Colo205 and HT-29), lung (NCI-H226 and H460), breast (MDA-MB-435), prostate (PC3-3M-luc), and renal (786-O) cancers, and suppressing or delaying the growth of many others, including the C6 rat and SF763 T human glioma xenografts and B16 melanoma lung cancer. Sunitinib produces a primary active metabolite *in vivo* via CYP3A4-mediated *N*-mono-deethylation (23–37% of the total exposure). The primary metabolite exhibits similar *in vitro* potency compared to sunitinib in RTK inhibition assays and in cell proliferation assays. The pharmacokinetic parameters of sunitinib follow a dose-proportional course in the dosing range of 25–100 mg. Following oral administration, the peak plasma concentrations of sunitinib are generally achieved between 6 and 12 h. Food has no effect on the bioavailability of sunitinib. The apparent volume of distribution (Vd/F) is 2230 L. Sunitinib and its primary metabolite exhibit *in vitro* protein binding values of 95% and 90%, respectively. Elimination of sunitinib is predominantly via feces (61%) and urine (16%), with a total oral clearance of 34–62 L/h. Sunitinib and its primary active metabolite account for 75–90% of the drug-related compounds identified in plasma, urine, and feces. Minor metabolites are found in urine and feces but generally not found in plasma. Following administration of a single oral dose in healthy volunteers, the terminal half-lives of sunitinib and its primary metabolite are approximately 40–60 h and 80–110 h, respectively. With repeated daily administration, sunitinib accumulates 3- to 4-fold while the primary metabolite accumulates 7- to 10-fold. Steady-state concentrations of sunitinib and its primary metabolite are achieved within 10–14 days. The recommended dosing regimen of sunitinib is 50 mg orally once daily for 4 consecutive weeks, followed by 2 weeks off. The efficacy of oral sunitinib in GIST and RCC patients has been demonstrated in multiple clinical studies. In a phase III, randomised, double-blind, placebo-controlled, multicenter trial in patients with metastatic and/or unresectable GIST following unsuccessful imatinib therapy, the median time to tumor progression and median progression-free survival time were $\geq$4-fold longer in patients receiving sunitinib 50 mg/day in 6-week cycles consisting of 4 weeks of treatment followed by a 2-week rest period than in those receiving placebo. Likewise, In two multicenter, single-arm, phase II clinical trials in patients with cytokine-refractory metastatic RCC, partial responses were reported in 40% and 43% of patients receiving sunitinib 50 mg/day for 4 weeks followed by 2 weeks without treatment in 6-week cycles; 27% and 22% of patients achieved stable disease for $\geq$3 months. Sunitinib was more effective than interferon-α as a first-line therapy in patients with metastatic RCC. In a large phase III trial in previously untreated patients, progression-free survival was significantly longer in patients receiving sunitinib 50 mg/day in 6-week cycles (4 weeks of treatment followed by a 2-week rest period) compared with those receiving interferon-α 9MU three times weekly (47.3 vs. 24.9 weeks). In general, sunitinib was well tolerated in patients with GIST and RCC, with adverse events usually being of mild or moderate severity. The most commonly reported adverse reactions were GI-related and included diarrhea, nausea, mucositis/stomatitis,

dyspepsia, and vomiting. Other common adverse reactions were fatigue, swelling, taste disturbance, and skin discoloration. Sunitinib is chemically synthesized starting from 3,5-dimethyl-1*H*-pyrrole-2,4-dicarboxylic acid 2-tert-butyl ester 4-ethyl ester via acid catalyzed decarboxylation of the 2-tert-butyl ester, followed by Vilsmeyer formylation and hydrolysis of the ethyl ester to give 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid. Amidation of the carboxylic acid intermediate with 2-(diethylamino)ethylamine, and subsequent condensation with 5-fluoro-1,3-dihydro-indol-2-one produces sunitinib.

Telbivudine (Hepatitis B) [124–128]

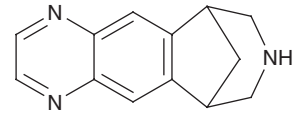
Country of origin:	US
Originator:	Idenix
First introduction:	Switzerland
Introduced by:	Idenix/Novartis
Trade name:	Sebivo
CAS registry no:	003424-98-4
Molecular weight:	242.23



Telbivudine is a β-L-thymidine nucleoside analog launched last year for the once-daily oral treatment of chronic hepatitis B virus (HBV) infection. It is the fourth nucleoside or nucleotide analog to be marketed for this indication. The previous drugs from this class include lamivudine, a deoxythiacytosine analog, adefovir, a nucleotide analog, and entecavir, a guanosine analog. Adefovir, entecavir, and telbivudine are specifically indicated for HBV, whereas lamivudine is indicated for both HBV and HIV infections. Telbivudine is efficiently phosphorylated by cellular kinases to the active triphosphate derivative, which inhibits HBV DNA polymerase by competing with the natural substrate, thymidine-5′-triphosphate. Incorporation of telbivudine-5′-triphosphate into viral DNA results in DNA chain termination, leading to inhibition of HBV replication. Telbivudine triphosphate has an intra-cellular half-life of 14 h. It is an inhibitor of both HBV first- and second-strand synthesis ($EC_{50} = 1.3 \pm 1.6\,\mu M$ and $0.2 \pm 0.2\,\mu M$, respectively), but does not inhibit human DNA polymerases α, β, or γ at concentrations up to 100 μM. In addition, no appreciable mitochondrial toxicity is observed in HepG2 cells treated with telbivudine at concentrations up to 10 μM. In HBV-expressing human hepatoma cell line 2.2.15, telbivudine inhibits HBV DNA synthesis with an EC_{50} of 0.2 μM. Telbivudine is not active against HIV type 1 ($EC_{50} = > 100\,\mu M$). The recommended dosage of oral telbivudine is 600 mg once daily. Following oral administration in healthy subjects, peak plasma concentrations of telbivudine are achieved in 1–4 h. Steady-state levels are achieved after approximately 5–7 days of once-daily administration with about 1.5-fold accumulation, suggesting an effective half-life of ~15 h. The *in vitro*

binding of telbivudine to human plasma proteins is low (3.3%). Telbivudine is not a substrate or inhibitor of the CYP450 isozymes, and it is minimally metabolized. Renal excretion in a bi-exponential manner is the predominant route of elimination, with approximately 42% of the dose unchanged. The elimination half-life of telbivudine was between 40 and 49 h. The clinical efficacy of telbivudine was evaluated in 1367 HBV patients in a phase III study, which included a lamivudine arm. The primary efficacy endpoint was therapeutic response at one year, a composite endpoint coupling viral suppression (serum HBV DNA suppression $<$100,000 copies/mL) with either improved liver disease markers (ALT normalization) or loss of detectable hepatitis B e-antigen (HBeAg). In HBeAg-positive patients, therapeutic response was 75% among patients treated with telbivudine and 67% for those patients treated with lamivudine, while the response for HBeAg-negative patients after one year was 75% vs. 77%, respectively. In addition, patients who achieved nondetectable HBV DNA levels at 24 weeks were more likely to undergo e-antigen seroconversion, achieve undetectable levels of HBV DNA, normalize ALT, and minimize resistance at one year. The most common adverse events reported in association with telbivudine treatment include fatigue and malaise, abdominal pain, headache, cough, nausea and vomiting, influenza-like symptoms, and diarrhea. Increases in creatine kinase levels also have been reported. Periodic monitoring of hepatic function during treatment is recommended. Telbivudine is chemically synthesized from the 3,5-bis-*p*-toluoyl derivative of 2-deoxy-L-ribofuranose, by first converting it into an α-L-ribofuranosyl chloride intermediate, which is subsequently condensed with thymine. Several methods have been reported for the preparation of the bis-*p*-toluoyl-2-deoxy-L-ribofuranose intermediate. The general strategy involves the stereoselective synthesis of 2-deoxy-L-ribono-1,4-lactone, followed by acylation of the hydroxyl groups with *p*-toluoyl chloride, and subsequent selective reduction of the lactone with diisobutylaluminum hydride.

Varenicline (Nicotine-dependence) [129–133]



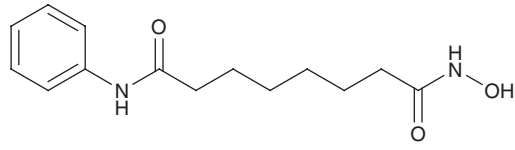
Country of origin:	US
Originator:	Pfizer
First introduction:	US
Introduced by:	Pfizer
Trade name:	Chantix
CAS registry no:	249296-44-4
Molecular weight:	211.26

Varenicline, a partial agonist of the α4β2 nicotinic receptor, is a first-in-class drug launched last year by Pfizer as an aid to smoking cessation treatment. Varenicline exhibits dual action by decreasing craving and withdrawal symptoms, and by decreasing the reinforcement associated with smoking. The addictive properties of nicotine are thought to be mediated in part through its action

as an agonist at α4β2 neuronal nicotinic acetylcholine receptors (nAChRs). Activation of α4β2 receptors by nicotine increases the release of dopamine in the mesolimbic system, an effect that is shared by most drugs of abuse. As nicotine levels decrease, dopamine levels decline, which in turn stimulates the urge to smoke. Additionally, a reduced dopaminergic tone due to abstinence from smoking stimulates craving and the withdrawal syndrome. A partial agonist of α4β2 receptors such as varenicline is expected to elicit a moderate and sustained increase in dopamine levels to relieve craving and withdrawal symptoms. In addition, by competitively binding to α4β2 receptors and inhibiting nicotine-induced dopaminergic activation, a partial agonist could attenuate the pharmacologic reward associated with smoking. Varenicline was discovered through the synthesis of a series of compounds inspired by the plant alkaloid natural product (–)-cytisine, which was previously known to have partial agonist activity at the α4β2 nAChR. Varenicline is synthesized starting from benzonorbornadiene in nine steps. Benzonorbornadiene is first converted to a bridged benzazepine intermediate in four sequential steps involving dihydroxylation with osmium tetroxide, periodate oxidation of the diol, reductive amination of the resultant dialdehyde with benzylamine, and removal of the benzyl group by hydrogenolysis. The bridged benzazepine is converted to varenicline by a five-step sequence involving *N*-trifluroacetylation, dinitration to an *o*-dinitro intermediate, reduction of nitro groups to amino groups, cyclization with glyoxal to build the pyrazine ring, and removal of the trifluoroacetyl protecting group by hydrolysis. Varenicline is commercially supplied for oral administration as its tartrate salt. Varenicline displays high affinity for human α4β2 receptors expressed in HEK-293 cells and from rat cortex ($K_i = 0.11$ and 0.06 nM, respectively). It binds more potently to α4β2 receptors than to other common nicotinic receptors (>500-fold vs. α3β4, >3500-fold vs. α7, >20000-fold vs. α1βγδ), or to non-nicotinic receptors and transporters (>20000-fold). In functional electrophysiological assays in *Xenopus* oocytes expressing human α4β2 nAChR, varenicline inhibits nicotine-induced current with an EC_{50} of 2.3 μM, and a maximal efficacy of 24% relative to nicotine. The recommended dose for varenicline is 1 mg twice daily following a one-week titration period. The titration schedule consists of 0.5 mg once daily for days 1–3 and 0.5 mg twice daily for days 4–7. Varenicline displays nearly 100% absorption after oral administration, and the bioavailability is not affected by food or time of administration. It exhibits linear pharmacokinetics over the recommended dosing range. Peak plasma concentrations occur typically within 3–4 hours after administration, and after multiple doses, a steady-state concentration is reached within 4 days. Varenicline has low plasma protein binding ($\leq 20\%$) regardless of patient's age and renal function. Renal elimination is the primary mechanism of varenicline clearance with 92% percent excreted unchanged in the urine. The elimination half-life is approximately 24 h. The efficacy of varenicline has been assessed in six different clinical trials involving chronic cigarette smokers (≥ 10 cigarettes per day). In all these trials, abstinence from smoking was determined by patient self-report and verified by measurement of exhaled carbon monoxide ($CO \leq 10$ ppm) at weekly visits. In two double-blind studies ($n = 2045$), more patients were able to achieve smoking cessation

