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PREFACE

Annual Reports in Medicinal Chemistry continues to focus on providing timely and critical reviews of important topics in medicinal chemistry together with an emphasis on emerging topics in the biological sciences, which are expected to provide the basis for entirely new future therapies.

Volume 38 mostly retains the familiar format of previous volumes, this year with 35 chapters. Sections I - IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 37 on Alzheimers' disease and antiviral agents. There has been a change with regard to the content of two of the sections. Thus the section on Cardiovascular and Pulmonary agents in Volume 37 has been modified to Cardiovascular and Metabolic diseases in Volume 38, as many of the latter are risk factors for cardiovascular diseases. The second change involves the section on Immunology, Endocrinology and Metabolic Diseases in Volume 37 to Inflammatory, Pulmonary and Gastrointestinal Diseases in Volume 38, there being many mechanistic links across these areas. As in past volumes, annual updates have been limited only to the most active areas of research in favor of specifically focussed and mechanistically oriented chapters, where the objective is to provide the reader with the most important new results in a particular field.

Sections V and VI continue to emphasize important topics in medicinal chemistry, biology, and drug design as well as the critical interfaces among these disciplines. Included in Section V, Topics in Biology, are chapters on obesity therapeutics, SNPs, RNAi and immune cell signaling. Chapters in Section VI, Topics in Drug Design and Discovery include enzyme induction, protein-protein interactions, virtual ligand screening, cheminformatics and chemogenomics, and SAR studies with bioisosteric groups.

Volume 38 concludes with To Market, To Market - a chapter on NCE and NBE introductions worldwide in 2002. Last, but not least, there are chapters on the impact of Climate Change on Health and another on Pharmaceutical productivity. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in Volumes 1-38 of this series.

Volume 38 *of Annual Reports in Medicinal Chemistry* was assembled again with the superb editorial assistance of Ms. Nadège Pingray and I would like to thank her for her hard work and enduring support. I have continued to work with innovative and enthusiastic section editors and my sincere thanks go to them again this year. I hope that you the reader will enjoy and profit from reading this volume.

Annette M. Doherty
Fresnes, France
May 2003

SECTION I. CENTRAL NERVOUS SYSTEM DISEASES

Editor: David W. Robertson, Pfizer Global Research & Development
Ann Arbor, MI 48105

Chapter 1. Current and Emerging Opportunities for the Treatment of Neuropathic Pain

John A. Butera and Michael R. Brandt
Wyeth Research
CN 8000, Princeton, NJ 08543-8000

Introduction - Neuropathic pain is characterized by abnormal pain sensations, including spontaneous pain, hyperalgesia (i.e., increased sensitivity to a noxious stimulus) and allodynia (i.e., increased sensitivity to a non-noxious stimulus) that typically lack an apparent physiologic function. In general, neuropathic pain is chronic and is refractory to current pharmacotherapies. Numerous recent advancements have contributed to a better, though still not complete understanding of the physiology and neurobiology of pain. It is now appreciated that many distinct mechanisms contribute to the development and maintenance of neuropathic pain. Some of these mechanisms have strong preclinical and clinical rationale as small molecule targets for neuropathic pain conditions. Compounds selective for these targets could potentially offer improved pain relief with fewer adverse effects compared to currently available treatments. The goal of the current review is to highlight small molecules with potential for treating neuropathic pain.

N-METHYL-D-ASPARTATE RECEPTOR (NMDAR) MODULATORS

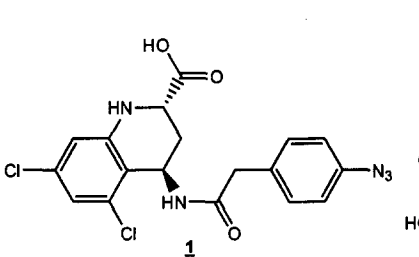
Numerous studies have demonstrated a role for excitatory amino acids in the development and maintenance of chronic neuropathic pain (1,2). Increased afferent input can lead to central sensitization via release of glutamate within the spinal cord (3,4). A significant medicinal chemistry effort has identified numerous molecules that either inhibit the release or block the effects of glutamate. The NMDAR is a ligand-gated ion channel containing numerous regulatory sites including glutamate, glycine, polyamine, Mg^{++} and PCP binding sites, all of which modulate channel activity. In addition to multiple regulatory sites, NMDARs are hetero-oligomers consisting of NR1 subunits, of which there are eight identified splice variants, plus a combination of NR2A-D subunits. Importantly, the pharmacology and ion gating properties of NMDAR channels are substantially altered with different combinations of NR1 and NR2 subunits (5). Recently NR3A and NR3B subunits have been identified, which confer distinct channel activity when combined with NR1 (6). Moreover, NMDAR subunits are differentially distributed among pain pathways in the central nervous system suggesting that specific subunits might preferentially modulate pain signaling (7). These aspects of NMDARs have provided numerous approaches for small molecule design (5).

NMDAR Glutamate Site Antagonists - Preclinical and clinical studies demonstrate that competitive glutamate antagonists reverse hypersensitivity associated with neuropathic pain states (8,9). In general, most competitive glutamate site antagonists contain phosphono amino acids separated from carboxyl groups by four or six atoms. One such example, selfotel (cis-4-(phosphono-methyl)-2-piperidine carboxylic acid), reversed mechanical hypersensitivity in a spinal cord ischemia model; however, these effects occurred at doses that also produced motor impairments. Although some glutamate antagonists (e.g., selfotel) were advanced

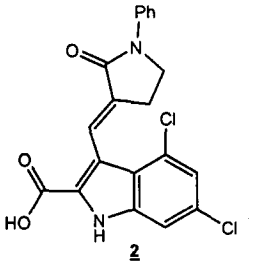
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to clinical studies for indications other than pain (i.e., stroke), adverse effects caused the discontinuation of these programs. The failure of glutamate antagonists might be due to the highly conserved glutamate-binding pocket on NR2 subunits and subsequent difficulty obtaining selectivity among NR2 subunits (10). However, studies indicate specificity among subtypes occurs with some molecules. For example, *D*-(-)-(*E*)-4-(3-phosphonoprop-2-enyl) piperazine-2-carboxylic acid (CPPene) and selfotel exhibit 20 to 40-fold subunit selectivity for NR1/NR2A or NR2B over NR1/NR2C or NR2D (11). However, little separation is typically observed between NR1/NR2A over NR1/NR2B, which might be important for obtaining improved adverse effect profiles. More recently, conantokin G, a peptide venom from the marine cone snail *Conus geographus*, was characterized as a NR1/NR2B selective glutamate antagonist (12). Characterization of the interaction of this peptide with specific residues of the NR2B subunit might lead to novel competitive glutamate antagonists (13).

NMDAR Glycine Site Antagonists – NMDARs require glutamate, as well as the co-agonist glycine, for channel activation. Thus, an alternative approach for modulating channel activity has been to develop selective glycine site antagonists (14). Although many glycine antagonists apparently lack NR2 subunit selectivity, which might be related to the glycine-binding site residing on the widely distributed NR1 subunit, the azido-probe **1** (CGP-51594) has been described as a glycine antagonist having 10-fold higher selectivity for NR1/NR2B subunits than for NR1/NR2A subunits (15).



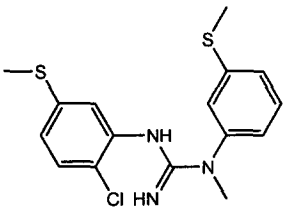
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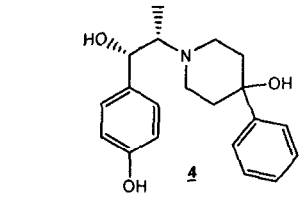
Activity of **1** for blocking pain has not been published, however GV-196771A (**2**) reversed chronic constriction injury (CCI) of the sciatic nerve-induced thermal and tactile hypersensitivity after p.o. doses of 1-10 mg/kg (16). In clinical trials, compound **2** significantly reduced static and dynamic allodynia associated with neuropathic pain yet did not reduce evoked pain intensity or produce pain relief (17). The reason for its lack of efficacy in humans is unclear.

NMDAR Channel Blockers - Clinically available NMDAR antagonists bind within the channel itself and block the flow of ions in a use-dependent manner. Ketamine is a dissociative anesthetic that reverses hypersensitivity in preclinical and clinical neuropathic pain states (8,9). However, the narrow separation between efficacy and adverse effects has hampered the utility of ketamine for the treatment of neuropathic pain. Memantine is a low affinity channel blocker that displaces [^{3}H]-MK-801 binding from rat membranes with a K_i of approximately 1 μM (18). Memantine was effective in reversing CCI-induced thermal and SNL-induced tactile hypersensitivity (18). Differences in the adverse effect profile of these non-competitive antagonists are attributed to the affinity and voltage dependency for which they bind within the channel (18). CNS 5161 (**3**) is another channel blocker that has a K_i of 1.8 nM in its ability to displace [^{3}H]-MK-801 binding from rat membranes (19). Although preclinical data of **3** are not published, its mechanism of action suggests it would be efficacious in neuropathic pain models.

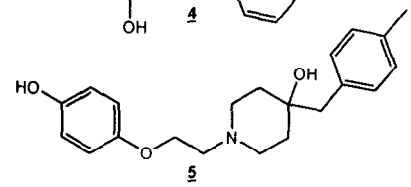


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NMDAR Polyamine-like Antagonists - Based on localization, biochemical and pharmacological data, it has been hypothesized that NR2B subunits preferentially mediate pain transmission. Traxoprodil (CP-101606, **4**) has a binding affinity (K_d) of 10 nM and dose-dependently reversed L5/L6 sciatic nerve ligation (SNL)-induced mechanical hypersensitivity (3 - 10 mg/kg; i.p.). A 10-fold higher dose did not produce disturbances in motor coordination (20,21). More recently synthesized benzamide derivatives have greater than 20,000-fold selectivity for recombinant NR1/NR2B receptors than for NR1/NR2A receptors (22). Related piperazine **5** had good oral bioavailability as indicated by ED_{50} values of 5.5 to 16 mg/kg for reversing carrageenan-induced hypersensitivity (23). These findings suggest that NR2B selective antagonists might have clinical utility in treating neuropathic pain with reduced adverse effects compared to non-selective antagonists.



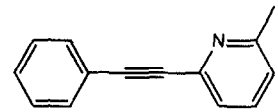
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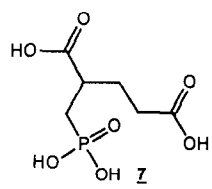
OTHER GLUTAMATE MECHANISMS

Metabotrophic Glutamate Receptor (mGluR) Modulators - Metabotropic glutamate receptors consist of eight different subunits of G protein coupled receptors that are classified into three groups [group I receptors (mGluR 1,5), group II (mGluR 2,3) and group III (mGluR 4,6,7,8)] based on sequence homology, coupling to intracellular messengers and pharmacologic profile (24,25). Recently, the mGluR5 selective compound **6** (MPEP) was identified and shown to reverse mechanical hypersensitivity in the Freund's complete adjuvant (FCA) inflammatory pain model without modifying the magnitude of edema at p.o. doses between 10 and 30 mg/kg (26). However, a higher dose of 100 mg/kg p.o. failed to reduce mechanical hypersensitivity in a neuropathic pain model (26). While these data suggest the potential for Group 1 antagonists in inflammatory pain, their weak effects for reversing established allodynia in neuropathic pain models suggest that they might have limited potential as clinical pharmacotherapies (27,28). Recently described mGluR1 and mGluR2 ligands might provide additional insight into their clinical utility (25).

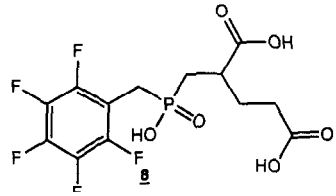


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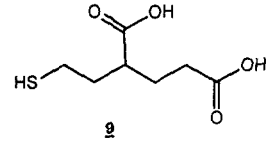
NAALADase Inhibitors - Glutamate carboxypeptidase II (GCP II; also termed N-acetylated-α-linked-acidic dipeptidase or NAALADase) is a membrane bound enzyme that hydrolyses N-acetyl-L-aspartyl-L-glutamate (NAAG) to form N-acetyl-aspartate and glutamate (29). In this regard, GCP II terminates the agonist activity of NAAG at mGluR3 and liberates glutamate. Importantly, the release of NAAG and hydrolysis to glutamate appears to increase under conditions of neuronal excitability observed in some neurological diseases. Selective GCP II inhibitors, such as 2-PMPA (**7**), have been shown to have antiallodynic and antihyperalgesic effects in a number of pain models following i.t. or i.v. administration (30). Another GCPII



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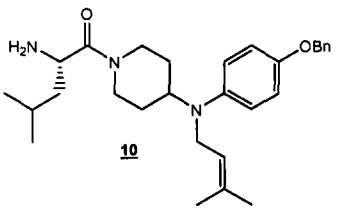
9

inhibitor, GPI-5232 (**8**) partially blocked the development of thermal hypersensitivity following daily dosing i.p. in a genetic model of insulin-dependent Type I diabetes (31). Other compounds such as thiol **9** were recently synthesized and inhibit GCP II activity (32,33).

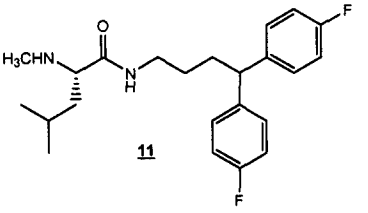
OTHER ION CHANNEL MECHANISMS

Ion channels, a family of diverse, membrane-bound proteins, provide a plethora of potential targets for design of novel pharmacotherapeutics. Modulation of these proteins by endogenous ligands or transmembrane voltage plays a predominant role in regulating cellular processes that govern excitability. The proven clinical efficacy of ion channel modulators coupled with recent studies demonstrating altered expression of channels in neuropathic pain models, has fueled efforts to design channel-based therapeutics for alleviating neuropathic pain.

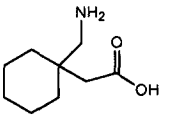
N-Type Calcium Channel Modulators – Voltage-gated calcium channels (VGCCs) modulate excitability of nociceptive sensory neurons in the dorsal horn of the spinal cord, and appear to be involved in the development and maintenance of neuropathic pain (34,35). VGCCs are classified into three major categories based upon their electrophysiologic and pharmacologic properties: high voltage-activated (L-, N-, P-, and Q-types), intermediate voltage-activated (R-type) and low voltage-activated (T-type) (36). N-type VGCCs are expressed mainly on dendrites and pre-synaptic terminals, suggesting a role for these channels in neuropathic pain. Consistent with this idea, knockout of the N-type $Ca_V2.2$ gene in mice decreased the magnitude of inflammatory and neuropathic pain behaviors (37). Ziconotide (SNX-111), an amino acid ω-conotoxin peptide, is a selective N-type VGCC blocker with preclinical and clinical effects (38). Related peptide toxins isolated from *Conus* venoms have recently been reported. For example, CNVIIA, a congener of ziconotide, binds selectively with a K_d of 36.3 pM (39). Another amino acid derivative (AM336), isolated from *Conus cactus*, evoked a dose-dependent antinociception (ED_{50} = 0.11 nmol) after i.t. administration in the rat hind paw model (40). Although its efficacy was comparable to SNX-111, AM336 did not exhibit a biphasic dose-response curve like SNX-111; most likely due to its enhanced selectivity for the N-type VGCC.



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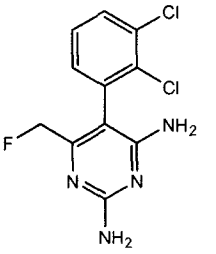


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Two recently reported non-peptidic N-type VGCC blockers (**10** and **11**) possessed antinociceptive effects in pain models. Compound **10** had anti-writhing effects with an ED_{50} = 6 mg/kg (i.t.) in rats. Compound **11** (IC_{50} = 1.5 μM in IMR32 assay) was efficacious in the anti-writhing (ED_{50} = 4.5 mg/kg, i.v.), SNL (ED_{50} = 23 μg, i.t.), and formalin (ED_{50} = 16 mg/kg, i.v.) pain models (41). However, both compounds also posses modest Na^+ channel blocking properties (42). Gabapentin (**12**) represents another class of VGCC modulators, although the mechanism of action is not fully understood. It appears to bind to α2/δ auxiliary subunits of VGCCs, thus down-regulating neurotransmitter release, an effect that might be related to its clinical utility in neuropathic pain states (43). More recently, pregabalin, ((*S*)-3-aminomethyl-5-methyl-hexanoic acid) a related α2/δ -selective binder, has shown efficacy for various conditions associated with pain, seizures, and anxiety (44).

Sodium Channel Modulators - Blockers of voltage-gated Na^+ channels (VGSC) have analgesic and anesthetic properties caused by inhibiting the initiation and propagation of action-potentials (35). Most inhibitors of VGSCs show a strong voltage-dependent block, meaning that they inhibit high-frequency repetitive activity without altering normal propagation of action potentials (45).

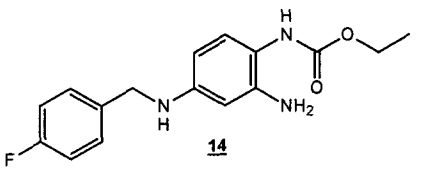
Many clinically used anticonvulsants (e.g., carbamazepine) that block VGSC also have utility for treating neuropathic pain (46). Although these compounds alleviate pain symptoms, they are not widely used due to limited separation between efficacy and adverse effects, likely related to their lack of selectivity among VGSC subtypes. Consequently, synthesis efforts have focused on identifying subtype-selective VGSC blockers. Multiple VGSC subtypes are expressed in DRG neurons including rapidly inactivating, TTX-sensitive (TTX-S), and slowly inactivating, TTX-resistant (TTX-R; $Na_V1.8$ and $Na_V1.9$) channels (47). Expression of TTX-R channels is restricted predominately to sensory neurons (48). Antisense oligodeoxynucleotide knockdown of the expression of $Na_V1.8$ reversed SNL-induced allodynia and hyperalgesia (49). $Na_V1.9$ has recently been implicated in hyperexcitability after nerve injury (50). Although highly selective TTX-R compounds have not been reported, BW 4030W92 (**13**) shows slight selectivity for this subtype of NaCh (51). Several recent patent applications have disclosed VGSC blockers that were active in neuropathic pain models (52). However, selectivity for other channels was not disclosed (53).



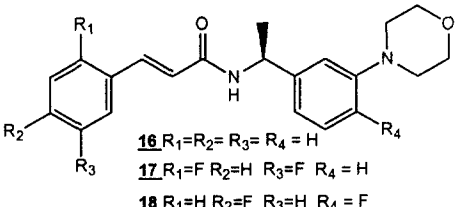
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Potassium Channel Modulators - Voltage gated K^+ channels play an important role in conditions of aberrant or excessive excitability, such as epilepsy and neuropathic pain. Activation of these channels results in hyperpolarization of the cell membrane and subsequent decrease in neuronal excitability. The role for K^+ channel modulators for treating CNS disorders has been recently reviewed in this series (54).

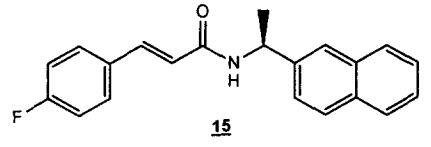
KCNQ channels are a family of channels containing at least five K^+ channel genes that have been linked to benign familial neonatal convulsions in humans (55). These channels regulate the "M-current", a K^+ current that is inhibited by muscarine thereby increasing neuronal excitability. The $KCNQ_2/KCNQ_3$ heteromultimeric channel is expressed predominately in neurons, whereas $KCNQ_3/KNCQ_4/KNCQ_5$ channels are found in other tissues. Retigabine (**14**) is a non-selective KCNQ channel opener that dose-dependently inhibited pain behaviors in models of hyperalgesia and neuropathic pain. A dose of 10 mg/kg of **14** was comparable to the effect caused by 60 mg/kg of oral gabapentin (56). Compounds **15**-**18** represent a novel series of cinnamide-based KCNQ openers, which significantly reversed SNL-induced hypersensitivity at i.v. doses between 3 and 10 mg/kg (57).