after 12 weeks of treatment with varenicline (44–51%) than with bupropion SR (30%), or with placebo (12–18%). The percent of patients with sustained abstinence 1 year after treatment initiation was also higher in the varenicline group (19–23%) than bupropion SR (14–16%), or placebo (4–10%) groups. In another clinical study ($n = 1927$), patients were treated with open-label varenicline for 12 weeks, and patients who had stopped smoking by week 12 were then randomized to double-blind treatment with either varenicline or placebo for an additional 12 weeks and then followed for 28 weeks post-treatment. The abstinence rate from week 13 through week 24 was 70% for patients continuing treatment with varenicline compared with 50% for patients switching to placebo. Superiority of varenicline over placebo was maintained during 28 weeks post-treatment follow-up, with 54% continuous abstinence rate in the varenicline group compared with 39% in the placebo group. The most common adverse effects of varenicline were nausea, headache, vomiting, flatulence, insomnia, abnormal dreams, and dysgeusia.

Vorinostat	(Anticancer)	[134–137]
Country of origin:	US	
Originator:	Columbia University	
First introduction:	US	
Introduced by:	Merck	
Trade name:	Zolinza	
CAS registry no:	149647-78-9	
Molecular weight:	264.32	



Vorinostat is the first drug in a new class of anti-cancer agents that inhibit histone deacetylases (HDAC). It was launched last year as an oral treatment for cutaneous manifestations in patients with cutaneous T-cell lymphoma (CTCL) who have progressive, persistent, or recurrent disease on or following two systemic therapies. HDACs are enzymes that catalyze the removal of the acetyl modification on lysine residues of proteins, including the core nucleosomal histones. Together with their counterpart histone acetyltransferases (HATs), HDACs regulate the acetylation level of the histones, which plays an important role in the regulation of chromatin plasticity and gene transcription. Hypo-acetylation of histones is associated with a condensed chromatin structure resulting in the repression of gene transcription, whereas acetylated histones are associated with a more open chromatin structure and activation of transcription. In some cancer cells, there is an overexpression of HDACs, resulting in hypo-acetylation of histones. Inhibitors of HDAC are thought to transcriptionally reactivate dormant tumor-suppressor genes by allowing for the accumulation of

acetyl groups on histones and an open chromatin structure. Vorinostat inhibits the enzymatic activity of HDAC1, HDAC2, HDAC3, and HDAC6 at nanomolar concentrations (IC_{50} <86 nM). *In vitro*, it induces growth arrest, differentiation or apoptosis in a variety of tumor cells. In addition, vorinostat inhibits tumor growth in animal models bearing solid tumors, including breast, prostate, lung and gastric cancers, as well as hematologic malignancies such as multiple myeloma and leukemias. The chemical synthesis of vorinostat has been accomplished by several methods. An efficient two-step synthesis involves the condensation of suberic acid monomethyl ester with aniline to produce methyl suberanilate, which is subsequently reacted with hydroxylamine to produce vorinostat. The recommended dose of vorinostat is 400 mg orally once daily with food. Vorinostat has an oral bioavailability of 48–56% in patients with relapsed or refractory solid tumors, lymphomas and leukemias. At steady state in a fed-state, the peak concentration following multiple 400-mg doses of oral vorinostat is 1.2 ± 0.53 μM, and the median time to peak is about 4 h. High-fat meal results in a 33% increase in the extent of absorption and a 2.5-hour delay in the rate of absorption compared to the fasted state. Vorinostat is approximately 71% bound to human plasma proteins. It is extensively metabolized via glucuronidation, and via hydrolysis and subsequent β-oxidation to provide the *O*-glucuronide and 4-anilino-4-oxobutanoic acid, respectively. Both metabolites are pharmacologically inactive. Approximately 40–60% of the oral dose is eliminated in the urine, mostly as metabolites, with less than 1% as unchanged vorinostat. The mean terminal half-life of vorinostat is approximately 2 h. The clinical efficacy of vorinostat in the treatment of CTCL has been assessed in two open-label studies ($n = 107$). In the first study, patients with advanced CTCL that was progressive, persistent, or recurrent on or following two systemic therapies were treated with 400 mg once-daily vorinostat. The primary end point was response rate as determined by Severity Weighted Assessment Tool (SWAT) measuring the percentage of total body surface area involvement. Response was defined as ≥50% decrease in the SWAT score, and disease progression as ≥50% increase in the score from the nadir. In this study, 30% experienced responses. The estimated median response duration was 168 days, and the median time to tumor progression was 202 days. In a second study in patients with CTCL who were refractory or intolerant to at least one treatment, a comparison of 400 mg vorinostat once daily, 300 mg twice daily 3 days per week, and 300 mg twice daily for 14 days followed by a 7-day rest indicated that 300 mg twice-daily regimen had higher toxicity with no additional clinical benefit over the 400 mg once-daily regimen. The most common adverse events associated with vorinostat therapy include diarrhea, fatigue, nausea, thrombocytopenia, anorexia, and dysgeusia.

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[1] The collection of new therapeutic entities first launched in 2006 originated from the following sources: (a) Integrity®, Prous Science database; (b) Iddb, Current Drugs database; (c) IMS R&D Focus; (d) Adis Business Intelligence R&D Insight; (e) Pharmaprojects.

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GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
abacavir sulfate	antiviral	1999	35, 333
abarelix	anticancer	2004	40, 446
abatacept	rheumatoid arthritis	2006	42, 509
acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
acemannan	wound healing agent	2001	37, 259
acetohydroxamic acid	hypoammonuric	1983	19, 313
acetorphan	antidiarrheal	1993	29, 332
acipimox	hypolipidemic	1985	21, 323
acitretin	antipsoriatic	1989	25, 309
acrivastine	antihistamine	1988	24, 295
actarit	antirheumatic	1994	30, 296
adalimumab	rheumatoid arthritis	2003	39, 267
adamantanium bromide	antiseptic	1984	20, 315
adefovir dipivoxil	antiviral	2002	38, 348
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
agalsidase alfa	fabry's disease	2001	37, 259
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
alefacept	plaque psoriasis	2003	39, 267
alemtuzumab	anticancer	2001	37, 260
alendronate sodium	osteoporosis	1993	29, 332
alfentanil HCl	analgesic	1983	19, 314
alfuzosin HCl	antihypertensive	1988	24, 296
alglucerase	enzyme	1991	27, 321
alglucosidase alfa	Pompe disease	2006	42, 511
alitretinoin	anticancer	1999	35, 333
alminoprofen	analgesic	1983	19, 314
almotriptan	antimigraine	2000	36, 295
anakinra	antiarthritic	2001	37, 261
anidulafungin	antifungal	2006	42, 512
alosetron hydrochloride	irritable bowel syndrome	2000	36, 295
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
alpidem	anxiolytic	1991	27, 322
alpiropride	antimigraine	1988	24, 296
alteplase	thrombolytic	1987	23, 326
amfenac sodium	antiinflammatory	1986	22, 315
amifostine	cytoprotective	1995	31, 338
aminoprofen	topical antiinflammatory	1990	26, 298

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
amisulpride	antipsychotic	1986	22, 316
amlexanox	antiasthmatic	1987	23, 327
amlodipine besylate	antihypertensive	1990	26, 298
amorolfine HCl	topical antifungal	1991	27, 322
amosulalol	antihypertensive	1988	24, 297
ampiroxicam	antiinflammatory	1994	30, 296
amprenavir	antiviral	1999	35, 334
amrinone	cardiotonic	1983	19, 314
amrubicin HCl	antineoplastic	2002	38, 349
amsacrine	antineoplastic	1987	23, 327
amtolmetin guacil	antiinflammatory	1993	29, 332
anagrelide HCl	hematological	1997	33, 328
anastrozole	antineoplastic	1995	31, 338
angiotensin II	anticancer adjuvant	1994	30, 296
aniracetam	cognition enhancer	1993	29, 333
anti-digoxin polyclonal antibody	antidote	2002	38, 350
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
aprepitant	antiemetic	2003	39, 268
APSAC	thrombolytic	1987	23, 326
aranidipine	antihypertensive	1996	32, 306
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arglabin	anticancer	1999	35, 335
aripiprazole	neuroleptic	2002	38, 350
arotinolol HCl	antihypertensive	1986	22, 316
arteether	antimalarial	2000	36, 296
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atazanavir	antiviral	2003	39, 269
atomoxetine	attention deficit hyperactivity disorder	2003	39, 270
atorvastatin calcium	dyslipidemia	1997	33, 328
atosiban	preterm labor	2000	36, 297
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 143
azacitidine	anticancer	2004	40, 447
azelnidipine	antihypertensive	2003	39, 270
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
balofloxacin	antibacterial	2002	38, 351
balsalazide disodium	ulcerative colitis	1997	33, 329
bambuterol	bronchodilator	1990	26, 299

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
barnidipine HCl	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
belotecan	anticancer	2004	40, 449
benazepril HCl	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
benidipine HCl	antihypertensive	1991	27, 322
beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betamethasone butyrate prospinate	topical antiinflammatory	1994	30, 297
betaxolol HCl	antihypertensive	1983	19, 315
betotastine besilate	antiallergic	2000	36, 297
bevacizumab	anticancer	2004	40, 450
bevantolol HCl	antihypertensive	1987	23, 328
bexarotene	anticancer	2000	36, 298
biapenem	antibacterial	2002	38, 351
bicalutamide	antineoplastic	1995	31, 338
bifemelane HCl	nootropic	1987	23, 329
bimatoprost	antiglaucoma	2001	37, 261
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene HCl	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bivalirudin	antithrombotic	2000	36, 298
bopindolol	antihypertensive	1985	21, 324
bortezomib	anticancer	2003	39, 271
bosentan	antihypertensive	2001	37, 262
brimonidine	antiglaucoma	1996	32, 306
brinzolamide	antiglaucoma	1998	34, 318
brodimoprin	antibiotic	1993	29, 333
bromfenac sodium	NSAID	1997	33, 329
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budipine	antiParkinsonian	1997	33, 330
budralazine	antihypertensive	1983	19, 315
bulaquine	antimalarial	2000	36, 299
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butenafine HCl	topical antifungal	1992	28, 327
butibufen	antiinflammatory	1992	28, 327
butoconazole	topical antifungal	1986	22, 318
butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cabergoline	antiprolactin	1993	29, 334
cadexomer iodine	wound healing agent	1983	19, 316

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323
camostat mesylate	antineoplastic	1985	21, 325
candesartan cilexetil	antihypertension	1997	33, 330
capecitabine	antineoplastic	1998	34, 319
captopril	antihypertensive agent	1982	13, 086
carboplatin	antibiotic	1986	22, 318
carperitide	congestive heart failure	1995	31, 339
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
caspofungin acetate	antifungal	2001	37, 263
cefbuperazone sodium	antibiotic	1985	21, 325
cefcapene pivoxil	antibiotic	1997	33, 330
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil HCl	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefoselis	antibiotic	1998	34, 319
cefotetan disodium	antibiotic	1984	20, 317
cefotiam hexetil HCl	antibiotic	1991	27, 324
cefozopran HCl	injectable cephalosporin	1995	31, 339
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpirome sulfate	antibiotic	1992	28, 328
cefpodoxime proxetil	antibiotic	1989	25, 310
cefprozil	antibiotic	1992	28, 328
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
ceftibuten	antibiotic	1992	28, 329
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celecoxib	antiarthritic	1999	35, 335
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cerivastatin	dyslipidemia	1997	33, 331
cetirizine HCl	antihistamine	1987	23, 331
cetrorelix	female infertility	1999	35, 336
cetuximab	anticancer	2003	39, 272
cevimeline hydrochloride	anti-xerostomia	2000	36, 299
chenodiol	anticholelithogenic	1983	19, 317
CHF-1301	antiparkinsonian	1999	35, 336
choline alfoscerate	nootropic	1990	26, 300

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cibenzoline	antiarrhythmic	1985	21, 325
ciclesonide	asthma, COPD	2005	41, 443
cicletanine	antihypertensive	1988	24, 299
cidofovir	antiviral	1996	32, 306
cilazapril	antihypertensive	1990	26, 301
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cinacalcet	hyperparathyroidism	2004	40, 451
cinildipine	antihypertensive	1995	31, 339
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
cisatracurium besilate	muscle relaxant	1995	31, 340
citalopram	antidepressant	1989	25, 311
cladribine	antineoplastic	1993	29, 335
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
clofarabine	anticancer	2005	41, 444
clopidogrel hydrogensulfate	antithrombotic	1998	34, 320
cloricromen	antithrombotic	1991	27, 325
clospipramine HCl	neuroleptic	1991	27, 325
colesevelam hydrochloride	hypolipidemic	2000	36, 300
colestimide	hypolipidaemic	1999	35, 337
colforsin daropate HCl	cardiotonic	1999	35, 337
conivaptan	hyponatremia	2006	42, 514
crotelidae polyvalent immune fab	antidote	2001	37, 263
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
dalfopristin	antibiotic	1999	35, 338
dapiprazole HCl	antiglaucoma	1987	23, 332
daptomycin	antibiotic	2003	39, 272
darifenacin	urinary incontinence	2005	41, 445
darunavir	HIV	2006	42, 515
dasatinib	anticancer	2006	42, 517
decitabine	myelodysplastic syndromes	2006	42, 519
defeiprone	iron chelator	1995	31, 340
deferasirox	chronic iron overload	2005	41, 446
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
delavirdine mesylate	antiviral	1997	33, 331
denileukin diftitox	anticancer	1999	35, 338
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
desflurane	anesthetic	1992	28, 329
desloratadine	antihistamine	2001	37, 264
dexfenfluramine	antiobesity	1997	33, 332
dexibuprofen	antiinflammatory	1994	30, 298
dexmedetomidine hydrochloride	sedative	2000	36, 301
dexmethylphenidate HCl	psychostimulant	2002	38, 352
dexrazoxane	cardioprotective	1992	28, 330
dezocine	analgesic	1991	27, 326
diacerein	antirheumatic	1985	21, 326
didanosine	antiviral	1991	27, 326
dilevalol	antihypertensive	1989	25, 311
dirithromycin	antibiotic	1993	29, 336
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317
docarpamine	cardiostimulant	1994	30, 298
docetaxel	antineoplastic	1995	31, 341
dofetilide	antiarrhythmic	2000	36, 301
dolasetron mesylate	antiemetic	1998	34, 321
donepezil HCl	anti-Alzheimer	1997	33, 332
dopexamine	cardiostimulant	1989	25, 312
doripenem	antibiotic	2005	41, 448
dornase alfa	cystic fibrosis	1994	30, 298
dorzolamide HCL	antiglaucoma	1995	31, 341
dosmalfate	antiulcer	2000	36, 302
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxercalciferol	vitamin D prohormone	1999	35, 339
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
drospirenone	contraceptive	2000	36, 302
drotrecogin alfa	antisepsis	2001	37, 265
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
duloxetine	antidepressant	2004	40, 452
dutasteride	5a reductase inhibitor	2002	38, 353
duteplase	anticougulant	1995	31, 342
eberconazole	antifungal	2005	41, 449
ebastine	antihistamine	1990	26 302
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
edaravone	neuroprotective	2001	37, 265
efalizumab	psoriasis	2003	39, 274
efavirenz	antiviral	1998	34, 321
efonidipine	antihypertensive	1994	30, 299
egualen sodium	antiulcer	2000	36, 303
eletriptan	antimigraine	2001	37, 266

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
emtricitabine	antiviral	2003	39, 274
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enfuvirtide	antiviral	2003	39, 275
enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
entacapone	antiparkinsonian	1998	34, 322
entecavir	antiviral	2005	41, 450
epalrestat	antidiabetic	1992	28, 330
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epinastine	antiallergic	1994	30, 299
epirubicin HCl	antineoplastic	1984	20, 318
eplerenone	antihypertensive	2003	39, 276
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eprosartan	antihypertensive	1997	33, 333
eptazocine HBr	analgesic	1987	23, 334
eptilfibatide	antithrombotic	1999	35, 340
erdosteine	expectorant	1995	31, 342
erlotinib	anticancer	2004	40, 454
ertapenem sodium	antibacterial	2002	38, 353
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
escitalopram oxolate	antidepressant	2002	38, 354
esmolol HCl	antiarrhythmic	1987	23, 334
esomeprazole magnesium	gastric antisecretory	2000	36, 303
eszopiclone	hypnotic	2005	41, 451
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
etoricoxibe	antiarthritic/analgesic	2002	38, 355
everolimus	immunosuppressant	2004	40, 455
exemestane	anticancer	2000	36, 304
exenatide	anti-diabetic	2005	41, 452
exifone	nootropic	1988	24, 302
ezetimibe	hypolipidemic	2002	38, 355
factor VIIa	haemophilia	1996	32, 307
factor VIII	hemostatic	1992	28, 330
fadrozole HCl	antineoplastic	1995	31, 342
falecalcitriol	vitamin D	2001	37, 266
famciclovir	antiviral	1994	30, 300
famotidine	antiulcer	1985	21, 327
fasudil HCl	neuroprotective	1995	31, 343

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenoldopam mesylate	antihypertensive	1998	34, 322
fenticonazole nitrate	antifungal	1987	23, 334
fexofenadine	antiallergic	1996	32, 307
filgrastim	immunostimulant	1991	27, 327
finasteride	5a-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flurithromycin ethylsuccinate	antibiotic	1997	33, 333
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutrimazole	topical antifungal	1995	31, 343
flutropium bromide	antitussive	1988	24, 303
fluvastatin	hypolipaemic	1994	30, 300
fluvoxamine maleate	antidepressant	1983	19, 319
follitropin alfa	fertility enhancer	1996	32, 307
follitropin beta	fertility enhancer	1996	32, 308
fomepizole	antidote	1998	34, 323
fomivirsen sodium	antiviral	1998	34, 323
fondaparinux sodium	antithrombotic	2002	38, 356
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
fosamprenavir	antiviral	2003	39, 277
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosfluconazole	antifungal	2004	40, 457
fosinopril sodium	antihypertensive	1991	27, 328
fosphenytoin sodium	antiepileptic	1996	32, 308
fotemustine	antineoplastic	1989	25, 313
fropenam	antibiotic	1997	33, 334
frovatriptan	antimigraine	2002	38, 357
fudosteine	expectorant	2001	37, 267
fulveristrant	anticancer	2002	38, 357
gabapentin	antiepileptic	1993	29, 338
gadoversetamide	MRI contrast agent	2000	36, 304

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
galsulfase	mucopolysaccharidosis VI	2005	41, 453
ganciclovir	antiviral	1988	24, 303
ganirelix acetate	female infertility	2000	36, 305
gatilfloxacin	antibiotic	1999	35, 340
gefitinib	antineoplastic	2002	38, 358
gemcitabine HCl	antineoplastic	1995	31, 344
gemeprost	abortifacient	1983	19, 319
gemifloxacin	antibacterial	2004	40, 458
gemtuzumab ozogamicin	anticancer	2000	36, 306
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
glatiramer acetate	Multiple Sclerosis	1997	33, 334
glimepiride	antidiabetic	1995	31, 344
glucagon, rDNA	hypoglycemia	1993	29, 338
GMDP	immunostimulant	1996	32, 308
goserelin	hormone	1987	23, 336
granisetron HCl	antiemetic	1991	27, 329
guanadrel sulfate	antihypertensive	1983	19, 319
gusperimus	immunosuppressant	1994	30, 300
halobetasol propionate	topical antiinflammatory	1991	27, 329
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
histrelin	precocious puberty	1993	29, 338
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibandronic acid	osteoporosis	1996	32, 309
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
ibutilide fumarate	antiarrhythmic	1996	32, 309
ibritunomab tiuxetan	anticancer	2002	38, 359
idarubicin HCl	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
idursulfase	mucopolysaccharidosis II (Hunter syndrome)	2006	42, 520
iloprost	platelet aggreg. inhibitor	1992	28, 332
imatinib mesylate	antineoplastic	2001	37, 267
imidapril HCl	antihypertensive	1993	29, 339
imiglucerase	Gaucher's disease	1994	30, 301
imipenem/cilastatin	antibiotic	1985	21, 328
imiquimod	antiviral	1997	33, 335
incadronic acid	osteoporosis	1997	33, 335
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indinavir sulfate	antiviral	1996	32, 310
indisetron	antiemetic	2004	40, 459
indobufen	antithrombotic	1984	20, 319
influenza virus (live)	antiviral vaccine	2003	39, 277

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
insulin lispro	antidiabetic	1996	32, 310
interferon alfacon-1	antiviral	1997	33, 336
interferon gamma-1b	immunostimulant	1991	27, 329
interferon, gamma	antiinflammatory	1989	25, 314
interferon, gamma-1a	antineoplastic	1992	28, 332
interferon, b-1a	multiple sclerosis	1996	32, 311
interferon, b-1b	multiple sclerosis	1993	29, 339
interleukin-2	antineoplastic	1989	25, 314
ioflupane	diagnosis CNS	2000	36, 306
ipriflavone	calcium regulator	1989	25, 314
irbesartan	antihypertensive	1997	33, 336
irinotecan	antineoplastic	1994	30, 301
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itopride HCl	gastroprokinetic	1995	31, 344
itraconazole	antifungal	1988	24, 305
ivabradine	angina	2006	42, 522
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
ketorolac tromethamine	analgesic	1990	26, 304
kinetin	skin photodamage/ dermatologic	1999	35, 341
lacidipine	antihypertensive	1991	27, 330
lafutidine	gastric antisecretory	2000	36, 307
lamivudine	antiviral	1995	31, 345
lamotrigine	anticonvulsant	1990	26, 304
landiolol	antiarrhythmic	2002	38, 360
lanoconazole	antifungal	1994	30, 302
lanreotide acetate	acromegaly	1995	31, 345
lansoprazole	antiulcer	1992	28, 332
laronidase	mucopolysaccaridosis I	2003	39, 278
latanoprost	antiglaucoma	1996	32, 311
lefunomide	antiarthritic	1998	34, 324
lenalidomide	myelodysplastic syndromes, multiple myeloma	2006	42, 523
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
lepirudin	anticoagulant	1997	33, 336
lercanidipine	antihyperintensive	1997	33, 337
letrazole	anticancer	1996	32, 311
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levalbuterol HCl	antiasthmatic	1999	35, 341
levetiracetam	antiepileptic	2000	36, 307
levobunolol HCl	antiglaucoma	1985	21, 328
levobupivacaine hydrochloride	local anesthetic	2000	36, 308