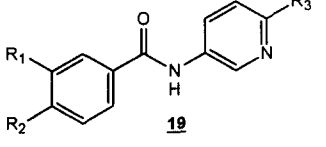
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16 $R_1=R_2=R_3=R_4=H$
17 $R_1=F$ $R_2=H$ $R_3=F$ $R_4=H$
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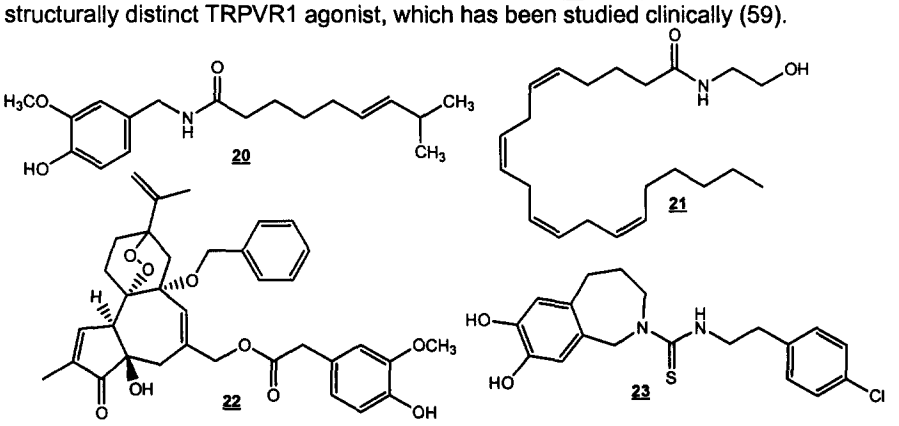
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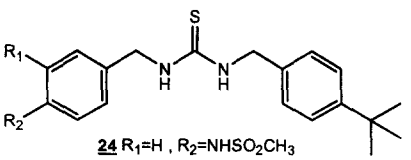
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Compounds in a series of pyridyl-benzamides (**19**) disclosed as selective $KCNQ_2/KCNQ_3$ openers were reported to be effective at prolonging the latency to lick in the rat hind paw model at p.o. doses between 10 and 100 mg/kg, although specific data for compounds were not revealed (58).

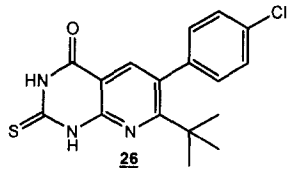
Vanilloid Receptor Modulators – The vanilloid receptor (VR1) is a member of the transient receptor potential (TRP) family of non-selective cation channels. Capsaicin (**20**), a natural product derived from hot peppers, acts as an agonist at vanilloid receptors (TRPVR1) located on primary afferent nociceptors (59). In addition to being sensitive to **20**, TRPVR1 is sensitive to protons and heat thus triggering a robust Ca^{2+} influx and subsequent depolarization. Prolonged activation can produce desensitization and a subsequent decrease in activity of nociceptor fibers. Thus, topical application of **20** has been used to treat pain, skin itch, and psoriasis, as well as other systemic diseases (60). Although specific ligand-binding domains for **20** have not been published and the endogenous ligands have not been definitively identified, recent studies demonstrate that the endogenous cannabinoid ligand anandamide (**21**) has agonist effects at TRPVR1 receptors (61). Numerous related fatty-acid analogs of **21**, which also have modulatory effects on TRPVR1 have recently been described (62). Resiniferatoxin (**22**) represents yet another, structurally distinct TRPVR1 agonist, which has been studied clinically (59).



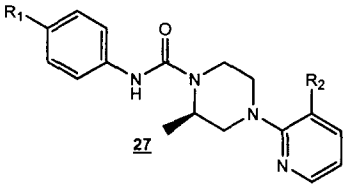
The physiology and pharmacology of TRPVRs suggest that both agonists and antagonists might be useful for treating painful conditions. Peripheral TRPVR1s are expressed primarily on unmyelinated C-fibers; however, reported increased expression of TRPVR1 on A-fibers following nerve injury suggests a greater role for these receptors for the modulation of neuropathic pain (63). Studies support the view that TRPVR1 antagonists modulate sensitization under pathophysiologic conditions of noxious stimuli (noxious heat and proton activation), but not under normal physiologic conditions (64). For example, capsazepine (**23**) has been shown to inhibit capsaicin-mediated nocifensive behaviors in rodents (65). Although early studies with **23** in rat neuropathic pain models suggested limited activity, more recent studies in guinea pig neuropathic pain models indicate greater activity of **23** (64,66). Numerous series of very closely related urea derivatives have recently been reported as TRPVR1 antagonists possessing activity in rodent pain models. Compounds **24** and **25** represent examples in a series of thioureas that reportedly decreased writhing by greater than 90% at i.p. doses between 3 and 10 mg/kg in the mouse writhing test (67). The pyrido-pyrimidinone compound **26** (IC_{50} = 65 nM) dose-dependently reduced mechanical hyperalgesia after p.o. doses between 0.3 and 30 mg/kg (68). Finally, a series of 2-pyridinyl-piperazine derived ureas and ethylene-diamine-derived ureas, illustrated by structures **27** and **28**, respectively, were claimed as potent TRPVR1 antagonists (69,70).



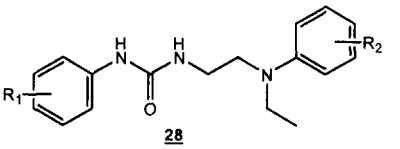
24 R_1=H , R_2=$NHSO_2CH_3$
25 R_1=Cl , R_2=$NHSO_2CH_3$



26

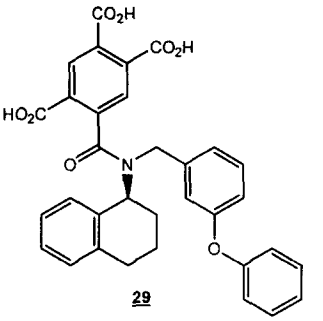


27



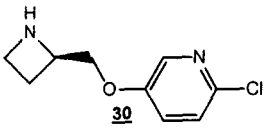
28

P2X Receptor Antagonists – P2X receptors are a family of ATP-gated non-selective ion channels of which seven P2X subunits ($P2X_{1-7}$) have been cloned (71). ATP is the endogenous ligand and produces intense pain when injected intradermally (72). Adenine nucleotide derivatives such as oxidized ATP and TNP-ATP have been reported to possess some P2X sub-type specificity and to produce antinociceptive effects in animal pain models (73,74). Although non-selective, P2 receptor antagonists such as suramin and PPADS were shown to produce antinociceptive responses in neuropathic pain models (71,72,75). The structurally novel, non-nucleotide $P2X_3$- and $P2X_{2/3}$-antagonist (K_i = 22-92 nM) A-317491 (**29**) was reported to reverse CCI-induced mechanical and thermal hypersensitivity with ED_{50} values between 10 and 15 μg/kg s.c (76). The effects were stereospecific, as the corresponding *R*-enantiomer (A-317344) was inactive.

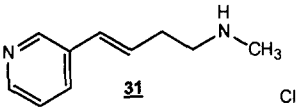


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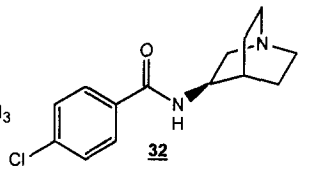
Nicotine Receptor Modulators – Activation of neuronal nicotinic acetylcholine receptors (nACHR) has been associated with analgesic effects in neuropathic pain models (77). The α4β2-selective nACHR agonist ABT-594 (**30**) had antinociceptive effects in animal pain models with a reduced side-effect profile compared to nicotine (35). Pyridinyl derivative TC-2403 (**31**) represents another potent α4β2-selective



30



31



32

nACHR agonist with potential for a better toxicity profile (78). Recent studies suggest that the α7 nACHR also might modulate pain transmission in rodent models (79). PNU-282987 (**32**) represents a new quinuclidine-derived α7 nACHR antagonist with a reported binding K_i of 26 nM, although no data were reported on its effects in pain models (80).

NON-ION CHANNEL MECHANISMS

Due to the overwhelming number of emerging neuropathic pain targets, it became necessary to focus and restrict the current review primarily to ion-channel classes of therapeutics. Other targets, some equally compelling as those highlighted above, are also emerging. Recent advances in the area of G-protein

coupled receptors (GPCRs) offer additional strategies for the design of effective agents. Galanin, muscarinic, prostanoid, adenosine, cannabinoid, opioid, neuropeptide Y, cholecystokinin, neurokinin, bradykinin, and calcitonin gene-related peptide receptors have been studied as neuropathic pain targets with varying degrees of success (81-91). Neurotrophins, a family of growth factors important for differentiation, growth and survival of neurons, have been shown to be neuroprotective in damaged sensory neurons and as such, might be attractive targets for the treatment of neuropathic pain (92). Studies have demonstrated that serotonin has antinociceptive effects under some conditions in normal animals and anti-allodynic/anti-hyperalgesic effects in some models of neuropathic pain (93-94). Together, these studies support a substantial role of GPCRs and intracellular signaling proteins in conditions of neuropathic pain.

Concluding Remarks – Neuropathic pain affects over 26 million patients worldwide, resulting in over $3 billion per year spent on drug therapies; most of which were developed for treating other conditions. Current approaches to treat refractory neuropathic pain are often minimally effective or limited in utility due to non-specificity at the molecular level, thereby causing serious adverse effects and reduced patient compliance. The ever increasing targets and approaches reviewed in this chapter are a result of an unprecedented increase in the understanding of the underlying pharmacology, physiology, and etiology of pain mechanisms and represent the current and future approaches in neuropathic pain management. There is still a substantial need for better-designed clinical studies and well-defined models of pain to more fully evaluate the potential of these emerging pharmaceuticals in terms of their long-term efficacy and potential side effects. As additional breakthroughs in pain biology evolve, we can look forward to a plethora of novel, more effective approaches for neuropathic pain management.

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Chapter 2. Neuropeptide Receptor Antagonists for CNS Disorders

John H. Kehne, George D. Maynard, Stéphane De Lombaert, and James E. Krause
Neurogen Corporation, 35 N.E. Industrial Road, Branford, CT 06405

Introduction – Drug discovery efforts have yielded an abundance of potent and selective non-peptidic ligands since the publication a decade ago of a report on promising neuropeptide receptor targets for CNS disorders (1). This chapter focuses on key neuropeptides which have attracted considerable attention for their potential utility in CNS disorders, including the neurokinin (NK) peptides, neuropeptide Y (NPY), corticotropin releasing factor (CRF), and melanin-concentrating hormone (MCH). As summarized in Table 1, efforts to produce antagonists have matured considerably for some of these peptide receptors, to the point of clinical evaluation and even registration, whereas for others, progress is still at the preclinical stage. Recent accomplishments and milestones in these drug design efforts will be highlighted in this chapter as well as progress for the emerging targets that hold considerable promise for the future.

Table 1. Non-peptidic antagonists for NK, CRF, NPY, and MCH receptors

Target	Therapeutic indication	Representative antagonists	Affinity (nM) (IC_{50} or K_i)	Status	Ref.
NK1	Emesis, Depression	Aprepitant (Emend™; MK-869; L-754030)	0.09	Reg.*	(2)
	Depression, IBS^	CP-122,721	0.14	Clin.	(2)
	IBD#	Nolpitanium besilate (SR-140333)	*0.04*	Clin.	(2)
NK2	Depression, Anxiety, IBS	Saredutant (SR-48,968)	*0.5*	Clin.	(2)
	Asthma, IBS	Nepadutant (MEN 11420)	2.6	Clin.	(2)
NK1/ NK2	Asthma, COPD+, Rhinitis	DNK-333	*4.8/ 5.5*	Clin.	(3)
NK3	Schizophrenia	Osanetant (SR-142,801)	0.42	Clin.	(2)
	Schizo., COPD	Talnetant (SB-223412)	1.0	Clin.	(2)
CRF1	Depression, Anxiety, Stress Disorders	R121919	2.8	Clin.#	(4)
		DMP696	1.7	Preclin.	(5)
		CP-154526	2.7	Preclin.	(6)
		Antalarmin	1.4	Preclin.	(6)
		NGD 98-1	4.2	Preclin.	(7)
		SSR125543A	2.0	Preclin.	(8)
NPY1	Obesity	J-115814	1.4	Preclin.	(9)
NPY5	Obesity	CGP 71683A	2.9	Preclin.	(10)
		FR252384	2.3	Preclin.	(11)
		NPY5RA-972	3.0	Preclin.	(12, 13)
MCH1	Obesity	SNAP-7941	0.18	Preclin.	(14)
		T-226296	*5.5*	Preclin.	(15)
MCH2	Obesity	None described			

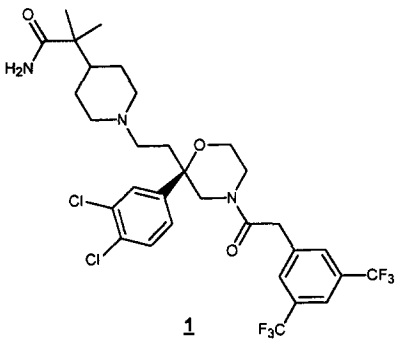
*Chemotherapy-induced nausea & emesis; ^Irritable bowel syndrome; #Inflammatory bowel disease; +Chronic obstructive pulmonary disorder; #No further development activity reported

NK Receptor Antagonists - The mammalian tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) bind and activate NK receptors with SP preferring NK1, NKA preferring NK2 and NKB preferring the NK3 receptor. Preclinical findings suggest utility of tachykinin receptor antagonists in diverse disorders including chemotherapy-induced emesis, anxiety and depression, migraine, pain, IBD, micturition disorder and asthma. Accordingly, a considerable effort has identified an impressive variety of subtype selective and non-selective

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tachykinin receptor antagonists (2). New preclinical and clinical studies with a subset of the available tachykinin receptor antagonists have significantly contributed to clarifying the therapeutic utility of tachykinin receptor antagonists. Further, several useful small molecule antagonists have been described recently.

A milestone accomplishment in tachykinin research was the U.S. registration of the NK1 antagonist, aprepitant, for treating chemotherapy-induced nausea and emesis (2). Recent clinical studies showed that aprepitant monotherapy is less effective than the 5-HT3 antagonist, ondansetron, in preventing acute emesis but more effective in reducing delayed emesis (16). Aprepitant, in combination with ondansetron and dexamethasone, provided superior relief from both acute and delayed emesis. The centrally-acting NK1 antagonist, CP-122,721, reduced post-operative nausea and emesis when used in combination with ondansetron (2, 17). Other centrally-acting NK1 antagonists include **1** (SSR240600), which shows high affinity (K_i = 0.006 nM) for the human NK1 receptor (18).



1

Efforts to advance NK1 antagonists for anxiety and depression continue, but have been complicated by the characteristic high failure rate of depression trials. Although the initial depression trial reported that aprepitant and paroxetine had similar efficacy, a follow up dose-finding study failed to differentiate either agent from placebo. Similar results were found for a backup compound with similar potency and brain penetration (19). The available data suggest that NK1 antagonist therapy requires a similar time to onset of action versus SSRIs, athough the reported low incidence of sexual side-effects with NK1 antagonists represents a potential advantage.

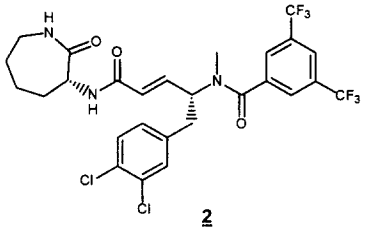
PET studies with aprepitant and [^{18}F]-SPA-RQ, a brain penetrant, selective, and highly potent (K_i = 0.04 nM) ligand, have demonstrated that high levels of NK1 receptor occupancy (>90%) were associated with significant antidepressant and antiemetic effects, whereas lower receptor occupancy was associated with reduced efficacy (20). These studies have also established that high levels of central NK1 receptor occupancy were achieved with aprepitant in earlier negative clinical trials for pain. A non-CNS penetrant NK1 antagonist, nolpitanium besilate (SR-140,333), is under investigation for several indications, including IBD and food allergy (2, 21).

New studies have further defined the therapeutic potential of NK2 receptor antagonists. In asthmatics, the NK2 antagonist, saredutant, was ineffective in reducing airway hyper-responsiveness to adenosine, while producing no benefit with respect to airway function (2, 22). Interestingly, preclinical studies suggest utility of saredutant in anxiety and depression (23, 24). Further new data support the presence of NK2 receptors in both rodent and human brain (25, 26).

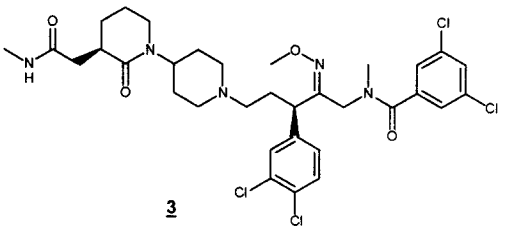
Additional NK2 receptor antagonists include nepadutant, presently pursued as a possible treatment for IBS and asthma. This compound was shown clinically to inhibit NKA-stimulated, but not basal, gastrointestinal motility (2, 27). Furthermore, oral doses of nepadutant have reduced diarrhea induced by bacterial toxins in mice, acetic acid irritation-induced motility in rats, and acetic acid-induced rectocolitis in guinea pigs (28, 29).

Dual NK1/NK2 receptor antagonists continue to be of interest, especially for respiratory diseases. Compound **2** (DNK-333), a potent dual antagonist (NK1/2 IC_{50}

= 4.8/5.5 nM) reduced NKA-induced bronchoconstriction in asthmatics following oral administration (3, 30). A new antagonist, **3** (SCH-206272), displays similar potency across NK1, NK2 and NK3 receptors with K_i = 1.3, 0.4, 0.3 nM, respectively (31).

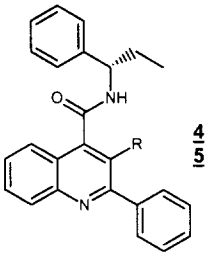


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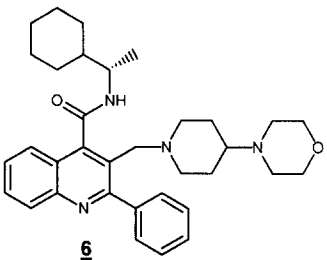
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Regarding the therapeutic utility of NK3 receptor antagonists, osanetant displayed antipsychotic activity in a recent clinical trial, in accord with preclinical data suggesting that central NK3 receptor blockade modulates dopaminergic, noradrenergic and serotonergic activity (2, 32-35). Furthermore, immunohistochemical studies demonstrated the presence of NK3 receptors in human brain regions associated with schizophrenia (36, 37).



4 R = CH_3
5 R = OCH_2CO_2H

Another clinically useful NK3 antagonist, talnetant, may define further the therapeutic utility of NK3 antagonists in a variety of indications (2). New talnetant analogs could be useful pharmacological tools: **4** (SB-222200) with a K_i of 4.4 nM, achieved enhanced brain penetration, while **5** (SB-235375), with a K_i of 2.2 nM, displayed reduced CNS exposure (38, 39). New dual NK2/NK3 receptor antagonists include **6** (SB-400238), with NK2 K_i = 0.8 nM, and NK3 K_i = 0.8 nM (40).



6

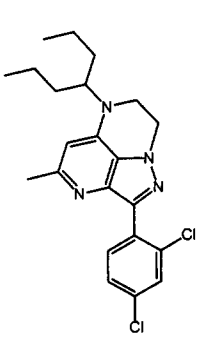
CRF Receptor Antagonists - CRF and CRF receptors play a key role in mediating in the body's response to stress, and as such, have provided compelling targets for novel pharmacological approaches to depression, anxiety, and stress disorders (7, 41). Major industry efforts have identified potent and selective antagonists for the CRF1 receptor, as summarized in recent reviews (4, 7, 41, 42).