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
levocabastine HCl	antihistamine	1991	27, 330
levocetirizine	antihistamine	2001	37, 268
levodropropizine	antitussive	1988	24, 305
levofloxacin	antibiotic	1993	29, 340
levosimendan	heart failure	2000	36, 308
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
linezolid	antibiotic	2000	36, 309
liranaftate	topical antifungal	2000	36, 309
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lodoxamide tromethamine	antiallergic ophthalmic	1992	28, 333
lomefloxacin	antibiotic	1989	25, 315
lomerizine HCl	antimigraine	1999	35, 342
lonidamine	antineoplastic	1987	23, 337
lopinavir	antiviral	2000	36, 310
loprazolam mesylate	hypnotic	1983	19, 321
loprinone HCl	cardiostimulant	1996	32, 312
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
lornoxicam	NSAID	1997	33, 337
losartan	antihypertensive	1994	30, 302
loteprednol etabonate	antiallergic ophthalmic	1998	34, 324
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
lulbiprostone	chronic idiopathic constipation	2006	42, 525
luliconazole	antifungal	2005	41, 454
lumiracoxib	anti-inflammatory	2005	41, 455
Lyme disease	vaccine	1999	35, 342
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatoprotective	1985	21, 329
manidipine HCl	antihypertensive	1990	26, 304
masoprocol	topical antineoplastic	1992	28, 333
maxacalcitol	vitamin D	2000	36, 310
mebefradil HCl	antihypertensive	1997	33, 338
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
meloxicam	antiarthritic	1996	32, 312
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
meropenem	carbapenem antibiotic	1994	30, 303
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
micafungin	antifungal	2002	38, 360
mifepristone	abortifacient	1988	24, 306

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
miglitol	antidiabetic	1998	34, 325
miglustat	gaucher's disease	2003	39, 279
milnacipran	antidepressant	1997	33, 338
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
mitiglinide	antidiabetic	2004	40, 460
mitoxantrone HCl	antineoplastic	1984	20, 321
mivacurium chloride	muscle relaxant	1992	28, 334
mivotilate	hepatoprotectant	1999	35, 343
mizolastine	antihistamine	1998	34, 325
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
moexipril HCl	antihypertensive	1995	31, 346
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
montelukast sodium	antiasthma	1998	34, 326
moricizine HCl	antiarrhythmic	1990	26, 305
mosapride citrate	gastroprokinetic	1998	34, 326
moxifloxacin HCL	antibiotic	1999	35, 343
moxonidine	antihypertensive	1991	27, 330
mozavaptan	hyponatremia	2006	42, 527
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
mycophenolate mofetil	immunosuppressant	1995	31, 346
mycophenolate sodium	immunosuppressant	2003	39, 279
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
naftifine HCl	antifungal	1984	20, 321
naftopidil	dysuria	1999	35, 344
nalmefene HCl	dependence treatment	1995	31, 347
naltrexone HCl	narcotic antagonist	1984	20, 322
naratriptan HCl	antimigraine	1997	33, 339
nartograstim	leukopenia	1994	30, 304
natalizumab	multiple sclerosis	2004	40, 462
nateglinide	antidiabetic	1999	35, 344
nazasetron	antiemetic	1994	30, 305
nebivolol	antihypertensive	1997	33, 339
nedaplatin	antineoplastic	1995	31, 347
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
nelarabine	anticancer	2006	42, 528
nelfinavir mesylate	antiviral	1997	33, 340

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
nepafenac	anti-inflammatory	2005	41, 456
neridronic acide	calcium regulator	2002	38, 361
nesiritide	congestive heart failure	2001	37, 269
neticonazole HCl	topical antifungal	1993	29, 341
nevirapine	antiviral	1996	32, 313
nicorandil	coronary vasodilator	1984	20, 322
nifekalant HCl	antiarrythmic	1999	35, 344
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nimotuzumab	anticancer	2006	42, 529
nipradilol	antihypertensive	1988	24, 307
nisoldipine	antihypertensive	1990	26, 306
nitisinone	antityrosinaemia	2002	38, 361
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norelgestromin	contraceptive	2002	38, 362
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
OCT-43	anticancer	1999	35, 345
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
olanzapine	neuroleptic	1996	32, 313
olimesartan Medoxomil	antihypertensive	2002	38, 363
olopatadine HCl	antiallergic	1997	33, 340
omalizumab	allergic asthma	2003	39, 280
omeprazole	antiulcer	1988	24, 308
ondansetron HCl	antiemetic	1990	26, 306
OP-1	osteoinductor	2001	37, 269
orlistat	antiobesity	1998	34, 327
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
oseltamivir phosphate	antiviral	1999	35, 346
oxaliplatin	anticancer	1996	32, 313
oxaprozin	antiinflammatory	1983	19, 322
oxcarbazepine	anticonvulsant	1990	26, 307
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
paclitaxal	antineoplastic	1993	29, 342
palifermin	mucositis	2005	41, 461
palonosetron	antiemetic	2003	39, 281

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
panitumumab	anticancer	2006	42, 531
pantoprazole sodium	antiulcer	1995	30, 306
parecoxib sodium	analgesic	2002	38, 364
paricalcitol	vitamin D	1998	34, 327
parnaparin sodium	anticoagulant	1993	29, 342
paroxetine	antidepressant	1991	27, 331
pazufloxacin	antibacterial	2002	38, 364
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaptanib	age-related macular degeneration	2005	41, 458
pegaspargase	antineoplastic	1994	30, 306
pegvisomant	acromegaly	2003	39, 281
pemetrexed	anticancer	2004	40, 463
pemirolast potassium	antiasthmatic	1991	27, 331
penciclovir	antiviral	1996	32, 314
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
perospirone HCL	neuroleptic	2001	37, 270
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide HCl	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimecrolimus	immunosuppressant	2002	38, 365
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340
pioglitazone HCL	antidiabetic	1999	35, 346
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
pitavastatin	hypocholesterolemic	2003	39, 282
pivagabine	antidepressant	1997	33, 341
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
posaconazole	antifungal	2006	42, 532
pramipexole HCl	antiParkinsonian	1997	33, 341
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pramlintide	anti-diabetic	2005	41, 460
pranlukast	antiasthmatic	1995	31, 347
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
pregabalin	antiepileptic	2004	40, 464
prezatide copper acetate	vulnery	1996	32, 314
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
propacetamol HCl	analgesic	1986	22, 325
propagermanium	antiviral	1994	30, 308
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine HCl	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
prulifloxacin	antibacterial	2002	38, 366
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quetiapine fumarate	neuroleptic	1997	33, 341
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
quinupristin	antibiotic	1999	35, 338
rabeprazole sodium	gastric antisecretory	1998	34, 328
raloxifene HCl	osteoporosis	1998	34, 328
raltitrexed	anticancer	1996	32, 315
ramatroban	antiallergic	2000	36, 311
ramelteon	insomnia	2005	41, 462
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranibizumab	age-related macular degeneration	2006	42, 534
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348
ranolazine	angina	2006	42, 535
rapacuronium bromide	muscle relaxant	1999	35, 347
rasagiline	parkinson's disease	2005	41, 464
rebamipide	antiulcer	1990	26, 308
reboxetine	antidepressant	1997	33, 342
remifentanil HCl	analgesic	1996	32, 316
remoxipride HCl	antipsychotic	1990	26, 308
repaglinide	antidiabetic	1998	34, 329
repirinast	antiallergic	1987	23, 341
reteplase	fibrinolytic	1996	32, 316
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
riluzole	neuroprotective	1996	32, 316
rimantadine HCl	antiviral	1987	23, 342
rimexolone	antiinflammatory	1995	31, 348
rimonabant	anti-obesity	2006	42, 537
risedronate sodium	osteoporosis	1998	34, 330
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
rizatriptan benzoate	antimigraine	1998	34, 330
rocuronium bromide	neuromuscular blocker	1994	30, 309
rofecoxib	antiarthritic	1999	35, 347
rokitamycin	antibiotic	1986	22, 325
romurtide	immunostimulant	1991	27, 332
ronafibrate	hypolipidemic	1986	22, 326
ropinirole HCl	antiParkinsonian	1996	32, 317
ropivacaine	anesthetic	1996	32, 318
rosaprostol	antiulcer	1985	21, 332
rosiglitazone maleate	antidiabetic	1999	35, 348
rosuvastatin	hypocholesterolemic	2003	39, 283
rotigotine	parkinson's disease	2006	42, 538
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin HCl	antibacterial	1992	28, 335
rupatadine fumarate	antiallergic	2003	39, 284
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin HCl	hyperphenylalaninemia	1992	8, 336
saquinavir mesvlate	antiviral	1995	31 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline HCl	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
sibutramine	antiobesity	1998	34, 331
sildenafil citrate	male sexual dysfunction	1998	34, 331
silodosin	dysuria	2006	42, 540
simvastatin	hypocholesterolemic	1988	24, 311
sitagliptin	antidiabetic	2006	42, 541
sitaxsentan	pulmonary hypertension	2006	42, 543
sivelestat	anti-inflammatory	2002	38, 366
SKI-2053R	anticancer	1999	35, 348
sobuzoxane	antineoplastic	1994	30, 310
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
solifenacin	pollakiuria	2004	40, 466
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorafenib	anticancer	2005	41, 466
sorivudine	antiviral	1993	29, 345

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
strontium ranelate	osteoporosis	2004	40, 466
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	b-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
sunitinib	anticancer	2006	42, 544
suplatast tosilate	antiallergic	1995	31, 350
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tacalcitol	topical antipsoriatic	1993	29, 346
tacrine HCl	Alzheimer's disease	1993	29, 346
tacrolimus	immunosuppressant	1993	29, 347
tadalafil	male sexual dysfunction	2003	39, 284
talaporfin sodium	anticancer	2004	40, 469
talipexole	antiParkinsonian	1996	32, 318
taltirelin	CNS stimulant	2000	36, 311
tamibarotene	anticancer	2005	41, 467
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347
tandospirone	anxiolytic	1996	32, 319
tasonermin	anticancer	1999	35, 349
tazanolast	antiallergic	1990	26, 309
tazarotene	antipsoriasis	1997	33, 343
tazobactam sodium	b-lactamase inhibitor	1992	28, 336
tegaserod maleate	irritable bowel syndrome	2001	37, 270
teicoplanin	antibacterial	1988	24, 311
telbivudine	hepatitis B	2006	42, 546
telithromycin	antibiotic	2001	37, 271
telmesteine	mucolytic	1992	28, 337
telmisartan	antihypertensive	1999	35, 349
temafloxacin HCl	antibacterial	1991	27, 334
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
temoporphin	antineoplastic/ photosensitizer	2002	38, 367
temozolomide	anticancer	1999	35, 349
tenofovir disoproxil fumarate	antiviral	2001	37, 271
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine HCl	antifungal	1991	27, 334
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
thymopentin	immunomodulator	1985	21, 333
tiagabine	antiepileptic	1996	32, 319
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tigecycline	antibiotic	2005	41, 468
tilisolol HCl	antihypertensive	1992	28, 337
tiludronate disodium	Paget's disease	1995	31, 350
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiopronin	urolithiasis	1989	25, 318
tiotropium bromide	bronchodilator	2002	38, 368
tipranavir	HIV	2005	41, 470
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine HCl	antiarrhythmic	1990	26, 310
tirilazad mesylate	subarachnoid hemorrhage	1995	31, 351
tirofiban HCl	antithrombotic	1998	34, 332
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
tolcapone	antiParkinsonian	1997	33, 343
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
topiramate	antiepileptic	1995	31, 351
topotecan HCl	anticancer	1996	32, 320
torasemide	diuretic	1993	29, 348
toremifene	antineoplastic	1989	25, 319
tositumomab	anticancer	2003	39, 285
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
travoprost	antiglaucoma	2001	37, 272
treprostinil sodium	antihypertensive	2002	38, 368
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
trimegestone	progestogen	2001	37, 273
trimetrexate glucuronate	Pneumocystis carinii pneumonia	1994	30, 312
troglitazone	antidiabetic	1997	33, 344
tropisetron	antiemetic	1992	28, 337
trovafloxacin mesylate	antibiotic	1998	34, 332
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
udenafil	erectile dysfunction	2005	41, 472
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
valaciclovir HCl	antiviral	1995	31, 352
vadecoxib	antiarthritic	2002	38, 369
vaglancirclovir HCL	antiviral	2001	37, 273
valrubicin	anticancer	1999	35, 350