Since the report of antidepressant activity with R121919 in an open-label, Phase 2 trial in depressed patients, no new clinical results with CRF1 antagonists have been published (7). However, advances in CRF neurobiology have further validated this target, including a strengthening of CRF's role in a primate model of depression and demonstrations of CRF interactions with known brain sites of action of antidepressants and anxiolytics such as the dorsal raphe and amygdala (43-45). In addition, preclinical studies expanded the profiles of key CRF1 antagonists in Table 1 (6, 8, 46-52). Findings gleaned from these studies which are relevant to drug discovery efforts include the following: (a) Consistent *in vivo* actions of acute CRF1 antagonists include attenuating the behavioral effects of icv CRF administration, and blunting HPA axis stimulation produced by stress or peripherally-administered CRF. (b) Efficacy of CRF1 antagonists in selected anxiolytic, antidepressant, antistress assays has been reported, though there are inconsistencies in outcomes across laboratories which may relate to experimental factors such as levels of stress, strain/species used, etc. (c) *Ex vivo* receptor binding

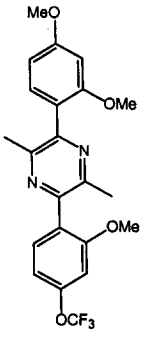
techniques have estimated CRF1 occupancy in rat cortex ranging from 50% to 85% at efficacious doses (51, 52). (d) Chronic dosing studies report sustained efficacy, but a tolerance to the acute effects of stress on the HPA axis, suggesting that chronic CRF1 antagonism may not adversely affect HPA axis (46, 53).

Preclinical studies provided further support for the utility of CRF1 antagonists in other therapeutic indications. These include stress-related gastrointestinal disorders (IBS, gastric ulceration), stroke, drug addiction, and inflammatory disorders (54-59).

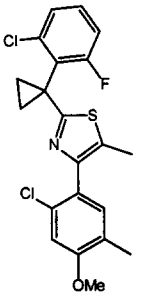
A topological description summarized drug design strategies for identifying potent, non-peptidic CRF1 antagonists (7). New analogs have resulted from a systematic effort aimed at conformationally constraining the top side-chain of known bicyclic templates. The tricyclic compound **7** is a representative example of this large class of structures (60). Chemical structures, such as **8-10**, which are beginning to diverge from the classical CRF1 receptor antagonist pharmacophore, have also emerged recently from the patent literature (61-63). The biological activities of these compounds have not been disclosed.



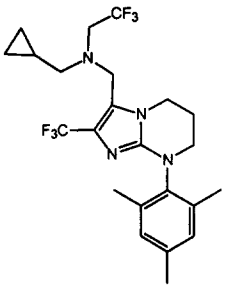
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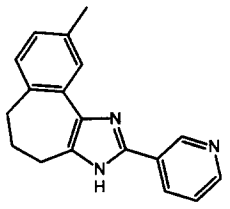


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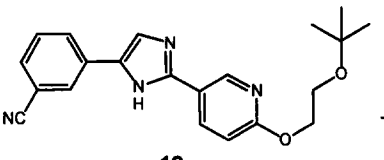
Progress has been reported in developing non-peptidic CRF1 radioligands (64, 65). *In vitro*, [^{3}H]-SN003 reveals specific CRF1 binding in rat brain tissues, which was consistent with known CRF1 receptor distributions (65).

NPY Receptor Antagonists - Since its discovery as a highly potent feeding stimulant, NPY has been considered an attractive target for the treatment of obesity. Pharmacological studies suggested that, among the six characterized NPY GPCR subtypes, the hypothalamic Y1 and Y5 receptors most likely played a critical role in the NPY regulation of appetite, food intake, and energy expenditure. However, experiments with genetic models have been inconclusive in this regard (66-70).

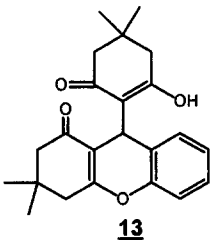
In the last year, several reports have challenged the importance of the Y5 receptor in NPY-induced feeding. The imidazole **11** (FR252384) is a potent (K_i = 2.3 nM), orally active, and CNS penetrant Y5 antagonist, but its levels in the brain poorly correlated with reductions in food intake (11).



11

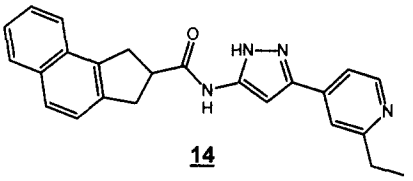


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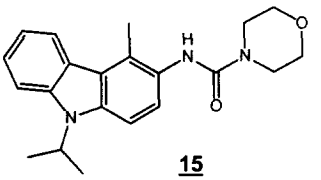


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Another imidazole, **12** (K_i = 1.2 nM) was orally active in blocking the orexigenic effect of a selective Y5 agonist (bPP) injected icv in rats. Again, despite achieving adequate plasma, CNS, and CSF exposure, **12** failed to demonstrate efficacy in natural feeding models (71). Similarly, the selective Y5 antagonists **13** and **14** (IC_{50} = 26 and 3.5 nM, respectively) prevented or attenuated bPP-induced feeding in rats, but remained ineffective in inhibiting the orexigenic effect of NPY (72, 73). The carbazole **15** (NPY5RA-972) is another potent (IC_{50} = 3 nM) and highly selective Y5 antagonist. When administered orally to rats, its plasma, brain and CSF concentrations largely exceeded its IC_{50}. While **15** blocked the feeding behavior elicited by icv injection of a selective peptidic Y5 agonist, it did not affect spontaneous feeding or fasting-induced feeding in rats (12, 13).



14



15

The specificity of the anorectic effect of CGP 71683A, known as the first potent (IC_{50} = 2.9 nM), non-peptidic Y5 antagonist to be disclosed, was recently challenged, considering its newly uncovered affinity at α2-adrenergic and muscarinic receptors, at the serotonin reuptake site, and its dose-related inflammatory side-effect in animals (74). Other Y5 antagonists reduced food consumption in a fasting-induced feeding model in rats, but the specificity of these effects is unclear (75, 76).

In contrast to the abundance of Y5 antagonists, only a few potent, selective, orally bioavailable, and brain penetrant Y1 antagonists have been characterized to date, though these agents encompass diverse chemical classes (77). The evidence pointing to an important role of the Y1 receptor in the regulation of food intake has been reviewed recently (77). J-115814, a potent (hY1 K_i = 1.4 nM), and selective Y1 antagonist, partially inhibited (~ 50%) icv NPY-induced food intake in satiated rats, and suppressed physiological feeding in lean and obese, but not in Y1 -/- mice. The incomplete inhibition of NPY-induced feeding is consistent with the extent of feeding suppression observed in Y1 -/-, suggesting an important, albeit not exclusive, role of the Y1 receptor in the orexigenic properties of NPY (9).

The lack of a pharmacokinetic/pharmacodynamic relationship with many selective Y5 antagonists, coupled with their inability to block the orexigenic effect of NPY in rats, and the finding that there was no reduction of NPY-induced feeding in Y5 -/- mice, have recently challenged the notion that the Y5 receptor is the NPY "feeding receptor" (78). While the Y1 receptor can still be considered an attractive drug target, additional receptor subtypes may need to be invoked to fully explain the neurobiology of NPY in the context of obesity. Interestingly, Y1 antagonists, attenuated the orexigenic action of MCH by 70%, suggesting a strong interrelationship between these two hypothalamic systems (79).

MCH Receptor Antagonists - The mammalian cyclic nondecapeptide MCH only recently emerged as a neuropeptide importantly related to feeding behavior, energy expenditure and the control of obesity. Reviews on MCH biology have appeared recently (80-82). This section will discuss compelling animal gene deletion and transgenic overexpression data documenting a key role of the MCH system in food intake and energy expenditure, and data from the initial discovery of small molecule MCHR1 antagonists.

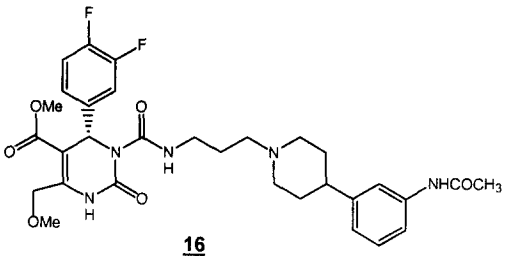
The MCH peptide was discovered over 20 years ago as a systemically released pituitary factor that concentrated melanin granules in melanophores located in scales of teleost fish, resulting in a refractory index change in the fish scale so that it appeared lighter in color (83). It was later found that the mammalian peptide paralogue was restrictively expressed in lateral hypothalamic area neurons with widespread axonal projections, particularly to brain regions involved in appetite control, energy balance, olfaction, food searching behavior, arousal and anxiety, and swallowing and mastication (84). Despite the historical understanding of the role of the lateral hypothalamic area in food intake and energy expenditure, a role for MCH in ingestive behavior was only recognized less than ten years ago based on initial work with the peptide and its mRNA. Expression of the MCH gene was increased two- to three-fold in hypothalami of *ob/ob* mice (85). In these studies preproMCH mRNA levels also increased with fasting, in both normal and *ob/ob* animals. ICV administration of MCH in rats led to an immediate, two- to three-fold increase in food intake. Also, targeted deletion of the MCH gene resulted in mice that had reduced body weight and increased energy expenditure (86). Consistent with this, the generation of transgenic mice that had a two-fold increase in the steady-state levels of preproMCH RNA levels within the same lateral hypothalamic area neurons resulted in animals that displayed diet-induced obesity, hyperphagia and insulin resistance, characteristics indicative of the obesity/diabetic phenotype in man (87).

The first MCH receptor was originally identified as an orphan G-protein coupled receptor, termed SLC-1(88). Efforts in the late 1990's to "deorphanize" the various putative G-protein coupled receptors yielded the MCHR1 as one of the success stories (89, 90). The MCHR1 binds to and is activated by nanomolar to subnanomolar MCH concentrations, and stimulates pertussis toxin-sensitive decreases in cAMP, inositol phosphate turnover, intracellular calcium mobilization, and stimulation of MAP kinase activity, consistent with coupling to multiple G-proteins including $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha q}$ (91). MCHR1 mRNA and protein are expressed in the same areas as the terminal fields of the lateral hypothalamic area MCH neurons (89, 90, 92). Paralogues of this MCHR1 gene are expressed in all mammalian species examined to date.

A second human MCH receptor (MCHR2) was recently identified in genomic databases and functional expression studies, and displays 32% identity to the human MCHR1 (93-95). The MCHR2 binds to and is activated by nanomolar to subnanomolar MCH concentrations, and activates inositol phosphate metabolism in a pertussis toxin insensitive manner, consistent with coupling to $G_{\alpha q}$. The MCHR2 is expressed in essentially the same CNS regions as MCHR1, though mRNA levels in the hypothalamus appear to be substantially lower than MCHR1. It is of interest that several mammalian species do not express MCHR2 or express a pseudogene, including rat, mouse, hamster, guinea pig or rabbit, while the MCHR2 is functionally expressed in canine, ferret, rhesus, macaque and man (95).

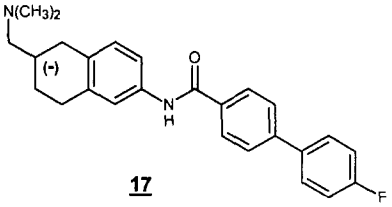
Two groups have independently generated homozygous null MCHR1 mice and the mice display hyperphagia, hypermetabolism and are resistant to diet-induced obesity (96, 97). These results clearly establish MCHR1 as a mediator of MCH effects on energy homeostasis and food intake in mice.

The first small molecule antagonist of the human MCHR1, **16** (SNAP-7941), inhibits MCH-mediated calcium responses and inositol phosphate accumulation in transfected cells (pA2 = 9.24), in a manner consistent with competitive inhibition (14). [^{3}H]-SNAP-7941 was used in auto-radiographic studies to map the location of the binding sites in rat brain (in the presence of unlabelled prazosin and dopamine), and displayed a similar localization to that of both MCHR1 mRNA and protein (89, 90, 92). Compound **16** binds to the human MCHR1 (Kd = 0.18 nM). Although not orally bioavailable, it antagonized MCH-stimulated palatable food intake and body weight gain in rats via ip injection. Moreover, **16** decreased body weight and food intake in a rat model of diet-induced obesity. Finally, **16** displayed antidepressant and anxiolytic properties in the rat forced swim test, in a maternal separation test in guinea pigs, and in a rat social interaction test. These results support additional utility of MCHR1 antagonists in anxiety and depression, consistent with MCHR1 brain localization.



16

Compound **17** (T-226296) is an orally active and selective MCHR1 competitive antagonist (15). It inhibited MCH binding (IC_{50} = 5.5 nM), reversed MCH-mediated inhibition of forskolin-stimulated cAMP accumulation, and blocked MCH-mediated intracellular calcium mobilization and arachidonic acid release, respectively. In the latter assay, **17** appeared to be a competitive antagonist. After oral dosing (30 mpk), **17** reduced icv MCH-stimulated food intake by approximately 90%.



17

Conclusions - Over the past decade, significant progress has been made identifying and advancing non-peptidic neuropeptide receptor antagonists, with a major success achieved in bringing an NK1 antagonist to registration. Recent animal genetic models point to the potential validity of several neuropeptide receptors as targets for drug discovery, though the need for safe, efficacious compounds cannot be underestimated. Many of the molecular targets discussed, despite being at different levels of maturity, have the potential to be future success stories.

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Chapter 3. Metabotropic Glutamate Receptors: Agonists, Antagonists and Allosteric Modulators

Corinne E. Augelli-Szafran and Roy D. Schwarz
Pfizer Global Research & Development
Ann Arbor Laboratories
2800 Plymouth Road, Ann Arbor, MI 48105

Introduction - The excitatory neurotransmitter glutamate acts at a variety of receptors in both the central and peripheral nervous systems, and these sites are broadly divided into two major types. Ionotropic glutamate receptors (iGluRs) are contained within ligand gated-ion channel complexes and include NMDA, AMPA, and kainate receptor subtypes with each subtype possessing a distinct pharmacology (1, 2). Metabotropic glutamate receptors (mGluRs) are members of the super family of G-protein coupled receptors (GPCRs) and the eight subtypes (mGluRs 1–8) are divided into three Groups/Families based upon sequence homology, common signal transduction mechanisms, and pharmacology. Group I receptors (mGluRs 1 and 5) couple to Gq and activate phospholipase C, while Group II receptors (mGluRs 2 and 3) and Group III receptors (mGluRs 4, 6, 7, and 8) couple to Gi/Go and result in the inhibition of cAMP formation. In general, it appears that Group I receptors are often located presynaptically and upon activation increase excitability, while Groups II/III have a greater postsynaptic localization and dampen excitability (3, 4).

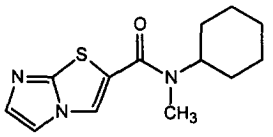
Early development of mGluR pharmacological agents focused predominantly on synthesizing amino acid-like structures that, in general, yielded relatively weak compounds (by today's standards) that often had activity at multiple subtypes and in some cases showed agonist activity at one receptor and antagonist activity at others (5). Subsequent chemistry focusing on specific subtypes created non-amino acid subtype selective compounds with low nanomolar affinity (6). Other reports in the literature have now shown that potent agonists and antagonists have been identified for many (but still not all) of the subtypes and that there are also positive and negative allosteric modulators of mGluR receptor function (7, 8). The purpose of this report is to provide an update on the pharmacology of mGluR receptors since the last review with an emphasis on selective ligands and allosteric modulators (6).

Like other members of Family/Type 3 GPCRs (which include GABA-B receptors, Ca^{++}-sensing receptors and pheromone receptors), mGluRs have a large amino terminal domain (ATD) that contains the recognition site for the endogenous ligand, which in this case is glutamate (9-11). There appears to be homology with bacterial periplasmic binding proteins that suggests the ATD is composed of two large globular domains linked by a hinge region. These domains exist in equilibrium between an open (inactive) and a closed (active) form with the overall confirmation altered by agonist binding (12). Additionally, there are sites within the seven transmembrane-spanning domains, which bind a variety of agents that either positively or negatively modulate responses produced by glutamate receptor activation. These sites present novel targets for drug discovery and are now being actively pursued (13-15).

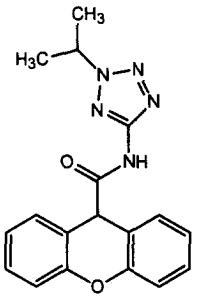
Agonists and Positive Allosteric Modulators - A limited number of mGluR1 agonists and positive allosteric modulators have been identified recently. Various amido analogs such as **1** and **2** through **4** have been disclosed as mGluR1 agonists over the past few years (16-21). Compound **1** showed an IC_{50} value of 20 nM in a

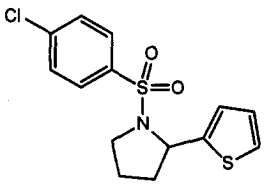
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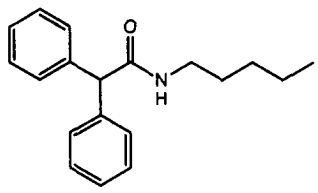
receptor binding assay and was suggested to be of use in the prevention and treatment of cerebral infarction.



1

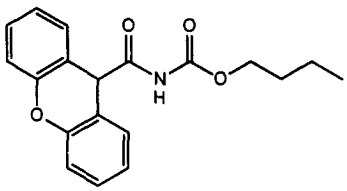




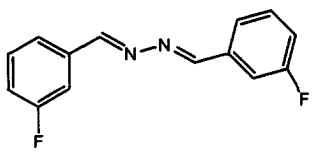


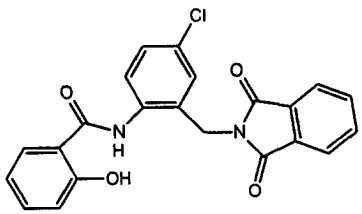
2 3 4

The selective positive allosteric modulators 2, 3 and 4 of mGluR1 receptors consist of tetrazole analogs, sulfonylpyrrolidines, and carbonylamino derivatives, respectively. A recent publication describes the effects of these compounds in recombinant and native mGluR1 receptor functional models (22). It was reported that 2 had an EC_{50} value of 0.045 nM and 4 and related analogs had EC_{50}'s ranging between 1.0 and 17.0 nM. An IC_{50} of 0.56 nM was reported for compound 3. Until recently, no compounds have been claimed specifically as mGluR5 receptor enhancers. In 2001, it was reported that (9H-xanthene-9-carbonyl)-carbamic acid butyl ester (5), a positive allosteric modulator, potentiated an mGluR5 agonist-stimulated functional response but to a lesser degree than at the mGluR1 receptor (22). This indicated that positive mGluR5 allosteric modulators were theoretically possible. Consequently, compounds 6 (DFB) and 7 (CPPHA) were recently presented as the first true examples of mGluR5 allosteric modulators (23,24).



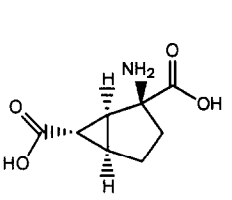
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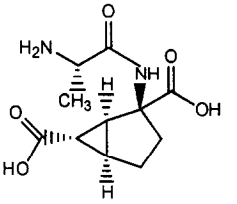


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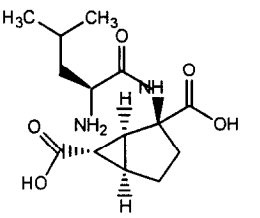
Over the past several years, Group II agonists have been highly illustrated in the literature (6). Recent claims include compounds that behave as positive allosteric potentiators of mGluR2 and mGluR3 receptors. These compounds are disclosed as prodrugs of a bicyclo-[3.1.0]-hexane derivative **8** (LY354740) and show improved oral potency. In studies that were done in the rat fear potentiated startle model, prodrugs **9** and **10** achieved plasmas levels at least 15 times higher in the pro-drug dosed animals than those receiving only **8** at doses of 5 mg/kg po each (25,26). Compound **9** had an MED value of 0.01mg/kg po compared to 3 mg/kg po for **8**.



8

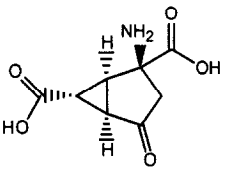


9

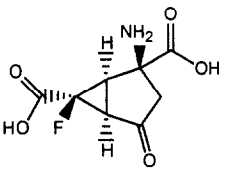


10

Minor structural modifications to the bicyclo-[3.1.0]-hexane core of **8** to give **11** and **12** were reported to have at least a 25-fold increase in ED_{50} values over **8**. These compounds inhibited forskolin-stimulated accumulation of cAMP in mGluR2-expressing CHO cells as an indicator of their effects (27,28).

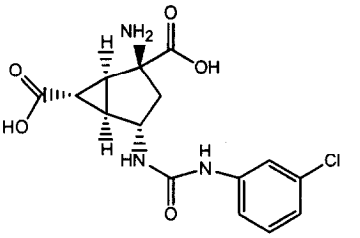


11



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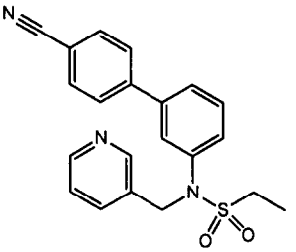
In contrast, analogs in which the keto group on the bicyclo-[3.1.0]-hexane core is replaced with a urea moiety appears to convert these types of molecules to an mGluR3 agonist. It is reported that selectivity of **13** is at least 53–fold for mGluR3 versus mGluR2 receptors, Ki = 9.54 nM vs Ki = 511.5 nM, respectively (29).



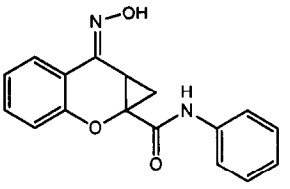
13

Group II and Group III positive allosteric potentiators are less common. Illustrated recently in the patent literature were substituted pyridine derivatives, such as **14**, that are claimed as mGluR2 and/or mGluR3 enhancers (30). It is noted that the compounds did not stimulate the mGluR2 receptor in the absence of an added agonist, but when co-applied, the compounds markedly enhanced the response

obtained with sub-stimulating concentrations of glutamate. With regard to Group III compounds, (+/-)-PHCCC was recently reported as an mGluR4 modulator **15**. It was shown that all of the activity of (+/-)-PHCCC resides in the (-)-enantiomer, which is inactive at mGluR2, 5a, 6, 7b and 8a, and shows partial antagonist activity at mGluR1b.

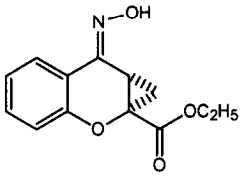


14

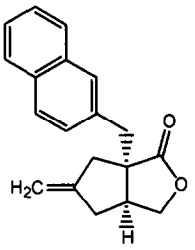


15

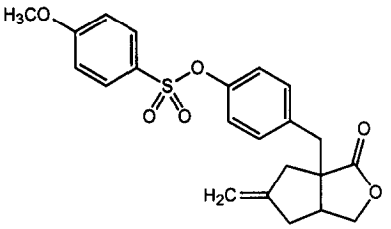
Antagonists and Negative Allosteric Modulators – Late in the 1990's, compound **16** (CPCCOOEt) emerged as an important pharmacological tool because it was demonstrated to be a low affinity, selective non-competitive antagonist that interacted within the mGluR1 receptor transmembrane domain (31,32). Over the past few years, several mGluR1 antagonists of new structural classes have been identified that are unique from **16**. A series of patents were published on **17** and **18**, which are alpha, beta-anellated butyrolactones (33-37). It has been reported that **17**, known as BAY36-7620, has shown a neuroprotective effect in animal models of cerebral infarction as well as being classified as more of an inverse agonist than **16** based on its inhibition of the mGluR1 constitutive activity and stabilizing the inactive state of the transmembrane domain (38). Compound **18** is from a recent patent in which more than 500 compounds are exemplified, with this particular analog being one of nine compounds specifically claimed (39). However, no biological data were presented.



16

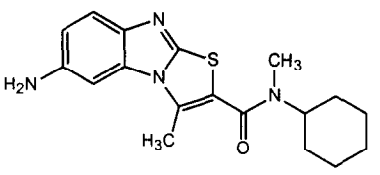


17

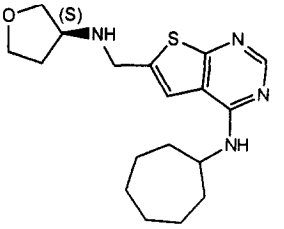


18

Similarly, at about the same time, several patents were issued that encompassed benzimidazole carboxamides, such as **19**, for use as neuroprotective agents, as described in a model of cerebral infarction (40,41). These compounds had reported affinities for the mGluR1 receptor in the low nanomolar range as well as dose-dependent effects between 10 and 100 mg/kg po in a model of spinal nerve ligation. However, more recently, a series of novel thienopyrimidines were described as mGluR1 antagonists such as **20** that had an IC_{50} value of 5 nM (42).

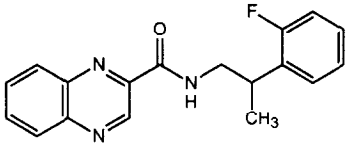


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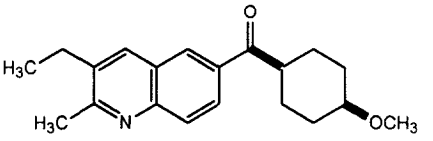


20

Several heterocyclic series of compounds have been recently disclosed as mGluR1 antagonists. Novel aryl carboxamide derivatives, specifically quinoxaline analogs such as **21**, were claimed as mGluR1 antagonists, but no biological data were disclosed (43). Quinoline analogs, such as **22**, had an IC_{50} value of 2.97 nM at the mGluR1 receptor as well as being efficacious in a cold allodynia test in rats with nerve ligation (Bennett model), exhibiting antagonism at a dose of 2.5 mg/kg (44).

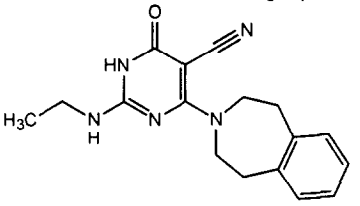


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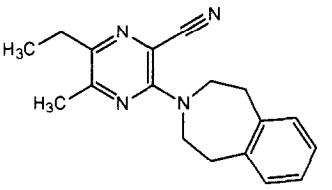


22

Azepine and pyrazine derivatives, such as **23** and **24**, exhibited functional IC_{50} values in the low nanomolar range (45-47).

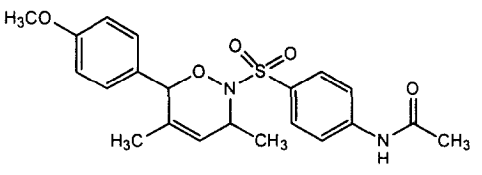


23

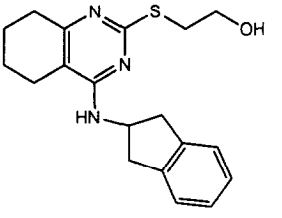


24

Oxazines analogs, such as **25**, were claimed as potentially effective against migraine and associated pain (48,49). Subsequently, a series of aminopyrimidines, e.g., **26**, was disclosed that exhibited picomolar activity for the mGluR1 receptor (IC_{50} value <1 nM) as well as activity against formalin-induced pain and in a migraine model of dural protein extravasion (50).

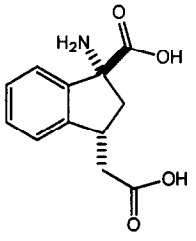


25



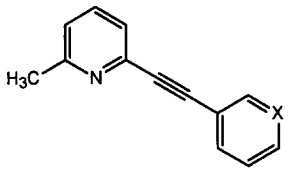
26

Lastly, a group of novel aminoindanes was cited as mGluR modulators that specifically exhibit antagonist activity in a phosphatidylinositol assay, with ED_{50} values in the low nanomolar range. A specific example is **27** in which the *trans* isomer had an EC_{50} value of 1.3 nM (51).

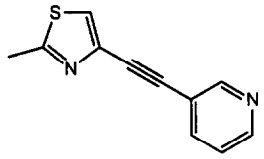


27

The most widely known pharmacological tool to explore the therapeutic potential of mGluR5 antagonists is **28** (MPEP), which was reported in 2000. This compound, claimed for the treatment of pain and anxiety, has good affinity for the mGluR5 receptor (IC_{50} = 36 nM, PI hydrolysis) and is highly selective against the mGluR1 receptor as well as being systemically active (52). Shortly thereafter, this particular compound was claimed for treatment of tolerance or dependency (53). More recently, a similar pyridine derivative, **29**, was specifically claimed for the treatment of pruritus (54). Based on the MPEP template, **30** (MTEP) was synthesized. This compound has affinity similar to MPEP at the mGluR5 receptor (IC_{50} = 5 nM vs IC_{50} = 2 nM, respectively), but has non-mGluR5 effects (e.g., MTEP has a Ki at the NE transporter of 43 uM whereas MPEP has a Ki = 0.37 uM) as well as higher CSF levels (1 uM vs 0.21 uM, respectively, measured at Tmax following a 30 mg/kg po dose, and greater *in vivo* potency than MPEP in both a receptor occupancy assay (ED_{50} = 1 mg/kg ip vs 3 mg/kg) and in the fear-potentiated model of anxiety (ED_{50} = 1mg/kg ip vs 5 mg/kg ip) (55,56).

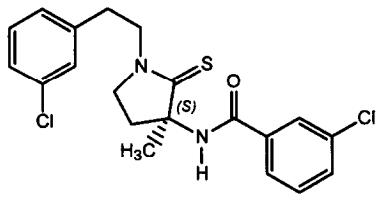


28: X = C; **29**: X = N

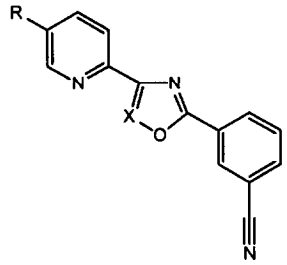


30

Analogs **31** and **32** represent additional structural templates that generated novel selective mGluR5 antagonists (57-60). These compounds have affinity for the receptor in the low nanomolar range and have the same utility as the compounds cited above.

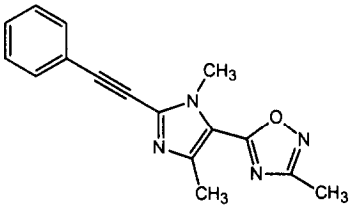


31

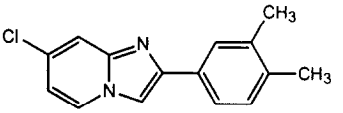


32: X = C, N; R = H, CH_3

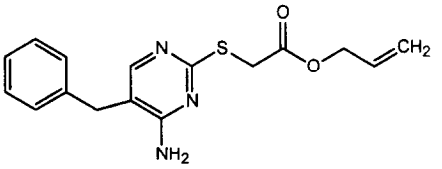
Lastly, additional structurally different mGluR5 antagonists such as phenylethynyl-, imazo-[1,2-a]-pyridine-, and 4-aminopyrimidine-derivatives, **33** (IC_{50} = 0.011 nM), **34** (IC_{50} = 0.11 nM), and **35** (IC_{50} = 0.12 nM), respectively, were disclosed as highly selective and potent mGluR5 antagonists (61-63).



33

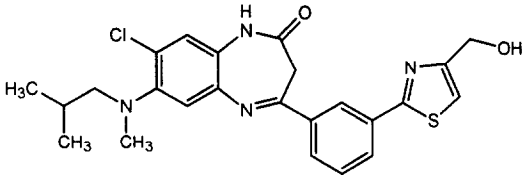


34

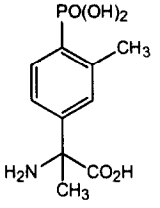


35

Group II and Group III antagonists are poorly represented in the literature compared to Group I compounds. An analog from the benzodiazepine chemotype, such as **36**, was reported to have very low nanomolar affinity (Ki value of 3 nM) for the mGluR2 receptor and the phenylglycine analog **37** is a representative of a Group III antagonist (64-66).



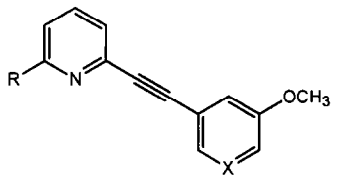
36

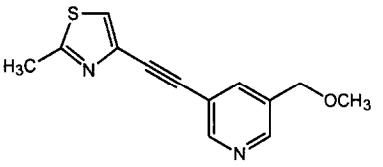


37

As exemplified in the limited disclosures in the above Group II/III category, non-competitive antagonist pharmacology is still in its infancy. However, with the widespread interest in this field, more subtype selective ligands will be appearing in the literature in the coming years.

Imaging Agents - Early mGluR binding studies used such ligands as [^{3}H]-glutamate or [^{3}H]-quisqualate. More recently, high affinity subtype selective radioligands have been synthesized. For example, there are several analogues of the mGluR5 antagonist, MPEP, that have been tritium-labeled for use in receptor binding and autoradiographic studies. These include: [^{3}H]-M-MPEP **38**, [^{3}H]-3-methoxy-PEPy **39**, and [^{3}H]-methoxymethyl-MTEP **40** (67-69). From these, and related compounds, imaging agents for use in humans are being developed.





38: R = CH_3, X=C; **39**: R = H; X=N **40**

The development of a PET ligand would be of value in early proof-of-concept Phase II clinical studies. It could directly measure penetration into the CNS by a novel compound. Additionally, it could aid in dose selection for efficacy trials by determining whether the drug is acting at the appropriate mGluR subtype and what level of receptor occupancy is achieved following administration of specific doses, efficacious doses as well as those producing unwanted side effects.

Clinical Indications - As previously reported, a variety of potential clinical indications have been suggested to be targets for the development of subtype selective mGluR agonists/antagonists as well as allosteric modulators. These include epilepsy, neuropathic and inflammatory pain, psychiatric disorders (e.g., anxiety and schizophrenia), movement disorders, (e.g. Parkinson's disease), neuroprotection (e.g., stroke and head injury), migraine, and addiction/drug dependency (2,3,70). Recently, the first human clinical data for any mGluR compound under development was presented. In a proof-of-concept study, the mGluR2/3 agonist, **8**, was shown to have positive effects in trials examining drug effects in patients suffering from panic attacks and in generalized anxiety disorder (GAD). Using a double blind trial design, a dose of 400 mg/day was found to reduce CO_2-induced panic attacks in 15 patients, although statistical significance was not achieved due to the low number of subjects. The drug was well tolerated and few side effects were observed (71). A second study showed the drug was active in 23 patients at 200 and 400 mg in a 6 week GAD study with lorazepam as a positive control. Again, the drug was well tolerated with no significant adverse events (72). These results are the first positive proof that compounds working at mGluRs have demonstrable clinical utility.

Conclusion - Clearly, there has been tremendous advancement in mGluR pharmacology in the two years since the last review in this field of research (6). Highly selective, non-amino acid agonists and antagonists with nanomolar potency have been identified for a number of the mGluR subtypes. In particular, non-competitive antagonists for mGluR1 and mGluR5 have been discovered and are under active clinical development by a number of pharmaceutical companies. Further, the identification of positive allosteric modulators offers an attractive alternative for enhancing glutamatergic function at mGluRs in contrast to the use of direct acting agonists binding to the extracellular-ligand recognition site (e.g. work on the mGluR2 subtype). With eight subtypes, there is much pharmacology yet to be discovered, particularly for Group III receptors. However, in the last two years there has been a tremendous increase in our knowledge of mGluR pharmacology and great strides have been made in the discovery and development of a new generation of novel therapeutic agents.

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Chapter 4. Melanocortin-4 Receptor Agonists and Antagonists: Chemistry and Potential Therapeutic Utilities

Iyassu Sebhat, Zhixiong Ye, Maria Bednarek, David Weinberg, Ravi Nargund, & Tung M. Fong
Merck Research Laboratories, Rahway, NJ 07065

Introduction - The study of melanocortin peptides traces back to the 1950s when pituitary peptides were shown to have skin darkening effects in an amphibian skin assay. Since that time, melanocortin peptides have been shown to have a wide array of biological effects *in vivo* (1-4), offering the hope for the development of therapeutic entities with utilities ranging from skin tanning to obesity treatment. Intensive research has greatly increased our biological understanding of the peptides and their effector systems. The challenge remains to develop novel chemical entities with the appropriate properties to be safe and efficacious drugs.

The melanocortin receptors are a family of five G protein-coupled receptor subtypes (Table 1). These melanocortin receptors are activated by α-MSH, β-MSH, γ-MSH or ACTH, all of which are derived from a single precursor peptide, proopiomelancortin (5).

Table 1: Five melanocortin receptor subtypes, their endogenous ligands and major functions

Receptor subtype	Primary Ligands	Major Function
MC1R	α-MSH, ACTH	Pigmentation
MC2R	ACTH	Steroidogenesis
MC3R	α-MSH, γ-MSH	Energy metabolism
MC4R	α-MSH, ACTH	Feeding, Erectile activity
MC5R	α-MSH, ACTH	Sebacious gland lipid secretion

Of these five subtypes, the melanocortin-4 receptor (MC4R) has been clearly linked to the regulation of energy homeostasis and will be the focus of this report (5). Based upon *in situ* hybridization studies, MC4R appears to be widely distributed throughout the brain with highest expression in the hypothalamus and dorsal motor nucleus of the vagus in the caudal brainstem (6-9). This localization correlates quite well with the brain sites where melanocortin-regulated feeding responses to exogenously applied ligands have been shown. The melanocortin agonist precursor POMC and an endogenous MC3R/4R antagonist, agouti related protein (AGRP) are also produced in the brain. Importantly, POMC and AGRP have been localized within the arcuate nucleus of the hypothalamus on separate populations of leptin-responsive neurons, where their levels are differentially regulated. Fasting or decreases in serum leptin levels gives rise to elevated AGRP and reduced POMC levels, while feeding or increased leptin levels has the opposite effect. Thus melanocortin signaling appears tightly coupled to the energetic state of the animal. *Mc4r* -/- mice were found to display hyperphagia, obesity, and metabolic perturbations (10). These data focused the attention on MC4R as the principal mediator of melanocortin-induced effects on energy homeostasis.

Pharmacological evidence that modulation of melanocortin receptors might provide a mechanism for regulation of energy balance has come from studies using agonist and antagonist peptides. Administration of melanocortin agonists to rodents decreases food intake and body weight, while antagonist administration produces the opposite effect (11). In addition to direct effects on food consumption, treatment with melanocortin agonists affects metabolic rate and may directly affect insulin

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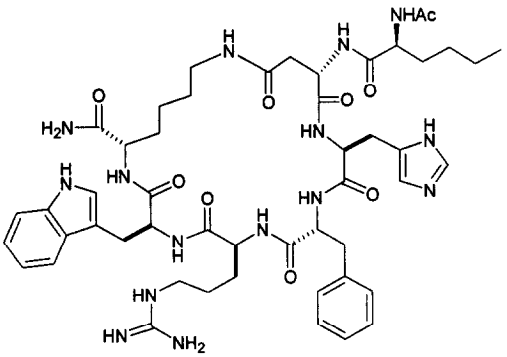
production and sensitivity (12-14). Much of the published pharmacological literature has employed nonselective MC3R/4R peptide agonists. However, these reagents fail to work in *Mc4r* -/- mice, suggesting that their mechanism of action is primarily through the MC4R (12). Genetic data have suggested that the insights gained from animal studies may extend to humans. Genetic analysis of human obese populations has revealed that up to 4% of severely obese humans have mutations in the MC4R gene (15-18). The identification of families in which both nonsense and frameshift mutations in MC4R led to dominantly inherited obesity provides perhaps the most convincing evidence. One potential adverse consequence of MC4R agonism was identified when a nonselective melanocortin agonist peptide was shown to be erectogenic in men with erectile dysfunction (19). It has been demonstrated that in rodents that this effect is mediated through MC4R (20). The pro-erectile consequences of chronic dosing with a selective MC4R agonist in man remain unknown. Both peptide and nonpeptide melanocortin agonists are under development for treatment of obesity.

Small molecule ligands offer the best hope of crossing the blood-brain-barrier and gaining access to the relevant receptor sites. In addition, melanocortin peptides delivered by intranasal administration reach the CSF and may be able to produce effects on body fat (21,22). The available data suggest that selective MC4R agonists may prove useful for the treatment of obesity and perhaps erectile dysfunction, while antagonists may prove valuable in the treatment of cachexia and wasting disorders. This report will detail the progress in the development of such molecules.

MC4R AGONISTS

Peptidyl MC4R Agonists - All endogenous ligands contain a conserved peptide fragment – His^6-Phe^7-Arg^8-Trp^9 (α-MSH numbering) – which has been identified as the "active core" or "message sequence" responsible for interaction with the melanocortin receptors. α-MSH is a 13 amino acid peptide agonist at all melanocortin receptors.

Structure-activity studies identified [Nle^4,$DPhe^7$]α-MSH (NDP-α-MSH, MT-I) - a more potent and enzyme-resistant analog with D-Phe substitution at position 7 (23). This and its radiolabelled iodo-analog have quickly become valuable tools in melanocortin receptor studies. A smaller, cyclized variant maintaining the active core of NDP-α-MSH was subsequently discovered (24). This lactam, MT-II (1), is a very potent and non-selective agonist at hMC1R, hMC3R, hMC4R and hMC5R.