GENERAL NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
valsartan	antihypertensive	1996	32, 320
vardenafil	male sexual dysfunction	2003	39, 286
varenicline	nicotine-dependence	2006	42, 547
venlafaxine	antidepressant	1994	30, 312
verteporfin	photosensitizer	2000	36, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
voriconazole	antifungal	2002	38, 370
vorinostat	anticancer	2006	42, 549
xamoterol fumarate	cardiotonic	1988	24, 312
ximelagatran	anticoagulant	2004	40, 470
zafirlukast	antiasthma	1996	32, 321
zalcitabine	antiviral	1992	28, 338
zaleplon	hypnotic	1999	35, 351
zaltoprofen	antiinflammatory	1993	29, 349
zanamivir	antiviral	1999	35, 352
ziconotide	severe chronic pain	2005	41, 473
zidovudine	antiviral	1987	23, 345
zileuton	antiasthma	1997	33, 344
zinostatin stimalamer	antineoplastic	1994	30, 313
ziprasidone hydrochloride	neuroleptic	2000	36, 312
zofenopril calcium	antihypertensive	2000	36, 313
zoledronate disodium	hypercalcemia	2000	36, 314
zolpidem hemitartrate	hypnotic	1988	24, 313
zomitriptan	antimigraine	1997	33, 345
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
gemeprost	ABORTIFACIENT	1983	19 (319)
mifepristone		1988	24 (306)
lanreotide acetate	ACROMEGALY	1995	31 (345)
pegvisomant		2003	39 (281)
pegaptanib	AGE-RELATED MACULAR DEGENERATION	2005	41 (458)
ranibizumab		2006	42 (534)
nitrefazole	ALCOHOL DETERRENT	1983	19 (322)
omalizumab	ALLERGIC ASTHMA	2003	39 (280)
tacrine HCl	ALZHEIMER'S DISEASE	1993	29 (346)
quinfamide	AMEBICIDE	1984	20 (322)
tibolone	ANABOLIC	1988	24 (312)
mepixanox	ANALEPTIC	1984	20 (320)
alfentanil HCl	ANALGESIC	1983	19 (314)
alminoprofen		1983	19 (314)
dezocine		1991	27 (326)
emorfazone		1984	20 (317)
eptazocine HBr		1987	23 (334)
etoricoxib		2002	38 (355)
flupirtine maleate		1985	21 (328)
fosfosal		1984	20 (319)
ketorolac tromethamine		1990	26 (304)
meptazinol HCl		1983	19 (321)
mofezolac		1994	30 (304)
parecoxib sodium		2002	38 (364)
propacetamol HCl		1986	22 (325)
remifentanil HCl		1996	32 (316)
sufentanil		1983	19 (323)
suprofen		1983	19 (324)
desflurane	ANESTHETIC	1992	28 (329)
propofol		1986	22 (325)
ropivacaine		1996	32 (318)
sevoflurane		1990	26 (309)
levobupivacaine hydrochloride	ANESTHETIC, LOCAL	2000	36 (308)
ivabradine	ANGINA	2006	42 (522)
ranolazine		2006	42 (535)
azelaic acid	ANTIACNE	1989	25 (310)
betotastine besilate	ANTIALLERGIC	2000	36 (297)
emedastine difumarate		1993	29 (336)
epinastine		1994	30 (299)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
fexofenadine		1996	32 (307)
nedocromil sodium		1986	22 (324)
olopatadine hydrochloride		1997	33 (340)
ramatroban		2000	36 (311)
repirinast		1987	23 (341)
suplatast tosilate		1995	31 (350)
tazanolast		1990	26 (309)
lodoxamide tromethamine	ANTIALLERGIC	1992	28 (333)
rupatadine fumarate		2003	39 (284)
loteprednol etabonate	OPHTHALMIC	1998	34 (324)
donepezil hydrochloride	ANTI-ALZHEIMERS	1997	33 (332)
rivastigmin		1997	33 (342)
gallopamil HCl	ANTIANGINAL	1983	19 (319)
cibenzoline	ANTIARRHYTHMIC	1985	21 (325)
dofetilide		2000	36 (301)
encainide HCl		1987	23 (333)
esmolol HCl		1987	23 (334)
ibutilide fumarate		1996	32 (309)
landiolol		2002	38 (360)
moricizine hydrochloride		1990	26 (305)
nifekalant HCl		1999	35 (344)
pilsicainide hydrochloride		1991	27 (332)
pirmenol		1994	30 (307)
tiracizine hydrochloride		1990	26 (310)
anakinra	ANTIARTHRITIC	2001	37 (261)
celecoxib		1999	35 (335)
etoricoxib		2002	38 (355)
meloxicam		1996	32 (312)
leflunomide		1998	34 (324)
rofecoxib		1999	35 (347)
valdecoxib		2002	38 (369)
amlexanox	ANTIASTHMATIC	1987	23 (327)
emedastine difumarate		1993	29 (336)
ibudilast		1989	25 (313)
levalbuterol HCl		1999	35 (341)
montelukast sodium		1998	34 (326)
pemirolast potassium		1991	27 (331)
seratrodast		1995	31 (349)
zafirlukast		1996	32 (321)
zileuton		1997	33 (344)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
balofloxacin	ANTIBACTERIAL	2002	38 (351)
biapenem		2002	38 (351)
ciprofloxacin		1986	22 (318)
enoxacin		1986	22 (320)
ertapenem sodium		2002	38 (353)
fleroxacin		1992	28 (331)
gemifloxacin		2004	40 (458)
norfloxacin		1983	19 (322)
ofloxacin		1985	21 (331)
pazufloxacin		2002	38 (364)
pefloxacin mesylate		1985	21 (331)
pranlukast		1995	31 (347)
prulifloxacin		2002	38 (366)
rifabutin		1992	28 (335)
rifapentine		1988	24 (310)
rufloxacin hydrochloride		1992	28 (335)
teicoplanin		1988	24 (311)
temafloxacin hydrochloride		1991	27 (334)
tosufloxacin tosylate		1990	26 (310)
arbekacin	ANTIBIOTIC	1990	26 (298)
aspoxicillin		1987	23 (328)
astromycin sulfate		1985	21 (324)
azithromycin		1988	24 (298)
aztreonam		1984	20 (315)
brodimoprin		1993	29 (333)
carboplatin		1986	22 (318)
carumonam		1988	24 (298)
cefbuperazone sodium		1985	21 (325)
cefcapene pivoxil		1997	33 (330)
cefdinir		1991	27 (323)
cefepime		1993	29 (334)
cefetamet pivoxil hydrochloride		1992	28 (327)
cefixime		1987	23 (329)
cefmenoxime HCl		1983	19 (316)
cefminox sodium		1987	23 (330)
cefodizime sodium		1990	26 (300)
cefonicid sodium		1984	20 (316)
ceforanide		1984	20 (317)
cefoselis		1998	34 (319)
cefotetan disodium		1984	20 (317)
cefotiam hexetil hydrochloride		1991	27 (324)
cefpimizole		1987	23 (330)
cefpiramide sodium		1985	21 (325)
cefpirome sulfate		1992	28 (328)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
cefpodoxime proxetil		1989	25 (310)
cefprozil		1992	28 (328)
ceftazidime		1983	19 (316)
cefteram pivoxil		1987	23 (330)
ceftibuten		1992	28 (329)
cefuroxime axetil		1987	23 (331)
cefuzonam sodium		1987	23 (331)
clarithromycin		1990	26 (302)
dalfopristin		1999	35 (338)
dirithromycin		1993	29 (336)
doripenem		2005	41 (448)
erythromycin acistrate		1988	24 (301)
flomoxef sodium		1988	24 (302)
flurithromycin ethylsuccinate		1997	33 (333)
fropenam		1997	33 (334)
gatifloxacin		1999	35 (340)
imipenem/cilastatin		1985	21 (328)
isepamicin		1988	24 (305)
lenampicillin HCl		1987	23 (336)
levofloxacin		1993	29 (340)
linezolid		2000	36 (309)
lomefloxacin		1989	25 (315)
loracarbef		1992	28 (333)
miokamycin		1985	21 (329)
moxifloxacin HCl		1999	35 (343)
quinupristin		1999	35 (338)
rifaximin		1985	21 (332)
rifaximin		1987	23 (341)
rokitamycin		1986	22 (325)
RV-11		1989	25 (318)
sparfloxacin		1993	29 (345)
sultamycillin tosylate		1987	23 (343)
telithromycin		2001	37 (271)
temocillin disodium		1984	20 (323)
tigecycline		2005	41 (468)
trovafloxacin mesylate		1998	34 (332)
meropenem	ANTIBIOTIC,	1994	30 (303)
panipenem/ betamipron	CARBAPENEM	1994	30 (305)
mupirocin	ANTIBIOTIC, TOPICAL	1985	21 (330)
nadifloxacin		1993	29 (340)
abarelix	ANTICANCER	2004	40 (446)
alemtuzumab		2001	37 (260)
alitretinoin		1999	35 (333)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
arglabin		1999	35 (335)
azacitidine		2004	40 (447)
belotecan		2004	40 (449)
bevacizumab		2004	40 (450)
bexarotene		2000	36 (298)
bortezomib		2003	39 (271)
cetuximab		2003	39 (272)
clofarabine		2005	41 (444)
dasatinib		2006	42 (517)
denileukin diftitox		1999	35 (338)
erlotinib		2004	40 (454)
exemestane		2000	36 (304)
fulvestrant		2002	38 (357)
gemtuzumab ozogamicin		2000	36 (306)
ibritumomab tiuxetan		2002	38 (359)
letrazole		1996	32 (311)
nelarabine		2006	42 (528)
nimotuzumab		2006	42 (529)
OCT-43		1999	35 (345)
oxaliplatin		1996	32 (313)
panitumumab		2006	42 (531)
pemetrexed		2004	40 (463)
raltitrexed		1996	32 (315)
SKI-2053R		1999	35 (348)
sorafenib		2005	41 (466)
sunitinib		2006	42 (544)
talaporfin sodium		2004	40 (469)
tamibarotene		2005	41 (467)
tasonermin		1999	35 (349)
temozolomide		1999	35 (350)
topotecan HCl		1996	32 (320)
tositumomab		2003	39 (285)
valrubicin		1999	35 (350)
vorinostat		2006	42 (549)
angiotensin II	ANTICANCER ADJUVANT	1994	30 (296)
chenodiol	ANTICHOLELITHOGENIC	1983	19 (317)
duteplase	ANTICOAGULANT	1995	31 (342)
lepirudin		1997	33 (336)
parnaparin sodium		1993	29 (342)
reviparin sodium		1993	29 (344)
ximelagatran		2004	40 (470)
lamotrigine	ANTICONVULSANT	1990	26 (304)
oxcarbazepine		1990	26 (307)
progabide		1985	21 (331)
vigabatrin		1989	25 (319)
zonisamide		1989	25 (320)
bupropion HCl	ANTIDEPRESSANT	1989	25 (310)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
citalopram		1989	25 (311)
duloxetine		2004	40 (452)
escitalopram oxalate		2002	38 (354)
fluoxetine HCl		1986	22 (320)
fluvoxamine maleate		1983	19 (319)
indalpine		1983	19 (320)
medifoxamine fumarate		1986	22 (323)
metapramine		1984	20 (320)
milnacipran		1997	33 (338)
mirtazapine		1994	30 (303)
moclobemide		1990	26 (305)
nefazodone		1994	30 (305)
paroxetine		1991	27 (331)
pivagabine		1997	33 (341)
reboxetine		1997	33 (342)
setiptiline		1989	25 (318)
sertraline hydrochloride		1990	26 (309)
tianeptine sodium		1983	19 (324)
toloxatone		1984	20 (324)
venlafaxine		1994	30 (312)
acarbose	ANTIDIABETIC	1990	26 (297)
epalrestat		1992	28 (330)
exenatide		2005	41 (452)
glimepiride		1995	31 (344)
insulin lispro		1996	32 (310)
miglitol		1998	34 (325)
mitiglinide		2004	40 (460)
nateglinide		1999	35 (344)
pioglitazone HCl		1999	35 (346)
pramlintide		2005	41 (460)
repaglinide		1998	34 (329)
rosiglitazone maleate		1999	35 (347)
sitagliptin		2006	42 (541)
tolrestat		1989	25 (319)
troglitazone		1997	33 (344)
voglibose		1994	30 (313)
acetorphan	ANTIDIARRHEAL	1993	29 (332)
anti-digoxin polyclonal antibody	ANTIDOTE	2002	38 (350)
crotelidae polyvalent immune fab		2001	37 (263)
fomepizole		1998	34 (323)
aprepitant	ANTIEMETIC	2003	39 (268)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
dolasetron mesylate		1998	34 (321)
granisetron hydrochloride		1991	27 (329)
indisetron		2004	40 (459)
ondansetron hydrochloride		1990	26 (306)
nazasetron		1994	30 (305)
palonosetron		2003	39 (281)
ramosetron		1996	32 (315)
tropisetron		1992	28 (337)
felbamate	ANTIEPILEPTIC	1993	29 (337)
fosphenytoin sodium		1996	32 (308)
gabapentin		1993	29 (338)
levetiracetam		2000	36 (307)
pregabalin		2004	40 (464)
tiagabine		1996	32 (320)
topiramate		1995	31 (351)
centchroman	ANTIESTROGEN	1991	27 (324)
anidulafungin	ANTIFUNGAL	2006	42 (512)
caspofungin acetate		2001	37 (263)
eberconazole		2005	41 (449)
fenticonazole nitrate		1987	23 (334)
fluconazole		1988	24 (303)
fosfluconazole		2004	40 (457)
itraconazole		1988	24 (305)
lanoconazole		1994	30 (302)
luliconazole		2005	41 (454)
micafungin		2002	38 (360)
naftifine HCl		1984	20 (321)
oxiconazole nitrate		1983	19 (322)
posaconazole		2006	42 (532)
terbinafine hydrochloride		1991	27 (334)
terconazole		1983	19 (324)
tioconazole		1983	19 (324)
voriconazole		2002	38 (370)
amorolfine hydrochloride	ANTIFUNGAL, TOPICAL	1991	27 (322)
butenafine hydrochloride		1992	28 (327)
butoconazole		1986	22 (318)
cloconazole HCl		1986	22 (318)
liranaftate		2000	36 (309)