1

Acyclic Peptides - Truncated analogs of α-MSH bearing the "message sequence" have been used extensively to probe receptor binding and activation. The tetrapeptide sequence has been modified in various ways by replacing each of the amino acids and/or altering the N/C-terminal moieties. Of relevance to this review are alterations that have afforded improved MC4R selectivity (25-32).

Analogs containing the tetrapeptide core, modified by an additional amino acid and hydrophobic N-terminus have shown excellent selectivity against hMC3R and hMC5R. Ro27-3225 (**2**) and a pentapeptide (**3**) are both potent hMC4R/hMC1R agonists with little or no activity at hMC3R and hMC5R (25,26).

$CH_3CH_2CH_2CO$-His^6-DPhe^7-Arg^8-Trp^9-Sar^{10}-NH_2

2

Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2

3

Replacement of the His^6 residue in the linear tetrapeptide impacted mMC3R selectivity (27). Ac-Anc^6-DPhe^7-Arg^8-Trp^9-NH_2, containing an amino-2-naphthylcarboxylic acid in place of His^6, was reported to be a potent agonist at mMC4R with over 4700-fold selectivity against mMC3R. The compound is also selective against mMC1R but not against mMC5R. The use of rigid non-basic His^6 surrogates in the pentapeptide (**3**) dialed out hMC1R activity (32). Substituted 2-aminotetraline-2-carboxylic acid containing compounds were reported to be potent agonists at hMC4R and inactive at the other subtypes (e.g. Penta-5-BrAtc^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2). Improvements in selectivity over mMC3R have been realized by altering either Phe^7 , Arg^8 or Trp^9 residues or through the use of an aromatic N-terminal "cap" on the linear tetrapeptide (28-31).

Cyclic Peptides - Extensive structure-activity studies have also been carried out on the cyclic lactam **1**. Improvements in selectivity have again been afforded by alteration of the "core" amino acids as well as variation of the linker and/or N-teminus (33-37). Excision of the N-terminal fragment is effective in increasing hMC4R selectivity. Replacement of Nle^4 with Pro led to [Pro^4]MT-II with similar hMC4R activity to the parent lactam but 400-fold lower potency at hMC5R and 20-fold reduced potency at hMC3R (33).

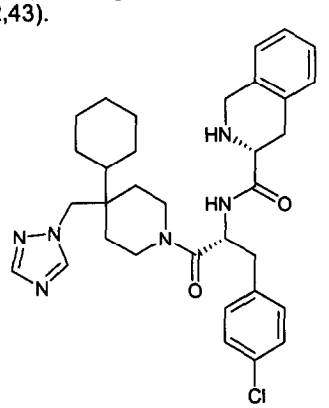
Large improvements in hMC4R selectivity have come from the introduction of two new templates: The first involves the use of a succinyl linker forming amide bonds between the α-amine of His^6 and the amino acid at position 10. For example, cyclo($COCH_2CH_2CO$-His^6-DPhe^7-Arg^8-Trp^9-Dab^{10})-NH_2 is a potent hMC4R agonist with 55-fold selectivity over hMC3R and >1000-fold selectivity against hMC5R (34,35). The second involves the formation of an amide bond between the γ-carboxyl group of Glu^{10} and the amino group of ω-amino acids. For example, cyclo($NHCH_2CH_2CO$-His^6-DPhe^7-Arg^8-Trp^9-Glu^{10})-NH_2 is potent at hMC4R with 90-fold selectivity over hMC3R and >2000-fold selectivity over hMC5R (24).

Replacement of the His^6 residue in **1** with Ala had little effect on activity at either of hMC3R-5R (36). However, using a related cyclic peptide, replacement of the amino acid with substituted 2-aminotetraline-2-carboxylic acids led to potent hMC4R agonists with inactive or weak agonist properties at hMC1R, hMC3R and hMC5R. One example is Penta-cyclo(Asp-5-ClAtc^6-DPhe^7-Arg^8-Trp^9-Lys^{10})-NH_2 (37).

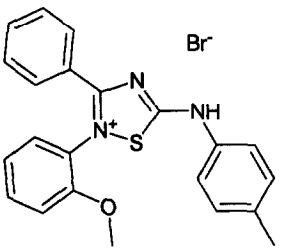
There have been a large number of patent applications issued covering peptide agonists of MC4R. These have been summarized in a recent review, and a few recent applications have been disclosed since then (38-41).

Small Molecule MC4R Agonists - To date there have been few publications reporting the discovery of potent and selective small molecule hMC4R agonists. Leads that have emerged come from both rational design and HTS with development proceeding *via* classical medicinal chemistry techniques (20,42-44).

Peptidyl-privileged structure based compounds have had significant success in recent years as agonists of G-protein coupled receptors. Their design is based upon the observation of commonly recurring structural elements in receptor ligands. This approach was extended to the development of an hMC4R agonist. Compound **4** is the first to be disclosed (20,42). It is a potent hMC4R agonist (EC_{50} = 2 nM) with >1300-fold selectivity over hMC1bR, >1100-fold selectivity over hMC3R and >350-fold selectivity over hMC5R. *In vivo* studies with **4** showed significant reductions in rat food intake. The compound has pro-erectile activity in a number of rodent models, providing evidence for the role of the MC4 receptor in sexual function (20,42,43).



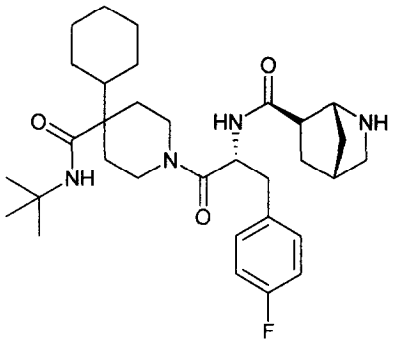
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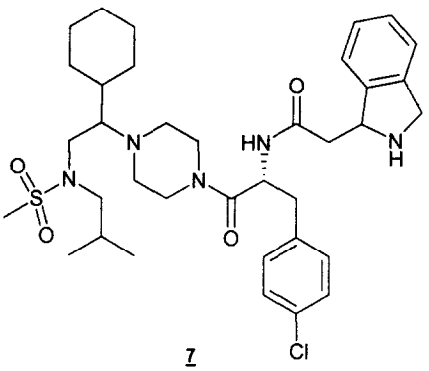
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A series of 2,3-diaryl-5-anilino[1,2,4]thiadiazoles were developed from an HTS lead (44). Optimization of the lead generated more potent compounds exemplified by **5** with an IC_{50} of 22 nM. While the compound was reported to be an agonist, no EC_{50} was given. Additionally, selectivity against the other subtypes was not reported. Compound **5** showed significant reductions of food intake in fasted rat when dosed *i.p.* but had no effect *p.o.*, reflecting its likely rapid metabolism. A more potent analog was also reported but showed no efficacy in the food intake assay.

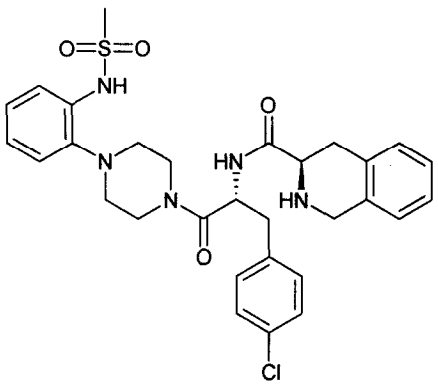
While there are few published papers describing small molecule MC4R agonists, there have been a large number of patent applications published in the area (38,45,46). Several applications, illustrated by compound **4**, cover MC4R agonists derived from the capped dipeptide template (47-57). Some focus exclusively on various substituted piperidine-based privileged structures, others focus on piperazine derivatives. The latest structure claimed in this series is compound **6** bearing a 2-azabicyclo[2.2.1]heptane-6-carboxylic acid (58) . Compound **7**, has a potency of 8.4 nM with 102% activation relative to α-MSH. More recently, an application covering piperazine-containing structure **8** was published (59). Six derivatives of compound **8** were reported to cause food intake reduction in fasted mice at doses of less than 30 mpk.



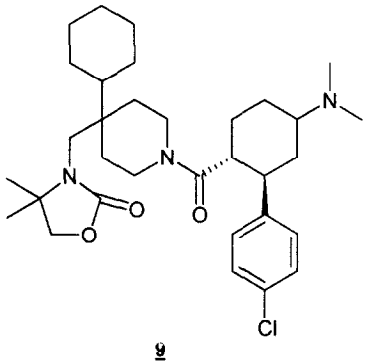
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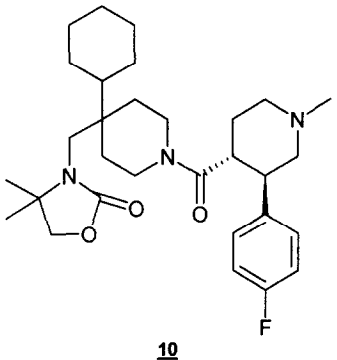


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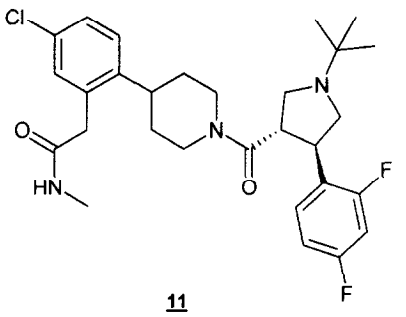


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Three new structure templates with piperidine bearing privileged structures have been claimed, exemplified by compounds **9**, **10** and **11**. However, no biological data were disclosed in the patent applications (60-62).



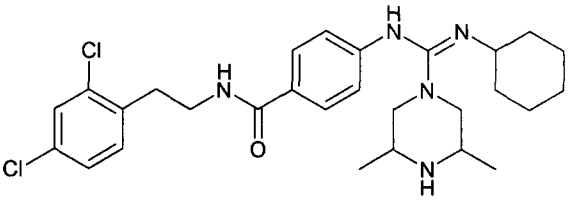
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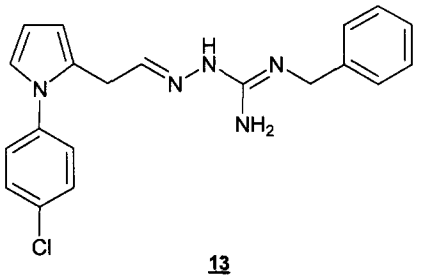
Additional applications cover various guanidines as MC4R agonists (63-65). In a 4-week study, 3 mpk, *b.i.d., i.p.* administration of compound **12** in *ob/ob* mice elicited a significant reduction in food intake and body weight. Recently, data were presented on the oral dosing of an MC4R agonist (EC_{50} = 23 nM) of undisclosed structure in *ob/ob* mice. At 10, 30, and 60 mpk, the compound caused a dose-dependent reduction in food intake. A three-week experiment with 20 or 60 mpk

b.i.d. dosing showed a reduction in food intake and body fat. The animals receiving the high dose also showed an increase in oxygen consumption (63).



12

A class of structures bearing aminoguanidines was claimed as MC4R agonists (66-72). Compound **13** was reported to be a full agonist with a K_i of 610 nM. The inventors have also recently reported a non-peptide MC4R partial agonist (IC_{50} = 12 nM) of undisclosed structure. This compound caused a reduction in cumulative feeding in a 10-day chronic study, although no effect was observed for the first three days of the experiment (66). Finally, there are a number of other companies and institutions who have recently claimed small molecules as MC4R agonists (65,73,74).

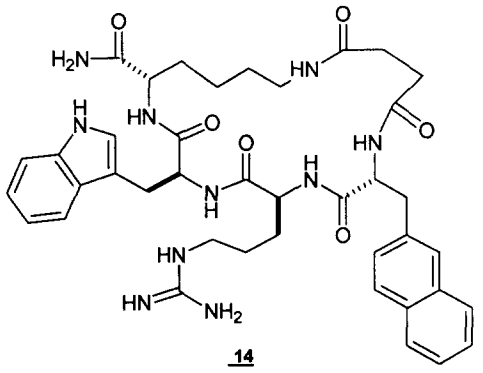


13

MC4R ANTAGONISTS

The development of selective, high affinity antagonists for MC4R trailed the development of MC4R agonists. Several structure-activity studies were reported on the lactam SHU9119, an analog of αMSH and a nonselective MC3R/MC4R antagonist (75,76). Analogs of SHU9119 with altered lactam ring sizes, amino acid substitutions and/or chirality changes in the "active core" were prepared in an effort to optimize receptor subtype selectivity (77-79). Replacement of His^6 with 1-amino-1-cyclopentane carboxylic acid resulted in an hMC4R antagonist with improved selectivity against hMC3R (78). All other compounds displayed slightly lower or similar antagonistic activities to SHU9119 at the human or mouse MC4R with little improvement in selectivity over other receptor subtypes.

Recognition that Ac-Nle^4 and His^6 only minimally affect the binding of SHU9119 to hMC4R led to the design of smaller cyclic peptides which were selective and potent antagonists for the human hMC4R (76). Compound MBP10 (**14**), bearing a succinyl moiety in place of Asp^5, displayed about 125-fold higher antagonist selectivity for hMC4R than hMC3R. This peptide did not activate hMC1bR, hMC3R and hMC4R even at micromolar concentrations and was only a weak agonist at hMC5R (EC_{50}=530 nM). Analogs of **14** with a glutaryl or o-phthalic group in place of the succinyl linker were recently reported to be less selective and less effective MC4R antagonists (35).

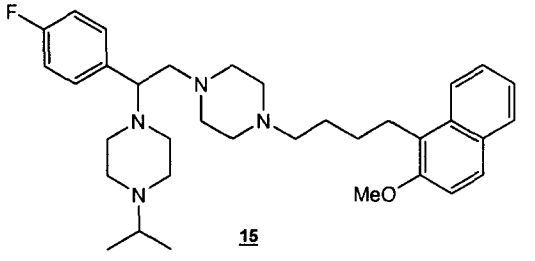


14

Several cyclic disulfide analogs of αMSH with ring sizes greater than 25 have been shown to be selective and effective antagonists at MC4R (80,81). HS014, Ac-cyclo(Cys-Glu-His-D-Nal(2')-Arg-Trp-Gly-Cys)-Pro-Pro-Lys-Asp-NH_2, is an antagonist at MC4R (20-fold selective over MC3R) and a partial agonist at MC1R and MC5R. HS024, with a larger ring size, Ac-cyclo(Cys-Nle-Arg-His-D-Nal(2')-Arg-Trp-Gly-Cys)-NH_2, did not activate MC1R, MC3R , MC4R or MC5R, and its affinity for MC4R was about 70-, 20- and 10-fold higher than for MC1R, MC3R and MC5R, respectively. These peptides stimulated feeding in rodents after *i.c.v.* injection. The latest MC4R antagonist from the same group is HS131, Ac-cyclo(Cys-Gly-D-Nal(2')-Arg-Trp-Cys)-NH_2. This antagonist increased food intake in rodents after *i.c.v.* and *s.c.* administration (82).

The tetrapeptide, Ac-His^6-D-Nal(2')^7-Arg^8-Trp^9-NH_2, which comprises the "active core" of α-MSH, but with D-Nal(2') in place of Phe^7, was found to be a moderately effective antagonist at the mouse MC4R with K_I of 17 nM (28). Its elongated analog (Ro27-4680), $CH_3CH_2CH_2CO$-His^6-D-Nal(2')^7-Arg^8-Trp^9-Sar^{10}-NH_2 is a weak partial agonist at MC4R, can antagonize NDP-α-MSH activation at high concentrations, and can increase food intake in rodents when administered centrally (25).

Anxiolytic-like and antidepressant-like activities were reported for MCL0129 (**15**), representing the first nonpeptide MC4R antagonist published (83). This compound inhibited [^{125}I]NDP-αMSH binding to the cloned human MC4R with high affinity and showed no affinity for the human MC1bR and rat MC3R even at a concentration of 10 μM.



15

Other patent applications have been published on the preparation and use of benzylideneamino guanidines and aromatic amides (as MC1R and MC4R selective agonists and/or antagonists) for the treatment of diseases related to the melanocortin receptors (68,70,71,84).

Conclusion - Significant progress has been made in the past several years in the development of selective MC4R modulators. In particular, the discovery of small molecule agonists and antagonists will provide the necessary tools to further explore the utilities of these ligands in the treatment of various pathological conditions, including obesity, erectile dysfunction, inflammatory diseases and CNS diseases. Furthermore, these selective tools will allow in-depth evaluation of the melanocortin system and the delineation of the functional roles of different MC receptor subtypes.

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Chapter 5. Secretase Inhibitors for Alzheimer's Disease

Anna Y. Kornilova and Michael S. Wolfe
Center for Neurologic Diseases
Brigham and Women's Hospital and Harvard Medical School
77 Avenue Louis Pasteur, Boston, MA 02115

Introduction - Brain deposits of the amyloid-β peptide (Aβ) in the form of plaques are a hallmark of Alzheimer's disease (AD), and formation and aggregation of this peptide are strongly implicated in the etiology of the disease (1). Aβ is produced from the amyloid β-protein precursor (APP), a single-pass membrane protein of unknown function, by the sequential action of two proteases, β- and γ-secretases, as depicted in Figure 1 (2). Proteolysis by β-secretase leads to release of the large extracellular/luminal domain of APP, and the 99-residue C-terminal fragment that remains (C99) is then proteolyzed within its transmembrane domain by γ-secretase, producing a heterogeneous collection of Aβ peptides ranging from 38 to 43 residues long. Although the 42-residue version (Aβ42) is a minor species, this peptide is particularly prone to self-assembly into fibrils and is the major Aβ isoform found in AD plaques (3-4).

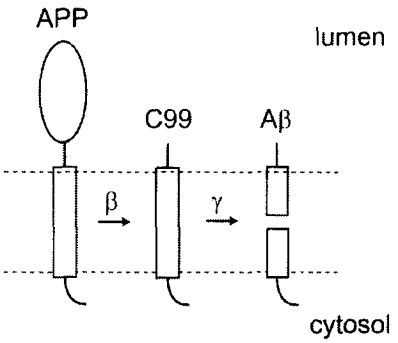


Figure 1 APP processing scheme

Dominant genetic mutations known to cause early-onset AD all increase the production of Aβ42 (5). These mutations occur in three genes: those encoding APP and the multi-pass membrane proteins presenilin-1 (PS1) and presenilin-2 (PS2). AD-causing missense mutations in APP are found near the β- and γ-secretase cleavage sites that generate Aβ. A double mutation found at the P2-P1 position of the β-secretase cleavage site is more efficiently processed by this protease, increasing production of C99 and therefore of all Aβ peptides, including Aβ42. Various single mutations near the γ-secretase cleavage sites lead to specific increases in the levels of Aβ42. Nearly 100 AD-causing missense mutations in the presenilins have been identified to date, and all those examined likewise specifically increase Aβ42 production (6). These findings provided strong genetic evidence for the amyloid hypothesis of AD and particularly pointed to Aβ42 as the primary molecular culprit.

Aβ-based strategies for the discovery and development of AD therapeutics can focus on the production, assembly, or neurotoxicity of this peptide (7). Although each of these three approaches has its unique advantages and is being actively pursued,

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this chapter deals strictly with inhibitors for β- and γ-secretases reported since 2000. Inhibitors identified prior to this are discussed in a previous Report (8).

β-SECRETASE

β-Secretase characterization - Most cell types produce Aβ, indicating broad expression of β-secretase (also called beta-site APP-cleaving enzyme or BACE, BACE1, asp2, and memapsin 2 by the various groups that first reported its discovery). However, considerably more Aβ is generated in primary brain cultures than in peripheral cells (9). β-secretase cuts at the sequence EVKM↓DAEF, but a KM-to-NL double mutation found in a Swedish family immediately adjacent to the β-secretase cleavage site causes AD and leads to increased Aβ production by enhancing proteolysis at the β-site (10). β-secretase is a membrane-tethered aspartyl protease in the pepsin family and contains a signal sequence and pro-peptide region at the N-terminus as shown in Figure 2 (11-15). Two aspartates, D93 and D289, are required for activity, and β-secretase shows a pH optimum of 4-4.5 for the cleavage of both wild type and Swedish mutant peptides (11, 12, 15). Use of decameric FRET substrates revealed that β-secretase shows relatively poor kinetic constants for its known substrates, the wild-type and Swedish mutant sequences (16). In common with several other aspartyl proteases (e.g., cathepsin D), β-secretase prefers a leucine residue at position P1. However, unlike other aspartyl proteases, BACE accepts polar or acidic residues at position P2' and P1 but prefers bulky hydrophobic residues at position P3 (16).

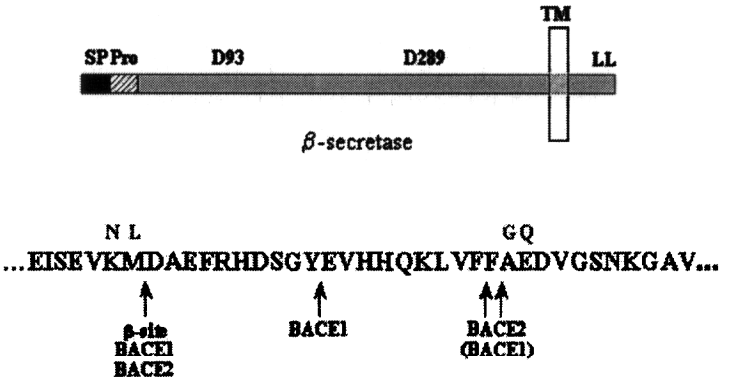


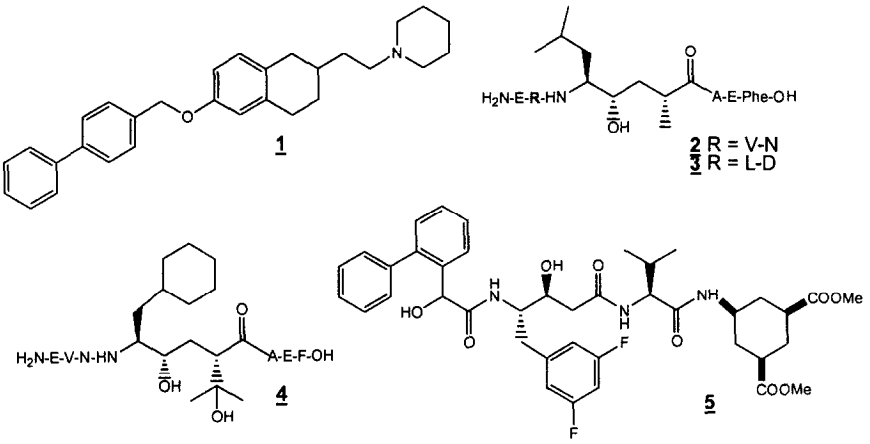
Figure 2. Structure of b-secretase, BASE1/2 cleavage sites on APP and disease-causing mutations

β-secretase (BACE1) and its homolog, BACE2, exhibit 52% amino acid sequence identity and 68% similarity, and BACE2 cleaves APP and short peptides in a β-secretase-like manner: activity is similarly increased at Asp1 by the Swedish double mutation and prevented by a P1 Met-to-Val mutation (17). However, BACE2 is not expressed well in the brain, suggesting that it may play little, if any role in AD plaque formation. Moreover, unlike BACE1, BACE2 generates little Glu11-Aβ (Figure 2) but efficiently performs an additional proteolysis in the middle of the Aβ region, resulting in the production of Phe20-Aβ and Ala21-Aβ, with the implication that BACE2 might limit the production of pathogenic forms of Aβ. The biological role of BACE2 is unknown. If BACE2's role is critical, inhibitors selective for BACE1 over this homolog may be needed to minimize toxicity.

A crystal structure of β–secretase bound to a hydroxyethyl transition-state analog inhibitor at 1.9 Å resolution shows that the bilobal structure of β-secretase has the conserved general folding found in many other aspartyl proteases (18, 19). The six cysteine residues in the ectodomain form three disulfide bonds. The inhibitor is located in the substrate binding cleft between the amino- and carboxy-terminal lobes, and as expected, the transition-state mimicking hydroxyethyl moiety is coordinated with the two active site aspartates. As with a number of other aspartyl proteases, β-secretase possesses a "flap" that partially covers the cleft, and the backbone of the inhibitor is mostly in an extended conformation. However, β-secretase does display some structural differences, at least compared with pepsin, that may be turned to advantage toward the development of selective inhibitors.

Several reports on BACE1 knockout mice concluded that this enzyme is the major β-secretase in the brain (20-22). The BACE1 knockout mice were healthy, viable, and appeared normal in gross anatomy, tissue histology, hematology, and clinical chemistry. These findings indicate that inhibition of BACE should lead to dramatic decreases in brain Aβ levels and that such inhibition may not lead to mechanism-based toxicity. Despite the promising results from the BACE1 knockout studies, this protease likely has other normal substrates, and it remains to be seen whether long-term β-secretase inhibition is tolerable in aging adult humans. Most recently, BACE1 expression and β-secretase activity have been reported to be elevated in the AD brain, further emphasizing the connection between this protease and the disease (23, 24).

β-Secretase inhibitors - β-secretase is an attractive pharmacological target for AD, and the search for potent and selective inhibitors of this enzyme has intensified during the past few years. Despite the availability of X-ray structures of the enzyme, most of the reported β-secretase inhibitors are still substrate-based peptidomimetics. The large active site of β-secretase apparently presents a challenge for the development of nonpeptidic small molecule inhibitors. One exception is the tetralin derivative **1**, which inhibits activity of recombinant β-secretase with an IC_{50} of 0.35 μM (25). Identifying nonpeptidic compounds is critical for obtaining effective clinical agents, because peptide analogs typically do not display good enough pharmacokinetic properties (e.g., the ability to cross the blood-brain barrier) to become drugs.



Reported SARs have involved variations on the original peptide structure based on the sequence EVNLDAEF, which is derived from the APP β-secretase site

containing the Swedish P2-P1 double mutation. This sequence was used to design potent first-generation β-secretase inhibitors, such as hydroxyethylene isostere transition-state analogues **2** (Ki = 1.6 nM) and later **3** (Ki = 0.2 nM) (19, 26). A recently disclosed version of **2** is **4**, which inhibits recombinant β-secretase with an IC_{50} of 49 nM (27). Importantly, a recent kinetic study using synthetic peptide libraries and mass spectrometric analysis revealed a peptide EIDLMVLD which was cleaved with a k_{cat}/K_M value 14-fold better than EVNLDAEF (28). This finding should aid the development of more potent peptidic inhibitors. At the same time, it suggests that other better substrates for β-secretase may exist. Cross-inhibition with the homologous BACE2 has not been reported (29). Such inhibition may cause unforeseen toxicity. BACE2 knockout mice are being generated to address this issue.

Additionally, continual work on the development of statine–based peptidomimetics selective against BACE has resulted recently in the identification of the cell-permeable **5** (IC_{50} = 0.12 μM), which is being evaluated *in vivo* (30).

γ-SECRETASE

γ-Secretase characterization - This enzyme has been considered central to understanding the molecular basis of AD, because it determines the proportion of the highly aggregation-prone $A\beta_{42}$ peptide. γ-Secretase has also been of interest because it somehow hydrolyzes within the middle of the transmembrane region of APP. The enzyme is inhibited by classical transition-state mimicking motifs for aspartyl proteases, suggesting that it falls into this mechanistic category of proteases (31-33). Knocking out PS in mice showed that it mediates γ-secretase cleavage of APP (34-36), begging the question: What is the role of this protein in γ-secretase activity?

PS is cut into two pieces that remain associated (37, 38). This heterodimer is thought to be the active form of the protein, because it is metabolically stable and its formation is tightly regulated by complexation with other cellular factors (37, 39, 40). PS also contains two conserved transmembrane aspartates, each contributed by one of the PS subunits, that are predicted to lie the same distance within the membrane and to roughly align with the γ-secretase cleavage site in APP. Mutation of either asparate prevented the formation of PS subunits and blocked γ-secretase processing of APP (41).

These results suggested that PS might be the catalytic component of γ-secretase: upon interaction with other, limiting cellular factors, PS undergoes autoproteolysis via the two aspartates, and the two PS subunits remain together, each contributing one aspartate to the active site of γ-secretase. More direct evidence that PS is the catalytic component of γ-secretase came from affinity labeling studies using transition-state analogue inhibitors: the heterodimeric form of PS was specifically tagged (42, 43). Also, a non-transition state analog inhibitor of γ-secretase likewise crosslinked presenilin subunits (44).

PS is not only involved in the proteolytic processing of the transmembrane domain of APP but is also critical for processing of the transmembrane region of the Notch receptor, a signaling molecule crucial for cell-fate determinations during embryogenesis (45). Release of the intracellular domain of Notch, a process mediated by PS, is essential for Notch signaling (46, 47). The responsible PS-dependent protease that cleaves Notch appears to be identical to the one that cleaves APP (35, 36, 48, 49). Because Notch is also required for cell differentiation during adulthood (e.g., hematopoiesis), concerns have been raised about

mechanism-based toxicities (e.g., immunosuppression) arising from γ-secretase inhibition (50, 51).

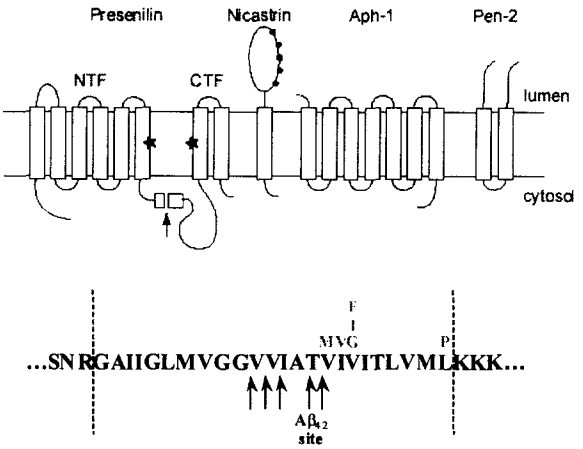
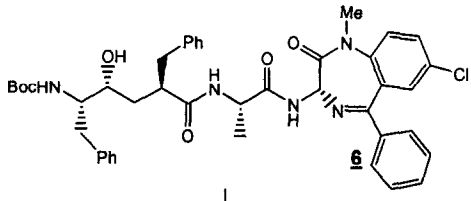


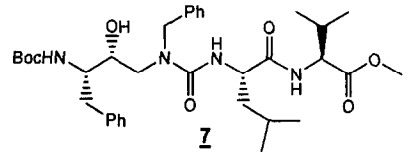
Figure 3. Scheme of γ-secretase complex, γ-secretase cleavage sites on APP and sites of AD-causing mutations

Although PS appears to be the catalytic component of γ-secretase, it does not work alone, requiring complexation with at least three other recently identified membrane proteins: nicastrin, Aph-1, and Pen-2 as shown in Figure 3 (52-54). Expression of all four proteins together results in increased formation of PS subunits and elevated γ-secretase activity (55-57). Moreover, these components assemble together and bind to an immobilized γ-secretase inhibitor (55, 56). These findings are consistent with the hypothesis that, after complexation with its three partners, PS undergoes autoproteolysis to become the catalytic component of γ-secretase, the active site lying at the interface between the two PS subunits. This is a major advance: the complete identification of γ-secretase should greatly facilitate our understanding of its mechanism and expedite the search for effective inhibitors. Moreover, from the perspective of basic biochemistry, γ-secretase appears to be a founding member of a new class of proteases containing membrane-embedded active sites (58).

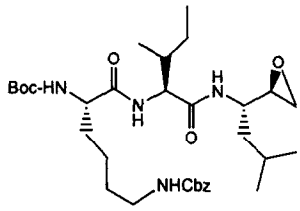
γ-Secretase inhibitors - Although the biochemistry and mechanism of γ-secretase are still not completely understood, many new γ-secretase inhibitors have been identified as a result of cell-based screening of both diverse and focused libraries. Aspartyl protease transition-state mimics, such as hydroxyethylene-based **6** and (hydroxyethyl)urea-based **7**, which are closely related to previously reported hydroxyethylene-based peptidomimetics, potently inhibit $A\beta_{40}$ and $A\beta_{42}$ production in cell-based and/or cell-free assays (32, 59, 60). A photoactivatable and biotinylated derivative of **7** has been used to assess the mechanism of action of structurally diverse γ-secretase inhibitors, and most them apparently act by binding or allosterically affecting the γ-secretase active site (61). Epoxide **8** has been shown to affect the active site in a time-dependent manner, suggesting it irreversibly inactivates the enzyme, possibly by reacting with one of the active site aspartates (61, 62). SAR studies have resulted in identification of fenchylamine sulfonamide **9** (IC_{50} = 1 μM), α-hydroxyacid-based **10** (IC_{50} = 160 nM) and dipeptide ester **11** (IC_{50} = 130 nM) (63-65).



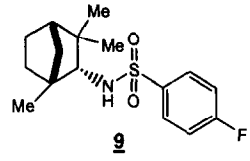
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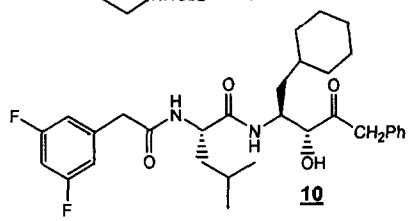
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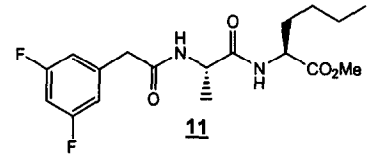
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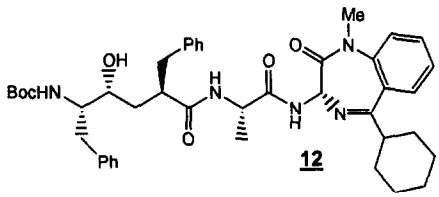


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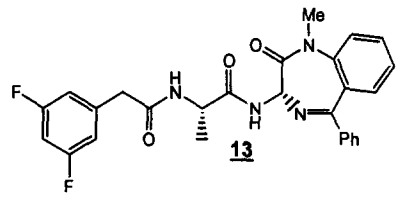


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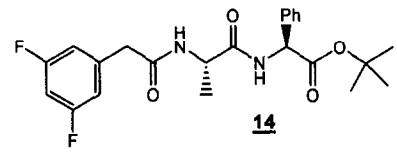
Compound **12** was reported to inhibit the formation of presenilin heterodimers with an IC_{50} of 60 nM (66). Recently disclosed highly active γ-secretase inhibitors have included benzodiazepine **13** (IC_{50} = 300 pM), dipeptide analogue **14** and second generation analogue benzocaprolactam **15** (44, 67, 68).



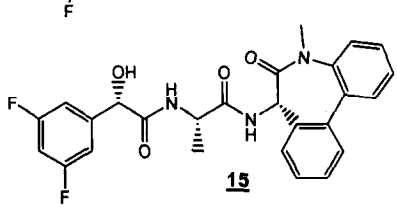
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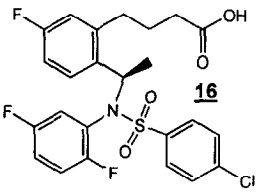


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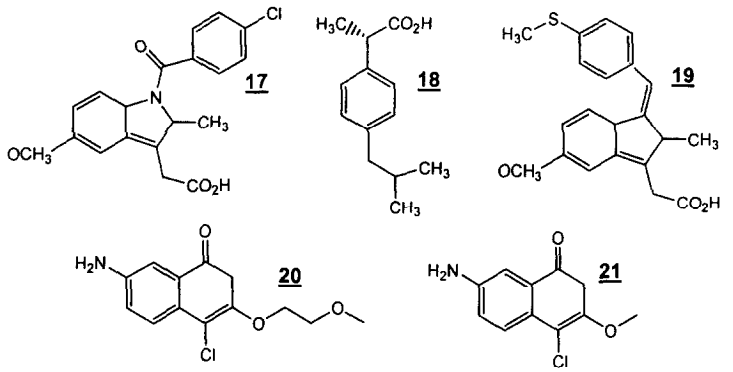
Compound **14** has been reported to lower Aβ levels in the brains of APP transgenic mice after acute administration (69). Although high concentrations of **13** and **14** were shown to inhibit Notch processing in *Drosophila* (70), their derivative **15** did not cause any apparent toxicity when administered to APP transgenic mice over 3 months (71). Compound **15** exhibits low nM potency and some selectivity for APP over Notch, suggesting that it may be possible to decrease Aβ levels without adversely affecting critical Notch signaling events. Compound **15** as well as certain sulphonamides, exemplified by **16**, are candidates for clinical trials (72).



16

Currently, efforts are being made to find γ-secretase inhibitors specific for APP over Notch or other known substrates. Other possible side effects of using γ-secretase inhibitors as well as their cross-inhibition with other proteases remain to be examined. In this regard, several γ-secretase inhibitors, including **6** and **15**, have been recently reported to inhibit signal peptide peptidase (SPP), a multi-pass membrane presenilin-like aspartyl protease (59).

Inhibition of Aβ Production by Unknown Mechanisms - Several non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin **17**, (*S*)-ibuprofen **18** and sulindac sulfide **19**, have been reported to selectively lower $A\beta_{42}$ levels in mammalian cells and in mice without affecting Notch proteolysis (73). Although the mechanism of action of these compounds is unclear, they have easily become candidates for clinical trials for AD prevention. Also, certain isocoumarin compounds **20** and **21** have been reported to be modest γ-secretase inhibitors, which lower Aβ in cells without affecting Notch processing (74). However, these compounds do not inhibit γ-secretase directly, but through some other unknown mechanism (75).



17 18 19

20 21

Conclusion - During the past few years, great progress has been made in identifying new β- and γ-secretase inhibitors. Although β-secretase has become a more attractive target for AD treatment because of the lack of evident side effects in knockout mice, the search for effective nonpeptidic compounds has been challenged by the large active site of this protease. Many more potent γ-secretase inhibitors have been disclosed with a few being tested *in vivo*. Although knockout of presenilins is lethal because of elimination of Notch signaling, it may nevertheless be possible to lower γ-secretase activity pharmacologically without undue toxicity.

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Chapter 6. Urinary Incontinence: Neuropharmacological Approaches

Matthew O. Fraser, Edward C. Burgard, and Karl B. Thor
Dynogen Pharmaceuticals, Inc.
Durham, NC 27713

Introduction - The most remarkable breakthrough in incontinence medicines across the last year was the submission of a New Drug Application to the FDA for duloxetine hydrochloride as a treatment of stress urinary incontinence (for which there are no currently approved medicines in the US). In urge incontinence, reformulations of oxybutynin (e.g. extended release and patch formulations) and other muscarinic cholinergic receptor antagonists continue to dominate the market and late stage clinical development programs. Enterprising clinicians have taken matters into their own hands and applied off label-use of various toxins (such as botulinum toxin A injected into the bladder or urethra and intravesical administration of capsaicin) as they try to find remedies for their patients, but safe, effective, and convenient therapy is obviously needed to compliment the anticholinergics.

This chapter describes the neural reflex pathways that control urine storage and micturition and describes pharmacological targets and drug discovery strategies within the context of these reflexes. However, it does not provide detailed discussions of species differences in drug effects, and the reader should be aware that major differences do exist. For example, the role of 5-HT1A receptors in control of micturition appear to be completely opposite in rat and cat (1). Furthermore, within the same species, drug effects can differ with different stages of development or under various pathological conditions that are accompanied by changes in the neural substrate or receptor expression (2-4). Thus, drug candidates should be studied in various clinically-relevant pathological models and in multiple animal species prior to committing extensive resources toward clinical development.

In some cases, the location of targets is not precisely known because drugs were administered intracerebroventricularly or intrathecally and thus could affect either the sensory or motor component of the reflex. Thus, the site of action is inferred from studies of somatic sensory or motor systems in which the site of drugs' effects have been documented.

FUNCTION AND DYSFUNCTION OF THE LOWER URINARY TRACT

The function of the lower urinary tract (LUT) is to store and periodically release urine. This requires the orchestration of storage and micturition reflexes involving both the sympathetic and parasympathetic components of the autonomic nervous system and somatic motor pathways. Key components include the end organs themselves - the bladder smooth muscle and the urethral smooth and striated muscles - as well as the peripheral and central nervous system neural circuits described below.