flutrimazole		1995	31 (343)
neticonazole HCl		1993	29 (341)
sertaconazole nitrate		1992	28 (336)
sulconizole nitrate		1985	21 (332)
apraclonidine HCl	ANTIGLAUCOMA	1988	24 (297)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
befunolol HCl		1983	19 (315)
bimatroprost		2001	37 (261)
brimonidine		1996	32 (306)
brinzolamide		1998	34 (318)
dapiprazole HCl		1987	23 (332)
dorzolamide HCl		1995	31 (341)
latanoprost		1996	32 (311)
levobunolol HCl		1985	21 (328)
travoprost		2001	37 (272)
unoprostone isopropyl ester		1994	30 (312)
acrivastine	ANTIHISTAMINE	1988	24 (295)
astemizole		1983	19 (314)
azelastine HCl		1986	22 (316)
cetirizine HCl		1987	23 (331)
desloratadine		2001	37 (264)
ebastine		1990	26 (302)
levocabastine hydrochloride		1991	27 (330)
levocetirizine		2001	37 (268)
loratadine		1988	24 (306)
mizolastine		1998	34 (325)
setastine HCl		1987	23 (342)
alacepril	ANTIHYPERTENSIVE	1988	24 (296)
alfuzosin HCl		1988	24 (296)
amlodipine besylate		1990	26 (298)
amosulalol		1988	24 (297)
aranidipine		1996	32 (306)
arotinolol HCl		1986	22 (316)
azelnidipine		2003	39 (270)
barnidipine hydrochloride		1992	28 (326)
benazepril hydrochloride		1990	26 (299)
benidipine hydrochloride		1991	27 (322)
betaxolol HCl		1983	19 (315)
bevantolol HCl		1987	23 (328)
bisoprolol fumarate		1986	22 (317)
bopindolol		1985	21 (324)
bosentan		2001	37 (262)
budralazine		1983	19 (315)
bunazosin HCl		1985	21 (324)
candesartan cilexetil		1997	33 (330)
carvedilol		1991	27 (323)
celiprolol HCl		1983	19 (317)
cicletanine		1988	24 (299)
cilazapril		1990	26 (301)
cinildipine		1995	31 (339)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
delapril		1989	25 (311)
dilevalol		1989	25 (311)
doxazosin mesylate		1988	24 (300)
efonidipine		1994	30 (299)
enalapril maleate		1984	20 (317)
enalaprilat		1987	23 (332)
eplerenone		2003	39 (276)
eprosartan		1997	33 (333)
felodipine		1988	24 (302)
fenoldopam mesylate		1998	34 (322)
fosinopril sodium		1991	27 (328)
guanadrel sulfate		1983	19 (319)
imidapril HCl		1993	29 (339)
irbesartan		1997	33 (336)
isradipine		1989	25 (315)
ketanserin		1985	21 (328)
lacidipine		1991	27 (330)
lercanidipine		1997	33 (337)
lisinopril		1987	23 (337)
losartan		1994	30 (302)
manidipine hydrochloride		1990	26 (304)
mebefradil hydrochloride		1997	33 (338)
moexipril HCl		1995	31 (346)
moxonidine		1991	27 (330)
nebivolol		1997	33 (339)
nilvadipine		1989	25 (316)
nipradilol		1988	24 (307)
nisoldipine		1990	26 (306)
olmesartan medoxomil		2002	38 (363)
perindopril		1988	24 (309)
pinacidil		1987	23 (340)
quinapril		1989	25 (317)
ramipril		1989	25 (317)
rilmenidine		1988	24 (310)
spirapril HCl		1995	31 (349)
telmisartan		1999	35 (349)
temocapril		1994	30 (311)
terazosin HCl		1984	20 (323)
tertatolol HCl		1987	23 (344)
tiamenidine HCl		1988	24 (311)
tilisolol hydrochloride		1992	28 (337)
trandolapril		1993	29 (348)
treprostinil sodium		2002	38 (368)
trimazosin HCl		1985	21 (333)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
valsartan		1996	32 (320)
zofenopril calcium		2000	36 (313)
captopril	ANTIHYPERTENSIVE AGENT	1982	13 (086)
daptomycin	ANTI INFECTIVE	2003	39 (272)
aceclofenac	ANTIINFLAMMATORY	1992	28 (325)
AF-2259		1987	23 (325)
amfenac sodium		1986	22 (315)
ampiroxicam		1994	30 (296)
amtolmetin guacil		1993	29 (332)
butibufen		1992	28 (327)
deflazacort		1986	22 (319)
dexibuprofen		1994	30 (298)
droxicam		1990	26 (302)
etodolac		1985	21 (327)
flunoxaprofen		1987	23 (335)
fluticasone propionate		1990	26 (303)
interferon, gamma		1989	25 (314)
isofezolac		1984	20 (319)
isoxicam		1983	19 (320)
lobenzarit sodium		1986	22 (322)
loxoprofen sodium		1986	22 (322)
lumiracoxib		2005	41 (455)
nabumetone		1985	21 (330)
nepafenac		2005	41 (456)
nimesulide		1985	21 (330)
oxaprozin		1983	19 (322)
piroxicam cinnamate		1988	24 (309)
rimexolone		1995	31 (348)
sivelestat		2002	38 (366)
tenoxicam		1987	23 (344)
zaltoprofen		1993	29 (349)
fisalamine	ANTIINFLAMMATORY,	1984	20 (318)
osalazine sodium	INTESTINAL	1986	22 (324)
alclometasone dipropionate	ANTIINFLAMMATORY,	1985	21 (323)
aminoprofen	TOPICAL	1990	26 (298)
betamethasone butyrate propionate		1994	30 (297)
butyl flufenamate		1983	19 (316)
deprodone propionate		1992	28 (329)
felbinac		1986	22 (320)
halobetasol propionate		1991	27 (329)
halometasone		1983	19 (320)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
hydrocortisone aceponate		1988	24 (304)
hydrocortisone butyrate propionate		1983	19 (320)
mometasone furoate		1987	23 (338)
piketoprofen		1984	20 (322)
pimaprofen		1984	20 (322)
prednicarbate		1986	22 (325)
pravastatin	ANTILIPIDEMIC	1989	25 (316)
arteether	ANTIMALARIAL	2000	36 (296)
artemisinin		1987	23 (327)
bulaquine		2000	36 (299)
halofantrine		1988	24 (304)
mefloquine HCl		1985	21 (329)
almotriptan	ANTIMIGRAINE	2000	36 (295)
alpiropride		1988	24 (296)
eletriptan		2001	37 (266)
frovatriptan		2002	38 (357)
lomerizine HCl		1999	35 (342)
naratriptan hydrochloride		1997	33 (339)
rizatriptan benzoate		1998	34 (330)
sumatriptan succinate		1991	27 (333)
zolmitriptan		1997	33 (345)
dronabinol	ANTINAUSEANT	1986	22 (319)
amrubicin HCl	ANTINEOPLASTIC	2002	38 (349)
amsacrine		1987	23 (327)
anastrozole		1995	31 (338)
bicalutamide		1995	31 (338)
bisantrene hydrochloride		1990	26 (300)
camostat mesylate		1985	21 (325)
capecitabine		1998	34 (319)
cladribine		1993	29 (335)
cytarabine ocfosfate		1993	29 (335)
docetaxel		1995	31 (341)
doxifluridine		1987	23 (332)
enocitabine		1983	19 (318)
epirubicin HCl		1984	20 (318)
fadrozole HCl		1995	31 (342)
fludarabine phosphate		1991	27 (327)
flutamide		1983	19 (318)
formestane		1993	29 (337)
fotemustine		1989	25 (313)
geftimib		2002	38 (358)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
gemcitabine HCl		1995	31 (344)
idarubicin hydrochloride		1990	26 (303)
imatinib mesylate		2001	37 (267)
interferon gamma-1a		1992	28 (332)
interleukin-2		1989	25 (314)
irinotecan		1994	30 (301)
lonidamine		1987	23 (337)
mitoxantrone HCl		1984	20 (321)
nedaplatin		1995	31 (347)
nilutamide		1987	23 (338)
paclitaxal		1993	29 (342)
pegaspargase		1994	30 (306)
pentostatin		1992	28 (334)
pirarubicin		1988	24 (309)
ranimustine		1987	23 (341)
sobuzoxane		1994	30 (310)
temoporphin		2002	38 (367)
toremifene		1989	25 (319)
vinorelbine		1989	25 (320)
zinostatin stimalamer		1994	30 (313)
porfimer sodium	ANTINEOPLASTIC ADJUVANT	1993	29 (343)
masoprocol	ANTINEOPLASTIC,	1992	28 (333)
miltefosine	TOPICAL	1993	29 (340)
dexfenfluramine	ANTIOBESITY	1997	33 (332)
rimonabant		2006	42 (537)
orlistat		1998	34 (327)
sibutramine		1998	34 (331)
atovaquone	ANTIPARASITIC	1992	28 (326)
ivermectin		1987	23 (336)
budipine	ANTIPARKINSONIAN	1997	33 (330)
CHF-1301		1999	35 (336)
droxidopa		1989	25 (312)
entacapone		1998	34 (322)
pergolide mesylate		1988	24 (308)
pramipexole hydrochloride		1997	33 (341)
ropinirole HCl		1996	32 (317)
talipexole		1996	32 (318)
tolcapone		1997	33 (343)
lidamidine HCl	ANTIPERISTALTIC	1984	20 (320)
gestrinone	ANTIPROGESTOGEN	1986	22 (321)
cabergoline	ANTIPROLACTIN	1993	29 (334)
tamsulosin HCl	ANTIPROSTATIC HYPERTROPHY	1993	29 (347)
acitretin	ANTIPSORIATIC	1989	25 (309)
calcipotriol		1991	27 (323)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
tazarotene		1997	33 (343)
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29 (346)
amisulpride	ANTIPSYCHOTIC	1986	22 (316)
remoxipride hydrochloride		1990	26 (308)
zuclopenthixol acetate		1987	23 (345)
actarit	ANTIRHEUMATIC	1994	30 (296)
diacerein		1985	21 (326)
octreotide	ANTISECRETORY	1988	24 (307)
adamantanium bromide	ANTISEPTIC	1984	20 (315)
drotecogin alfa	ANTISEPSIS	2001	37 (265)
cimetropium bromide	ANTISPASMODIC	1985	21 (326)
tiquizium bromide		1984	20 (324)
tiropramide HCl		1983	19 (324)
argatroban	ANTITHROMBOTIC	1990	26 (299)
bivalirudin		2000	36 (298)
defibrotide		1986	22 (319)
cilostazol		1988	24 (299)
clopidogrel hydrogensulfate		1998	34 (320)
cloricromen		1991	27 (325)
enoxaparin		1987	23 (333)
eptifibatide		1999	35 (340)
ethyl icosapentate		1990	26 (303)
fondaparinux sodium		2002	38 (356)
indobufen		1984	20 (319)
limaprost		1988	24 (306)
ozagrel sodium		1988	24 (308)
picotamide		1987	23 (340)
tirofiban hydrochloride		1998	34 (332)
flutropium bromide	ANTITUSSIVE	1988	24 (303)
levodropropizine		1988	24 (305)
nitisinone	ANTITYROSINAEMIA	2002	38 (361)
benexate HCl	ANTIULCER	1987	23 (328)
dosmalfate		2000	36 (302)
ebrotidine		1997	33 (333)
ecabet sodium		1993	29 (336)
egualen sodium		2000	36 (303)
enprostil		1985	21 (327)
famotidine		1985	21 (327)
irsogladine		1989	25 (315)
lansoprazole		1992	28 (332)
misoprostol		1985	21 (329)
nizatidine		1987	23 (339)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
omeprazole		1988	24 (308)
ornoprostil		1987	23 (339)
pantoprazole sodium		1994	30 (306)
plaunotol		1987	23 (340)
polaprezinc		1994	30 (307)
ranitidine bismuth citrate		1995	31 (348)
rebamipide		1990	26 (308)
rosaprostol		1985	21 (332)
roxatidine acetate HCl		1986	22 (326)
roxithromycin		1987	23 (342)
sofalcone		1984	20 (323)
spizofurone		1987	23 (343)
teprenone		1984	20 (323)
tretinoin tocoferil		1993	29 (348)
troxipide		1986	22 (327)
abacavir sulfate	ANTIVIRAL	1999	35 (333)
adefovir dipivoxil		2002	38 (348)
amprenavir		1999	35 (334)
atazanavir		2003	39 (269)
cidofovir		1996	32 (306)
delavirdine mesylate		1997	33 (331)
didanosine		1991	27 (326)
efavirenz		1998	34 (321)
emtricitabine		2003	39 (274)
enfuvirtide		2003	39 (275)
entecavir		2005	41 (450)
famciclovir		1994	30 (300)
fomivirsen sodium		1998	34 (323)
fosamprenavir		2003	39 (277)
foscarnet sodium		1989	25 (313)
ganciclovir		1988	24 (303)
imiquimod		1997	33 (335)
indinavir sulfate		1996	32 (310)
interferon alfacon-1		1997	33 (336)
lamivudine		1995	31 (345)
lopinavir		2000	36 (310)
nelfinavir mesylate		1997	33 (340)
nevirapine		1996	32 (313)
oseltamivir phosphate		1999	35 (346)
penciclovir		1996	32 (314)
propagermanium		1994	30 (308)
rimantadine HCl		1987	23 (342)
ritonavir		1996	32 (317)
saquinavir mesylate		1995	31 (349)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
sorivudine		1993	29 (345)
stavudine		1994	30 (311)
tenofovir disoproxil fumarate		2001	37 (271)
valaciclovir HCl		1995	31 (352)
zalcitabine		1992	28 (338)
zanamivir		1999	35 (352)
zidovudine		1987	23 (345)
influenza virus live	ANTIVIRAL VACCINE	2003	39 (277)
cevimeline hydrochloride	ANTI-XEROSTOMIA	2000	36 (299)
alpidem	ANXIOLYTIC	1991	27 (322)
buspirone HCl		1985	21 (324)
etizolam		1984	20 (318)
flutazolam		1984	20 (318)
flutoprazepam		1986	22 (320)
metaclazepam		1987	23 (338)
mexazolam		1984	20 (321)
tandospirone		1996	32 (319)
ciclesonide	ASTHMA, COPD	2005	41 (443)