Urinary incontinence (UI) occurs when the lower urinary tract does not store urine properly and there is involuntary loss of urine. There are 3 types of UI: urge, stress, and mixed. Urge UI is considered to be due to an overactive bladder, while stress UI is considered to be due to decreased urethral outlet resistance. Mixed UI contains both components. Unlike most other autonomic control mechanisms, conscious control plays a major role in normal LUT function, allowing for discrete voiding under environmentally appropriate conditions. When conscious control of the parasympathetic micturition reflex is compromised, conditions of overactive bladder (OAB) and/or UI arise, creating serious health and social concerns.

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REFLEX CONTROL OF THE LOWER URINARY TRACT

Supraspinal Micturition Reflex - Figure 1 shows the "normal" supraspinal, parasympathetic micturition reflex responsible for determining when micturition occurs under normal conditions (without underlying pathology). This reflex is initiated by stretch receptors in the detrusor muscle (the smooth muscle of the bladder body) which passively stretches during filling and actively contracts during micturition. The stretch receptors are terminal specializations of thinly myelinated Aδ afferent fibers that traverse the pelvic nerve to reach the spinal cord. The cell bodies of these fibers are medium sized primary afferent neurons located in the sacral dorsal root ganglia (DRG). The central branches of these primary afferent neurons project along Lissauer's tract and the lateral edge of the dorsal horn (the "lateral projection") to contact second-order neurons in the dorsal horn of the sacral spinal cord (5). Via a spinobulbospinal pathway, second-order neurons in the sacral spinal cord project to the periaqueductal grey matter of the brain, which in turn activate neurons in the pontine micturition center (PMC).

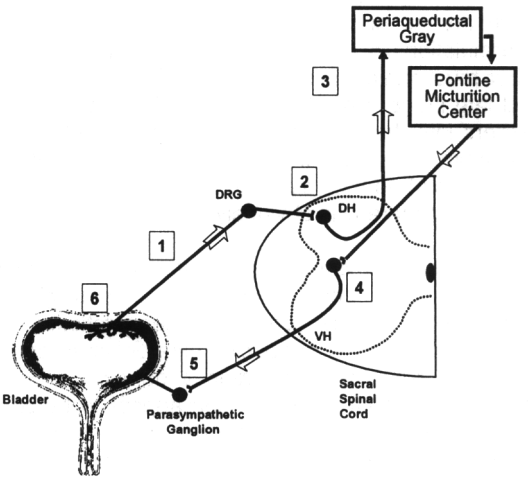


Figure 1. Schematic diagram depicting supraspinal micturition reflex pathway. Neuronal pathways between the bladder, spinal cord and brain are shown. The periaqueductal grey and pontine micturition center are supraspinal centers located in the brainstem. Numbers refer to the regions listed on Table 1. (DRG, dorsal root ganglion; DH, dorsal horn; VH, ventral horn)

The PMC (also called "Barrington's nucleus or "M" region) is located in the medial portion of the "dorsolateral tegmentum", medial to the locus coeruleus. Neurons in the PMC project directly to bladder preganglionic neurons located in the lateral band region of the sacral parasympathetic nucleus and to interneurons in the sacral dorsal gray commissure. The axons of cholinergic sacral parasympathetic preganglionic neurons traverse the pelvic nerve to activate pelvic parasympathetic post-ganglionic neurons in the pelvic plexus via nicotinic cholinergic receptors. The cholinergic postganglionic neurons, in turn, release acetylcholine resulting in detrusor smooth muscle contraction via stimulation of muscarinic M_2 or M_3 receptors.

Suprapontine (e.g. cortical or hypothalamic) control of the micturition reflex is crucial for ensuring that micturition occurs within the proper behavioral and environmental conditions. Compromise of these suprapontine controls is thought to play a role in the etiology of overactive bladder that accompanies, for example, cerebrovascular stroke.

C-fiber Spinal Micturition Reflex - Under normal conditions, primary afferent C-fibers do not respond to bladder distension (i.e. are not mechanosensitive) at normal bladder volumes. However, under pathological conditions, C-fibers become responsive to bladder distension at volume thresholds below those of Aδ fibers. In

contrast to the supraspinal organization of the bladder reflex initiated by myelinated Aδ fibers, the bladder reflex that is initiated by unmyelinated C-fibers is organized within the sacral spinal cord (not shown in Figure 1.) The bladder C-fibers also have their cell bodies in the sacral DRG and probably also project along Lissauer's tract and the lateral projection to activate second order neurons. However, unlike the supraspinal micturition reflex pathway, the C-fiber reflex does not depend on supraspinal communication to activate sacral parasympathetic preganglionic neurons. The parasympathetic efferent pathway of this C-fiber spinal reflex and the Aδ fiber supraspinal reflex are the same. The C-fiber spinal reflex, however, is not consistently demonstrable in animals with an intact spinal cord, unless the bladder outlet is obstructed for weeks or under conditions of bladder inflammation or irritation. Importantly, loss of descending control following spinal cord injury also allows for the C-fiber reflex to be revealed. This latter observation suggests a tonic descending inhibition of a C-fiber contribution to normal micturition and that interruption of this supraspinal inhibition reveals C-fiber reflex activity. For these reasons and others, sensitization of, and/or removal of inhibition from, the C-fiber reflex pathways are thought to contribute to the etiology of overactive bladder and urge incontinence that accompanies benign prostatic hyperplasia, spinal cord trauma or disease, and urinary tract infections.

Sympathetic Storage Reflex - Figure 2 shows the sympathetic storage reflex (pelvic-to-hypogastric reflex) pathway. This reflex is also initiated by bladder distension and activation of Aδ fibers of the pelvic nerve that, in turn, activate sacral dorsal horn interneurons. An intersegmental, polysynaptic pathway projects rostrally to activate efferent sympathetic preganglionic neurons, situated at L1-L3 levels. Their efferent axons travel along the inferior splanchnic nerve to the inferior mesenteric ganglion (IMG) where they either synapse (minor component) or continue along the hypogastric nerve to synapse in the pelvic plexus (major component). Postganglionic sympathetic neurons release norepinephrine, which facilitates urine storage by stimulating β_3 adrenergic receptors that relax bladder smooth muscle, by stimulating α_1 adrenergic receptors that contract urethral smooth muscle, and by stimulating α adrenergic receptors that inhibit ganglionic transmission (6). During micturition, this reflex pathway is markedly inhibited via supraspinal mechanisms to allow the bladder to contract and the urethra to relax.

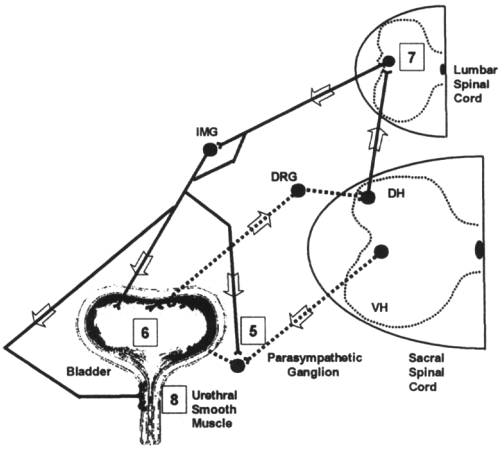


Figure 2. Schematic diagram depicting sympathetic storage pathways. Dotted lines represent afferent and efferent pathways previously shown in Figure 1. Solid lines represent novel sympathetic storage pathways. Numbers refer to the regions listed on Tables 1 and 2. (DRG, dorsal root ganglion; DH, dorsal horn; VH, ventral horn; SPN, sacral parasympathetic nucleus; IMG, inferior mesenteric ganglion)

Somatic Storage Reflex – Figure 3 shows the somatic storage reflex (pelvic-to-pudendal reflex) pathway. This reflex is also initiated by activation of Aδ fibers of the pelvic nerve that, in turn, activate sacral dorsal horn interneurons. An

intrasegmental path travels from the sacral dorsal horn to the sacral ventral horn via polysynaptic connections. Efferent somatic urethral sphincter motor neurons are located in the lateral subdivision of "Onuf's nucleus" (7). The axons from these motor neurons traverse the pudendal nerve and release acetylcholine which, in turn, activates nicotinic cholinergic receptors on striated muscle fibers of the urethra causing them to contract. During urine storage, this pathway is tonically active, and during micturition this reflex is strongly inhibited via spinal and supraspinal mechanisms to allow the urethral sphincter to relax and permit passage of urine through the urethra.

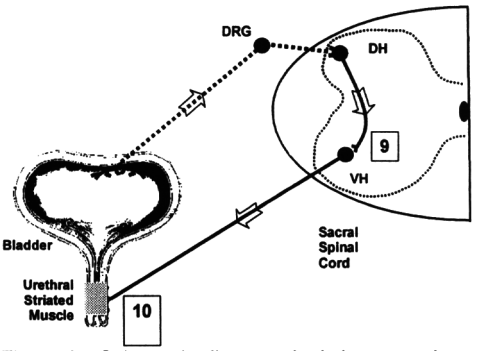


Figure 3. Schematic diagram depicting somatic storage pathways. Dotted lines represent the afferent pathway previously shown in Figure 1. Solid lines represent novel somatic storage pathways. Numbers refer to the regions listed on Table 3.

SITES OF DRUG ACTION FOR INHIBITION OF MICTURITION REFLEXES

Drugs that suppress the micturition reflex could be useful for treating overactive bladder and urge UI. Targets for suppressing the overactive bladder are divided into 6 sites (Figure 1, Table 1): primary afferent neurons (and their peripheral terminals in the bladder); the sacral dorsal horn; the periaqueductal gray/pontine micturition center; the sacral parasympathetic nucleus; the pelvic parasympathetic ganglion; and the detrusor smooth muscle neuroeffector junction.

Therapeutic agents that suppress action potential initiation and/or propagation along primary afferent fibers, through manipulation of ion channels or G protein-coupled receptors (GPCR), would be expected to increase the volume (i.e. stretch) threshold for activation of the micturition reflex and thus reduce bladder overactivity and urgency. Because C-fibers are responsible for pathological bladder activity, while Aδ-fibers are responsible for normal micturition, it would be beneficial to find targets that are preferentially associated with C-fibers (e.g. VR1 vanilloid receptors).

Once the action potential from the primary afferent fiber reaches its spinal synaptic terminal or "*en passant*" boutons, an influx of calcium is necessary to cause fusion of the synaptic vesicles with the plasma membrane and subsequent release of the primary afferent neurotransmitters. Thus, compounds that can reduce the flow of calcium through blockade of N-type calcium channels or through activation of GPCR receptors (e.g. opioid) will reduce release of transmitters and subsequent excitation of 2nd order neurons in the reflex pathway. Another strategy for blocking 2nd order neuron activation is to block the postsynaptic receptors on the 2nd order neurons that respond to the transmitters of the primary afferent neurons (e.g. NK1 receptor antagonists to block substance P-induced excitation). However, because there is a redundancy of transmitters in primary afferent neurons (e.g. glutamate, substance P, vasoactive intestinal polypeptide), it remains to be shown that blocking a single receptor subtype will completely prevent transmission of afferent input. A final way to block transmission is to directly activate inhibitory receptors on the 2nd order neurons (e.g. opioids). Because this would reduce their

Table 1. Sites and targets for inhibition of the micturition reflex.

Site	Target	Function (+ indicates excitatory; - indicates inhibitory)	References
1. Primary Afferent Neurons	Na^+ channels (Nav1.8)	+ peripheral	11
	VR1	+ peripheral	12, 13
	$P2X_3$	+ peripheral	14, 15
	NK_2	+ peripheral	16
	Nitric oxide	- peripheral	17
	K^+ channels	- peripheral	18
	δ, κ opioid	- peripheral	19, 20
2. Sacral Dorsal Horn	N-type Ca^{++} channel	+ presynaptic	17
	μ , δ opioid	-	21, 22, 23
	5-HT_1, 5-HT_3,	-	1, 24, 25
	α_2 adrenergic	-	26
	$GABA_A$, Glycine	-	27
	Nitric oxide	- presynaptic	28
	NK_1	+ postsynaptic	29
	AMPA, NMDA	+	30, 31
	A_1 adenosine	-	32
3. PAG / PMC sites	NMDA	+	33
	D_2 dopamine	+	34
	μ, δ opioid	-	35
	$GABA_A$	-	36
	muscarinic	-	37, 38
	D_1 dopamine	-	39
4. Sacral Parasympathetic Nucleus	AMPA, NMDA	+ postsynaptic	31, 40, 41
	α_1 adrenergic	+	42
	δ opioid	-	21, 43
	5-HT	-	44, 25
	$GABA_A$	- postsynaptic	45, 46
	Glycine	- postsynaptic	45, 47
5. Parasym-pathetic Ganglia	VIP	+ postsynaptic	48
	δ opioid	- presynaptic	49
	$GABA_A$	-	49, 50
	α adrenergic	-	51
	5-HT_{1A}	-	52
6. Bladder Smooth Muscle Neuroeffector Junction	M_1 muscarinic	+ prejunctional	53, 54
	α_1 adrenergic	+ prejunctional	55
	5-HT_4	+ prejunctional	56
	M_2 muscarinic	- prejunctional	57
	Botulinum toxin	- prejunctional	58
	M_2 / M_3 muscarinic	+ smooth muscle	59
	$P2X_1$	+ smooth muscle	60
	NK_2	+ smooth muscle	61
	L-type Ca^{++} channels	+ smooth muscle	62
	Ryanodine-sensitive Ca^{++} channels	+ smooth muscle	63, 37

Table 1. Sites and targets for inhibition of the micturition reflex (con't).

6. Bladder Smooth Muscle Neuroeffector Junction (con't)	AtII angiotensin	+ smooth muscle	64, 65
	ET_A endothelin	+ smooth muscle	66
	K_{ATP}, K^+ channels	- smooth muscle	67
	β_3 adrenergic	- smooth muscle	68
	Nitric Oxide	- smooth muscle	69
	VIP	- smooth muscle	70
	α_1 adrenergic	+ smooth muscle	42
	Botulinum toxin	Prejunctional neurotoxin	10

excitability regardless of the primary afferent transmitter, this strategy may be the more powerful for targeting the afferent limb. Moreover, if C-fiber reflex 2nd order neurons are different and express different receptor subtypes from 2nd order neurons involved in the supraspinal Aδ reflex, it may be possible to selectively block pathological bladder activity.

In cases where overactive bladder is due to loss of suprapontine inhibitory controls (e.g. stroke), it would be logical to target the periaqueductal gray (PAG) or PMC. Intracerebroventricular administration of various compounds, as well as direct injection into the PMC have shown that these supraspinal sites can be suppressed either through blockade of excitatory receptors or activation of inhibitory receptors. As an example, targeting supraspinal inhibitory controls has worked extremely well for treating Parkinson patients that suffer bladder overactivity using pergolide, a dopamine receptor agonist that substantially restores normal bladder activity.

Drugs that act on the efferent outflow in the sacral parasympathetic nucleus are unlikely to show a specific effect on pathological bladder conditions. However, in cases where bladder overactivity and bladder-sphincter dyssynergia co-exist (e.g. spinal cord injured patients) and intermittent catheterization must be used as a part of normal health maintenance, selectivity may not be a concern.

Similarly, drugs that work by suppressing either ganglionic transmission, transmission at the neuroeffector junction, or at the level of the smooth muscle are not likely to spare normal micturition. However, an advantage of targeting these sites is that the drugs do not have to cross the blood brain barrier and thus might offer a larger therapeutic window. Drugs that reduce transmission between the parasympathetic postganglionic neuron and the smooth muscle (i.e. reduce neuroeffector junction transmission) either reduce the presynaptic release of acetylcholine (e.g. M_1 receptor antagonists or M_2 receptor agonists), poison the cholinergic nerve terminal (e.g. botulinum A toxin), or block the excitatory smooth muscle receptor (e.g. M_3 receptor antagonists). Drugs that work directly on the smooth muscle (i.e. produce relaxation) can either activate inhibitory receptors (e.g. β_3 adrenergic receptor agonists), suppress influx of Ca^{+2}, or hyperpolarize the smooth muscle (e.g. K_{ATP} channel openers).

SITES OF DRUG ACTION FOR ENHANCING STORAGE REFLEXES

Drugs that enhance the storage reflexes may be useful for treating stress UI. There are a number of reasons for targeting the somatic storage reflex over the sympathetic reflex in stress UI. Since stress UI is episodic, often occurring with unanticipated, rapid increases in abdominal pressure (e.g. during a cough, laugh, or sneeze), a reflex to counteract these rapid increases in pressure should also

respond rapidly. The somatic storage reflex contains both a tonic and a rapid phasic component, while the sympathetic reflex only shows tonic or slower responses. This slower response is evident in both the central delays for the reflex (60 msec for pelvic-to-hypogastric versus 10 msec for pelvic-to-pudendal reflex) as well as the end organ response, where smooth muscle contracts much more slowly than striated muscle. In addition, striated muscle can generate a much more powerful phasic contraction than smooth muscle and thus offers greater resistance to urine loss. However, even a small pressure increase due to smooth muscle contraction distributed along the length of the urethra would be expected to contribute somewhat to an increase in urethral resistance.

An additional point to consider is that it has not yet been demonstrated that the Aδ primary afferent neurons that mediate the supraspinal micturition reflex are any different than the Aδ fibers that mediate the storage reflexes. In fact, it is possible that collateral branches from the same primary afferent neuron might project to multiple 2nd order neurons of both micturition and storage reflexes. Thus, primary afferent neurons or dorsal horn sites as drug targets for storage reflexes will not be discussed, as targeting these sites for this purpose may interfere with normal micturition reflexes.

Sympathetic Reflex Drug Targets - Only a few studies have examined pharmacology of sympathetic reflexes to the bladder, and these studies focused on central noradrenergic mechanisms. These studies showed that α_1 adrenergic receptor stimulation enhances hypogastric nerve reflexes, while α_2 adrenergic receptor stimulation suppresses the reflexes. Both seem to be tonically active in anesthetized cats since α_1 adrenergic receptor antagonists suppress hypogastric nerve activity, while α_2 adrenergic receptor antagonists facilitate activity. Norepinephrine reuptake inhibitors seem to increase activation of both receptor subtypes without a preference.

Table 2. Sites and targets for enhancing the sympathetic storage reflex.

Region	Target	Function	References
7. Sympathetic Preganglionic Nucleus	α_1 adrenergic	+	71
	α_2 adrenergic	-	71, 72, 73
8. Urethral Smooth Muscle Neuroeffector Junction	α_1 adrenergic	+ smooth muscle	8
	β adrenergic	- smooth muscle	8

Urethral smooth muscle is also an area of research for stress UI because α_1 adrenergic receptors produce an increase in urethral tone. However, α_1 adrenergic receptor agonists have yet to be proven clinically useful. Inhibitory β adrenergic receptors are also found in the urethra. Under experimental conditions, norepinephrine reuptake inhibitors produce an increase in β adrenergic receptor stimulation (which produces urethral smooth muscle relaxation) in urethra (8).

Somatic Reflex Drug Targets - The pharmacology of somatic reflex activity has received slightly more attention than sympathetic activity. Adrenergic responses of somatic pathways are similar to those on sympathetic pathways with α_1 adrenergic receptors mediating facilitation and α_2 adrenergic receptors mediating inhibition. In addition, 5-HT_2 receptors and the ionotropic excitatory amino acid receptors (NMDA and AMPA), appear excitatory, while κ opioid receptors appear inhibitory. Duloxetine hydrochloride, a serotonin norepinephrine reuptake inhibitor, enhanced somatic activation of the urethral sphincter by increasing activation of 5-HT_2 and α_1

adrenergic receptor stimulation by increasing levels of serotonin and norepinephrine, respectively.

Table 3. Sites and targets for enhancing the somatic storage reflex.

Region	Target	Function	References
9. Onuf's Nucleus	α_1 adrenergic	+	71, 74
	5-HT_2	+	75
	AMPA, NMDA	+	40, 76
	κ opioid	-	77
	α_2 adrenergic	-	71
10. Urethral Striated Muscle Neuroeffector Junction	Botulinum toxin	- prejunctional neurotoxin	78

Conclusions - Most previous pharmacological research and current therapies for overactive bladder and stress incontinence focus on the bladder and urethral smooth muscle, respectively. For overactive bladder there are a plethora of muscarinic cholinergic receptor antagonists on the market (oxybutynin, tolterodine, trospium, etc.) or in clinical development (darifenicin, YM905). As alluded to above, these agents suffer from blockade of normal micturition reflexes and result in increased residual urine, which can reduce functional bladder capacity (i.e. following micturition, the bladder begins filling again already partially full) and may provide favorable conditions for urinary tract infection. Numerous companies have also examined β3 adrenergic receptor agonists and K^+ channel openers to relax bladder smooth muscle without clinical success to date. Similarly for stress incontinence, focus has been on smooth muscle α_1 adrenergic receptor agonists in an attempt to increase urethral tone; this has not met with clinical success.