atomoxetine	ATTENTION DEFICIT HYPERACTIVITY DISORDER	2003	39 (270)
flumazenil	BENZODIAZEPINE ANTAG.	1987	23 (335)
bambuterol	BRONCHODILATOR	1990	26 (299)
doxofylline		1985	21 (327)
formoterol fumarate		1986	22 (321)
mabuterol HCl		1986	22 (323)
oxitropium bromide		1983	19 (323)
salmeterol hydroxynaphthoate		1990	26 (308)
tiotropium bromide		2002	38 (368)
APD	CALCIUM REGULATOR	1987	23 (326)
clodronate disodium		1986	22 (319)
disodium pamidronate		1989	25 (312)
gallium nitrate		1991	27 (328)
ipriflavone		1989	25 (314)
neridronic acid		2002	38 (361)
dexrazoxane	CARDIOPROTECTIVE	1992	28 (330)
bucladesine sodium	CARDIOSTIMULANT	1984	20 (316)
denopamine		1988	24 (300)
docarpamine		1994	30 (298)
dopexamine		1989	25 (312)
enoximone		1988	24 (301)
flosequinan		1992	28 (331)
ibopamine HCl		1984	20 (319)
loprinone hydrochloride		1996	32 (312)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
milrinone		1989	25 (316)
vesnarinone		1990	26 (310)
amrinone	CARDIOTONIC	1983	19 (314)
colforsin daropate HCL		1999	35 (337)
xamoterol fumarate		1988	24 (312)
cefozopran HCL	CEPHALOSPORIN, INJECTABLE	1995	31 (339)
cefditoren pivoxil	CEPHALOSPORIN, ORAL	1994	30 (297)
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22 (317)
nimodipine		1985	21 (330)
propentofylline		1988	24 (310)
succimer	CHELATOR	1991	27 (333)
trientine HCl		1986	22 (327)
fenbuprol	CHOLERETIC	1983	19 (318)
lulbiprostone	CHRONIC IDIOPATHIC CONSTIPATION	2006	42 (525)
deferasirox	CHRONIC IRON OVERLOAD	2005	41 (446)
auranofin	CHRYSOTHERAPEUTIC	1983	19 (314)
taltirelin	CNS STIMULANT	2000	36 (311)
aniracetam	COGNITION ENHANCER	1993	29 (333)
pramiracetam H_2SO_4		1993	29 (343)
carperitide	CONGESTIVE HEART	1995	31 (339)
nesiritide	FAILURE	2001	37 (269)
drospirenone	CONTRACEPTIVE	2000	36 (302)
norelgestromin		2002	38 (362)
nicorandil	CORONARY VASODILATOR	1984	20 (322)
dornase alfa	CYSTIC FIBROSIS	1994	30 (298)
neltenexine		1993	29 (341)
amifostine	CYTOPROTECTIVE	1995	31 (338)
nalmefene HCL	DEPENDENCE TREATMENT	1995	31 (347)
ioflupane	DIAGNOSIS CNS	2000	36 (306)
azosemide	DIURETIC	1986	22 (316)
muzolimine		1983	19 (321)
torasemide		1993	29 (348)
atorvastatin calcium	DYSLIPIDEMIA	1997	33 (328)
cerivastatin		1997	33 (331)
naftopidil	DYSURIA	1999	35 (343)
silodosin		2006	42 (540)
alglucerase	ENZYME	1991	27 (321)
udenafil	ERECTILE DYSFUNCTION	2005	41 (472)
erdosteine	EXPECTORANT	1995	31 (342)
fudosteine		2001	37 (267)
agalsidase alfa	FABRY'S DISEASE	2001	37 (259)
cetrorelix	FEMALE INFERTILITY	1999	35 (336)
ganirelix acetate		2000	36 (305)
follitropin alfa	FERTILITY ENHANCER	1996	32 (307)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
follitropin beta		1996	32 (308)
reteplase	FIBRINOLYTIC	1996	32 (316)
esomeprazole magnesium	GASTRIC ANTISECRETORY	2000	36 (303)
lafutidine		2000	36 (307)
rabeprazole sodium		1998	34 (328)
cinitapride	GASTROPROKINETIC	1990	26 (301)
cisapride		1988	24 (299)
itopride HCL		1995	31 (344)
mosapride citrate		1998	34 (326)
imiglucerase	GAUCHER'S DISEASE	1994	30 (301)
miglustat		2003	39 (279)
somatotropin	GROWTH HORMONE	1994	30 (310)
somatomedin-1	GROWTH HORMONE INSENSITIVITY	1994	30 (310)
factor VIIa	HAEMOPHILIA	1996	32 (307)
levosimendan	HEART FAILURE	2000	36 (308)
pimobendan		1994	30 (307)
anagrelide hydrochloride	HEMATOLOGIC	1997	33 (328)
erythropoietin	HEMATOPOETIC	1988	24 (301)
factor VIII	HEMOSTATIC	1992	28 (330)
telbivudine	HEPATITIS B	2006	42 (546)
malotilate	HEPATOPROTECTIVE	1985	21 (329)
mivotilate		1999	35 (343)
darunavir	HIV	2006	42 (515)
tipranavir		2005	41 (470)
buserelin acetate	HORMONE	1984	20 (316)
goserelin		1987	23 (336)
leuprolide acetate		1984	20 (319)
nafarelin acetate		1990	26 (306)
somatropin		1987	23 (343)
zoledronate disodium	HYPERCALCEMIA	2000	36 (314)
cinacalcet	HYPERPARATHYROIDISM	2004	40 (451)
sapropterin hydrochloride	HYPERPHENYL-ALANINEMIA	1992	28 (336)
quinagolide	HYPERPROLACTINEMIA	1994	30 (309)
cadralazine	HYPERTENSIVE	1988	24 (298)
nitrendipine		1985	21 (331)
binfonazole	HYPNOTIC	1983	19 (315)
brotizolam		1983	19 (315)
butoctamide		1984	20 (316)
cinolazepam		1993	29 (334)
doxefazepam		1985	21 (326)
eszopiclone		2005	41 (451)
loprazolam mesylate		1983	19 (321)
quazepam		1985	21 (332)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
rilmazafone		1989	25 (317)
zaleplon		1999	35 (351)
zolpidem hemitartrate		1988	24 (313)
zopiclone		1986	22 (327)
acetohydroxamic acid	HYPOAMMONURIC	1983	19 (313)
sodium cellulose PO4	HYPOCALCIURIC	1983	19 (323)
divistyramine	HYPOCHOLESTEROLEMIC	1984	20 (317)
lovastatin		1987	23 (337)
melinamide		1984	20 (320)
pitavastatin		2003	39 (282)
rosuvastatin		2003	39 (283)
simvastatin		1988	24 (311)
glucagon, rDNA	HYPOGLYCEMIA	1993	29 (338)
acipimox	HYPOLIPIDEMIC	1985	21 (323)
beclobrate		1986	22 (317)
binifibrate		1986	22 (317)
ciprofibrate		1985	21 (326)
colesevelam hydrochloride		2000	36 (300)
colestimide		1999	35 (337)
ezetimibe		2002	38 (355)
fluvastatin		1994	30 (300)
meglutol		1983	19 (321)
ronafibrate		1986	22 (326)
conivaptan	HYPONATREMIA	2006	42 (514)
mozavaptan		2006	42 (527)
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30 (303)
bucillamine	IMMUNOMODULATOR	1987	23 (329)
centoxin		1991	27 (325)
thymopentin		1985	21 (333)
filgrastim	IMMUNOSTIMULANT	1991	27 (327)
GMDP		1996	32 (308)
interferon gamma-1b		1991	27 (329)
lentinan		1986	22 (322)
pegademase bovine		1990	26 (307)
pidotimod		1993	29 (343)
romurtide		1991	27 (332)
sargramostim		1991	27 (332)
schizophyllan		1985	22 (326)
ubenimex		1987	23 (345)
cyclosporine	IMMUNOSUPPRESSANT	1983	19 (317)
everolimus		2004	40 (455)
gusperimus		1994	30 (300)
mizoribine		1984	20 (321)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
muromonab-CD3		1986	22 (323)
mycophenolate sodium		2003	39 (279)
mycophenolate mofetil		1995	31 (346)
pimecrolimus		2002	38 (365)
tacrolimus		1993	29 (347)
ramelteon	INSOMNIA	2005	41 (462)
defeiprone	IRON CHELATOR	1995	31 (340)
alosetron hydrochloride	IRRITABLE BOWEL	2000	36 (295)
tegasedor maleate	SYNDROME	2001	37 (270)
sulbactam sodium	b-LACTAMASE INHIBITOR	1986	22 (326)
tazobactam sodium		1992	28 (336)
nartograstim	LEUKOPENIA	1994	30 (304)
pumactant	LUNG SURFACTANT	1994	30 (308)
sildenafil citrate	MALE SEXUAL DYSFUNCTION	1998	34 (331)
gadoversetamide	MRI CONTRAST AGENT	2000	36 (304)
telmesteine	MUCOLYTIC	1992	28 (337)
laronidase	MUCOPOLYSACCARIDOSIS	2003	39 (278)
galsulfase	MUCOPOLYSACCHARIDOSIS VI	2005	41 (453)
idursulfase	MUCOPOLYSACCHARIDOSIS II (HUNTER SYNDROME)	2006	42 (520)
palifermin	MUCOSITIS	2005	41 (461)
interferon X-1a	MULTIPLE SCLEROSIS	1996	32 (311)
interferon X-1b		1993	29 (339)
glatiramer acetate		1997	33 (334)
natalizumab		2004	40 (462)
afloqualone	MUSCLE RELAXANT	1983	19 (313)
cisatracurium besilate		1995	31 (340)
doxacurium chloride		1991	27 (326)
eperisone HCl		1983	19 (318)
mivacurium chloride		1992	28 (334)
rapacuronium bromide		1999	35 (347)
tizanidine		1984	20 (324)
decitabine	MYELODYSPLASTIC SYNDROMES	2006	42 (519)
lenalidomide	MYELODYSPLASTIC SYNDROMES, MULTIPLE MYELOMA	2006	42 (523)
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20 (322)
tinazoline	NASAL DECONGESTANT	1988	24 (312)
aripiprazole	NEUROLEPTIC	2002	38 (350)
clospipramine hydrochloride		1991	27 (325)
nemonapride		1991	27 (331)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
olanzapine		1996	32 (313)
perospirone hydrochloride		2001	37 (270)
quetiapine fumarate		1997	33 (341)
risperidone		1993	29 (344)
sertindole		1996	32 (318)
timiperone		1984	20 (323)
ziprasidone hydrochloride		2000	36 (312)
rocuronium bromide	NEUROMUSCULAR BLOCKER	1994	30 (309)
edaravone	NEUROPROTECTIVE	1995	37 (265)
fasudil HCL		1995	31 (343)
riluzole		1996	32 (317)
varenicline	NICOTINE-DEPENDENCE	2006	42 (547)
bifemelane HCl	NOOTROPIC	1987	23 (329)
choline alfoscerate		1990	26 (300)
exifone		1988	24 (302)
idebenone		1986	22 (321)
indeloxazine HCl		1988	24 (304)
levacecarnine HCl		1986	22 (322)
nizofenzone fumarate		1988	24 (307)
oxiracetam		1987	23 (339)
bromfenac sodium	NSAID	1997	33 (329)
lornoxicam		1997	33 (337)
OP-1	OSTEOINDUCTOR	2001	37 (269)
alendronate sodium	OSTEOPOROSIS	1993	29 (332)
ibandronic acid		1996	32 (309)
incadronic acid		1997	33 (335)
raloxifene hydrochloride		1998	34 (328)
risedronate sodium		1998	34 (330)
strontium ranelate		2004	40 (467)
tiludronate disodium	PAGET'S DISEASE	1995	31 (350)
rasagiline	PARKINSON'S DISEASE	2005	41 (464)
rotigotine		2006	42 (538)
tadalafil	PDE5 INHIBITOR	2003	39 (284)
vardenafil		2003	39 (286)
temoporphin	PHOTOSENSITIZER	2002	38 (367)
verteporfin		2000	36 (312)
alefacept	PLAQUE PSORIASIS	2003	39 (267)
beraprost sodium	PLATELET AGGREG. INHIBITOR	1992	28 (326)
epoprostenol sodium		1983	19 (318)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
iloprost		1992	28 (332)
sarpogrelate HCl	PLATELET ANTIAGGREGANT	1993	29 (344)
trimetrexate glucuronate	PNEUMOCYSTIS CARINII PNEUMONIA	1994	30 (312)
solifenacin	POLLAKIURIA	2004	40 (466)
alglucosidase alfa	POMPE DISEASE	2006	42 (511)
histrelin	PRECOCIOUS PUBERTY	1993	29 (338)
atosiban	PRETERM LABOR	2000	36 (297)
gestodene	PROGESTOGEN	1987	23 (335)
nomegestrol acetate		1986	22 (324)
norgestimate		1986	22 (324)
promegestrone		1983	19 (323)
trimegestone		2001	37 (273)
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24 (297)
nafamostat mesylate		1986	22 (323)
adrafinil	PSYCHOSTIMULANT	1986	22 (315)
dexmethylphenidate HCl		2002	38 (352)
dutasteride		2002	38 (353)
efalizumab	PSORIASIS	2003	39 (274)
sitaxsentan	PULMONARY HYPERTENSION	2006	42 (543)
finasteride	5a-REDUCTASE INHIBITOR	1992	28 (331)
surfactant TA	RESPIRATORY SURFACTANT	1987	23 (344)
abatacept	RHEUMATOID ARTHRITIS	2006	42 (509)
Adalimumab		2003	39 (267)
dexmedetomidine hydrochloride	SEDATIVE	2000	36 (301)
ziconotide	SEVERE CHRONIC PAIN	2005	41 (473)
kinetin	SKIN PHOTODAMAGE/ DERMATOLOGIC	1999	35 (341)
tirilazad mesylate	SUBARACHNOID HEMORRHAGE	1995	31 (351)
APSAC	THROMBOLYTIC	1987	23 (326)
alteplase		1987	23 (326)
balsalazide disodium	ULCERATIVE COLITIS	1997	33 (329)
darifenacin	URINARY INCONTINENCE	2005	41 (445)
tiopronin	UROLITHIASIS	1989	25 (318)
propiverine hydrochloride	UROLOGIC	1992	28 (335)
Lyme disease	VACCINE	1999	35 (342)
clobenoside	VASOPROTECTIVE	1988	24 (300)
falecalcitriol	VITAMIN D	2001	37 (266)
maxacalcitol		2000	36 (310)
paricalcitol		1998	34 (327)
doxercalciferol	VITAMIN D PROHORMONE	1999	35 (339)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., (PAGE)
prezatide copper acetate	VULNERARY	1996	32 (314)
acemannan	WOUND HEALING AGENT	2001	37 (257)
cadexomer iodine		1983	19 (316)
epidermal growth factor		1987	23 (333)