Thus, this review focuses on neural mechanisms as targets for therapy. This strategy has met with clinical success through the use of duloxetine as a treatment for stress urinary incontinence (9). Limited success with this strategy has also been shown in the clinic with vanilloids, capsaicin and resiniferatoxin (10). These VR1 receptor agonists "over stimulate" the VR1 receptor and cause desensitization of C-fiber afferent terminals in the bladder. However, this intravesical therapy requires catheterization and administration directly into the bladder, thus it has limited applications.

Based upon the abundance of site-specific targets, it may be possible in the future to selectively modulate various components of the micturition and storage reflex pathways, in order to achieve the best therapeutic benefit for the patient.

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SECTION II. CARDIOVASCULAR AND METABOLIC DISEASES

Editor: William J. Greenlee, Schering Plough Research Institute, Kenilworth,NJ

Chapter 7. Recent Developments in Neuropeptide Y Receptor Modulators

Andrew W. Stamford, Joyce Hwa, Margaret van Heek
Schering-Plough Research Institute
2015 Galloping Hill Road, Kenilworth NJ 07033

Introduction - Neuropeptide Y (NPY) is a 36 amino acid neuropeptide that is widely distributed in both the central and peripheral nervous systems, and is one of the most abundant neuropeptides identified to date (1-5). NPY is a member of the pancreatic polypeptide family, which includes the structurally related peptides, peptide YY (PYY) and pancreatic polypeptide (PP). Scientific investigation in the last twenty years has generated a large body of knowledge about the biology of NPY, and thus it is now known that NPY is involved in a broad spectrum of physiological systems. The effects of NPY are mediated by several G protein-coupled receptors which have been designated Y_1, Y_2, Y_3, Y_4, Y_5, and y_6 (5,6). These receptors share only modest primary sequence homology (30-50%) and have unique tissue localizations. Due to the compelling biology, there has been a great deal of interest in identifying and developing drugs that modulate the NPY system, particularly in the area of body weight regulation. This review briefly describes the role that NPY and its receptors play in these physiological systems, and summarizes the status of drug discovery in the NPY field.

POTENTIAL THERAPEUTIC APPLICATIONS

Body Weight Regulation - NPY has potent effects on physiological and endocrine systems that modulate energy homeostasis (2,3,7). NPY has been identified as the most potent naturally occurring orexigenic peptide. Over the last few decades, it has been demonstrated in a number of species that administration of NPY to the brains of satiated animals induces a tremendous surge in food consumption (8,9). Although not entirely understood, this feeding response is thought to be mediated by the NPY Y_1, NPY Y_5 or both receptors, each of which is expressed in the hypothalamus. Central administration of NPY also lowers energy expenditure (10,11). Increased food consumption and decreased energy expenditure results in a state of positive energy balance that will promote adipose tissue accretion. Indeed, chronic central administration of NPY to normal rats results in a pathophysiological profile similar to that in human obesity, including hyperphagia, increased adiposity, hyperleptinemia, hyperinsulinemia and hypertriglyceridemia (12,13).

Several different lines of evidence indicate that endogenous NPY may play a key role in energy homeostasis. Fasting leads to a significant increase in NPY mRNA expression in the arcuate nucleus, and an increase in NPY itself in brain regions known to be involved in energy homeostasis, the arcuate nucleus and the paraventricular nucleus (14). This increased NPY synthesis and release may drive the hyperphagic response observed after fasting. The *ob/ob* mouse, the *db/db* mouse, and the Zucker rat, which are genetic models of early onset, spontaneous obesity, exhibit high levels of hypothalamic NPY mRNA and peptide (15,16). Thus, increased NPY transmission may be partially responsible for the extreme hyperphagia and morbid obesity in these rodents. Interestingly, the obese phenotype of the *ob/ob* mouse is attenuated when NPY is removed from the system, indicating that NPY is at least partially responsible for the massive obesity in the *ob/ob* mouse (17). However, results from other mutant mice in which either NPY or its receptors have been manipulated have been paradoxical. While mice overexpressing NPY

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have an expected profile only when maintained on certain diets which promote obesity (18), NPY deficient mice have the same food intake, body weight, plasma corticosterone, insulin, and glucose levels as their wildtype counterparts (19). Mice lacking NPY Y_1 receptors have slightly diminished nocturnal and NPY-stimulated feeding, but refeeding after fasting was significantly decreased in the Y_1 -/- mice (20,21). However, Y_1 receptor deficient mice develop mild late onset obesity and moderate hyperinsulinemia, which could be due to the reduced locomotor activity observed in these mice. Thus far, data generated in mice lacking NPY Y_5 receptors do not support a role for this receptor in mediating the effects of NPY on body weight regulation (22). Young NPY Y_5 deficient mice have normal growth, feeding behavior, and body temperature. NPY Y_5 deficient mice also unexpectedly develop mild late onset obesity, likely due to an increase in food intake. *Ob/ob* mice deficient for NPY Y_5 were as obese as *ob/ob* mice, indicating that the attenuation of obesity in the *ob/ob* mouse lacking NPY is not mediated through the NPY Y_5 receptor. Studies in the NPY Y_1 and Y_5 knockout mice do indicate a role for these receptors in the increase in food intake following centrally administered NPY.

More recently, the NPY Y_2 receptor has emerged as a potential player in energy homeostasis. Peripheral injection of PYY(3-36), an NPY Y_2 agonist, reduced food intake and weight gain in rats and mice, but not in Y_2 -/- mice. *Ob/ob* mice lacking the NPY Y_2 receptor have reduced adiposity, hyperglycemia, hyperinsulinemia and have increased HPA axis activity (23). In addition, peripheral administration of PYY(3-36) to humans significantly reduced appetite and food intake (24).

Recent studies suggest that one mechanism by which NPY promotes a state of positive energy balance is through its inhibitory effects on the HPT axis (7,25). Centrally administered NPY to rats decreased circulating levels of the thyroid hormones T_3 and T_4, and suppressed proTRH mRNA synthesis, thus mimicking the effects of fasting. The actions of NPY on the HPT axis are mediated by hypothalamic Y_1 and Y_5 receptors (26). Consistent with the inhibitory action of NPY on TRH release is the co-localization of GABA-ergic neurons expressing NPY immunoreactivity and TRH-producing neurons in the paraventricular nucleus (27).

Circadian Rhythm – The role of NPY in modulating circadian rhythm has recently been reviewed (28). NPY affects the mammalian circadian system in two ways: it can change the phase of the clock itself during the subjective day, and it can inhibit the phase shift normally caused by light during the subjective night. The change effected by NPY on circadian rhythm during the day is thought to be mediated by the NPY Y_2 receptor, while the inhibition by light-induced phase shift at night appears to be mediated by the NPY Y_5 receptor.

Anxiety and Depression - A large body of evidence has implicated a role for NPY and its receptors in psychiatric illnesses such as anxiety and depression, and this has been extensively reviewed (4,29). NPY is consistently reported to produce anxiolytic effects in a variety of anxiety/depression models, including punished responding tests, exploratory behavior models, social interaction and fear potentiated startle. The data support a strong role for the NPY Y_1 receptor in anxiety; however, involvement of other NPY receptors has not been ruled out.

Bone Formation - NPY is a down stream modulator of leptin action. Chronic central administration of NPY and leptin both have a similar inhibitory effect on bone mass. By comparing bone phenotypes of germline and selective hypothalamic Y_2 receptor - deficient mice, it was reported that hypothalamic Y_2 receptors are involved in a tonic inhibition of bone formation by alteration of autonomic activity in the bone (30). Y_2 receptor deficient mice have an increased rate of bone mineralization, as well as stimulated osteoblast activity which leads to a two fold increase in bone volume. This

rapid increase in bone volume after central deletion of Y_2 receptors suggests that a Y_2 receptor antagonist may be an effective treatment for the prevention of osteoporosis.

Congestive Heart Failure - Increased plasma levels of NPY are found in patients with cardiovascular disease, including hypertension and heart failure (31). Although a causal role for circulating NPY in these diseases has not been established, a correlation has been established between plasma NPY concentration and severity of left ventricular hypertrophy. NPY can contribute to cardiac hypertrophy via its hemodynamic effects on blood vessels through the NPY Y_1 receptor (32). However, NPY can also produce cardiac hypertrophy by acting as a growth factor by directly activating p38, ERK and JNK in primary cardiomyocytes (33), or by potentiating the alpha-adrenergic agonist-induced activation of mitogen-activated protein kinase via the NPY Y_5 receptor (33). It has also been demonstrated that NPY can increase protein synthesis and/or inhibit protein degradation via NPY Y_5 receptors in SHR cardiomyocytes (34). NPY Y_5 receptors may therefore represent a novel therapeutic target for drugs designed to prevent or regress left ventricular hypertrophy.

Rhinitis and Nasal Congestion - Nasal obstruction and rhinorrhea present in allergic rhinitis are at least partly influenced by neuropeptides released from sensory, parasympathetic, and sympathetic nerves. NPY is co-localized with norepinephrine in sympathetic perivascular nerves. NPY is released with norepinephrine on sympathetic nerve stimulation and produces long lasting vasoconstriction of the nasal vascular bed through postsynaptic NPY Y_1 receptors (35). In addition to direct vasoconstriction, there is evidence to suggest that NPY modulates the release of transmitters originating from parasympathetic and sensory nerves by acting on prejunctional NPY Y_2 receptors. This would then attenuate the vasodilator response to the subsequent parasympathetic nerve stimulation in the nasal mucosa via non-adrenergic and non-cholinergic mechanisms (36). Putative therapeutic application of NPY in rhinitis has been recently suggested because intranasal administration of exogenous NPY in human beings reduces nasal airway resistance and vascular permeability without affecting submucosal gland secretion (37). Intranasal or intrabronchial pretreatment with a NPY Y_2 receptor peptide agonist, (TASP-V), reduced nasal obstruction and bronchoconstriction evoked by histamine challenge in the pig; this agent also attenuated objective histamine-induced nasal obstruction in healthy volunteers (38). Therefore, NPY Y_2 receptor agonists may have therapeutic applications in allergic rhinitis and asthma.

Pain - It has been reported that NPY modulates nociception at different levels in the central nervous system. At the spinal level, it was shown that intrathecal injection of NPY can be anti-nociceptive and pronociceptive in uninjured animals models depending on the dose due to biphasic dose-effect curves (39,40). Experiments with Y_1 knockout mice suggest that Y_1 receptors contribute to the antinociceptive effects of intrathecal NPY in rodent models of acute nociception (41). After experimentally-induced nerve injury, NPY gene expression is upregulated within the population of medium- and large-diameter DRG neurons of the A beta-fiber class and follows a time course which is consistent with the development of tactile hypersensitivity-induced allodynia (42). NPY microinjection into the n. gracilis of uninjured rats induced reversible tactile allodynia, but not thermal hypersensitivity, in the ipsilateral hindpaw. In addition, NPY anti-serum and the NPY Y_1 receptor antagonist BIBO 3304 blocked nerve injury-induced tactile hypersensitivity, but not thermal hyperalgesia (43). These data suggest that NPY Y_1 receptor antagonists may be useful for the treatment of nerve injury-induced tactile allodynia.

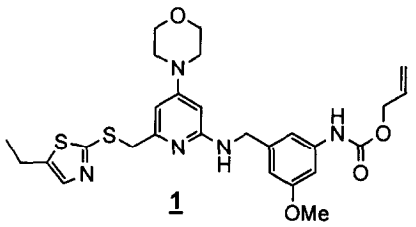
Alcohol Dependence - There is a growing body of evidence suggesting that NPY plays a major role in alcohol dependence. Alcohol consumption is elevated in NPY-

deficient mice, but decreased in transgenic mice that overexpress NPY (44). Alcohol consumption behavior was enhanced in mutant mice lacking the NPY Y_1 receptors, (45) but reduced in mutant mice deficient of NPY Y_2 receptors (46). In addition, mutant mice without the NPY Y_1 receptor were less sensitive to alcohol-induced sedation, whereas mutant mice lacking the NPY Y_5 receptor had increased sleeping time without altering voluntary alcohol consumption behavior (45). Recently, it has been confirmed that icv administration of the selective NPY Y_2 receptor antagonist, BIIE0246, can suppress alcohol self-administration in rodents, without affecting the consumption of a sweetened solution (47). These data suggest that a Y_2 receptor antagonist might be a novel treatment for alcoholism.

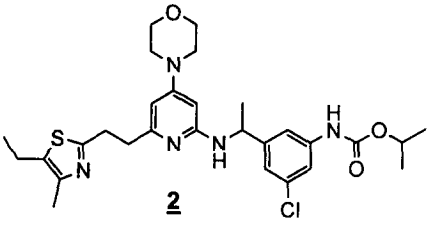
NPY RECEPTOR LIGANDS

This section will highlight structure-activity relationship (SAR) studies and pharmacological characterization of small molecule NPY receptor antagonists that have been reported recently. Most studies have focused on characterization of NPY Y_1 and Y_5 receptor antagonists as potential therapeutic agents for obesity management. There has been little recent activity with respect to selective NPY Y_2 and Y_4 receptor ligands.

NPY Y_1 Receptor Ligands – Acute in vivo studies with the potent, orally active aminopyridine Y_1 receptor antagonists J-104870 (**1**) and J-115814 (**2**) support a role for NPY Y_1 receptors in modulation of feeding behavior in rodents (48). Importantly, **2** suppressed feeding in wild-type mice, but not in NPY Y_1 receptor-deficient mice, supporting the Y_1 specificity of the compound (49). Recently **1** was evaluated chronically in obese Zucker rats (50). The compound is brain-penetrant, has high affinity for rat and human Y_1 receptors (K_i of 0.51 nM and 0.26 nM, respectively), no significant affinity for human Y_2, Y_4 and Y_5 receptors (K_i>6000 nM), and is a potent functional antagonist of Y_1 receptors (51). Administration of **1** to Zucker rats (100 mg/kg/day p.o.) for 2 weeks caused a reduction in food consumption which plateaued after 4 days, then gradually approached that of control animals. Body weight gain after 2 weeks was inhibited by 3% in treated animals relative to control animals. The inhibition in body weight gain was accompanied by a reduction in epididymal adipose cell size, normalization of plasma corticosterone levels, and reduced fat accumulation in the liver. Although these effects were observed at a relatively high dose, no overt adverse effects were discerned; however the difficulties in ruling out non-Y_1 receptor mediated mechanisms that might account for the anorectic effect were pointed out (50).



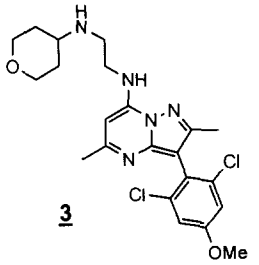
1



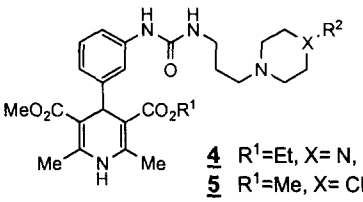
2

The structurally distinct pyrazolopyrimidine CP-671906 (**3**) displays high affinity for human and rat NPY Y_1 receptors (K_i of 1.5 nM and 3.5 nM, respectively) low affinity for NPY Y_2 and Y_5 receptors (K_i>1,000 nM) and no significant binding against a panel of other receptors, enzymes and ion channels (52). The compound is a functional antagonist of NPY Y_1 receptors, and is orally bioavailable and brain penetrant in rats. Administration of a 40 mg/kg p.o. dose of **3** to rats inhibited feeding induced by NPY, but not spontaneous feeding or feeding induced by a Y_5 receptor agonist (52). While the latter observations are consistent with a specific Y_1 receptor-

mediated anorectic effect, **3** inhibited food intake in obese Zucker rats and in overweight beagle dogs near doses that produced adverse effects, casting doubts on the Y_1 receptor specificity of its anorectic effects.



3

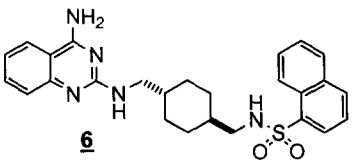


4 R^1=Et, X= N, R^2 = 2-MeOPh
5 R^1=Me, X= CH, R^2 = 3-MeOPh

SAR studies related to the high-throughput screening lead **4**, a moderately potent antagonist of human NPY Y_1 receptors (K_i=109 nM), have been reported (53,54). Modification of the piperazine and C3-ester functionalities afforded BMS-193885 (**5**), which exhibited a K_i of 3.3 nM for Y_1 receptors (53). This compound was demonstrated to be a functional antagonist of human Y_1 receptors (K_b=4.5 nM), and did not have significant affinity for NPY Y_2, Y_4 and Y_5 receptors. In rats, **5** (10 – 30 mg/kg) administered intraperitoneally (i.p.) dose-dependently inhibited NPY-induced feeding, and spontaneous nocturnal food consumption (53). Replacement of the dihydropyridine moiety by dihydropyrazine resulted in loss of Y_1 receptor binding affinity (54).

Taken together, the results from studies with the newer, structurally diverse, brain-penetrant NPY Y_1 receptor antagonists in wild-type and genetically modified rodent models confirm a role for NPY Y_1 receptors for mediating feeding, in particular for feeding following a fasting period.

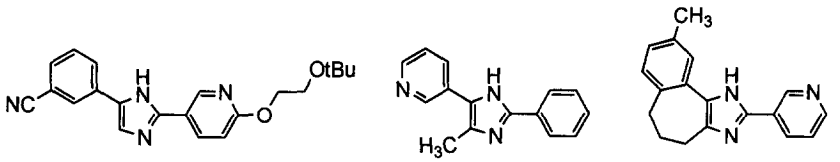
NPY Y_5 Receptor Ligands – Since the late 1990's there have been numerous reports of potent NPY Y_5 receptor antagonists, which have been the subject of recent reviews (55, 56). Activity in the area reflects both the high level of interest in NPY Y_5 receptor antagonists as potential anti-obesity agents, and the apparent ease of identifying tractable leads suitable for optimization. While reports of new NPY Y_5 receptor antagonists have slowed over the past year, there has been significant progress in the development of potent Y_5 receptor antagonists with optimized pharmacokinetic properties and their evaluation in feeding models. Studies with these newer, structurally distinct compounds are important in light of recent data reported for CGP 71683A (**6**), one of the first potent and selective Y_5 receptor antagonists. In studies that were interpreted as defining the pharmacological profile of a Y_5 antagonist, **6** produced marked anorectic effects in rodent feeding models (57). However, the Y_5 receptor specificity of these effects has been discredited. In addition to its affinity for Y_5 receptors, **6** has high affinity for serotonergic transporters and muscarinic receptors, and inhibits food intake in NPY Y_5 receptor deficient mice and NPY deficient rats (58,59).



6

Detailed characterization of the bis-aryl imidazole **7** in rat feeding models has recently been reported (60). Compound **7** displayed high affinity for human and rat NPY Y_5 receptors (hY_5 K_i=1.2 nM; rY_5 K_i=1.7 nM) and antagonized NPY-induced Ca^{2+} mobilization in cells expressing the human Y_5 receptor with an IC_{50} of 0.7 nM. In addition, **7** did not significantly bind to NPY Y_1 and Y_2 receptors (IC_{50}>1 μM), or to

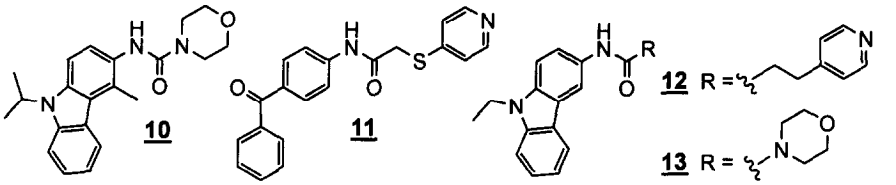
over 50 other receptors, ion channels and transporters (IC_{50}>1 μM). Compound **7** (30 mg/kg p.o.) achieved high CNS exposure in rats and inhibited food intake elicited by the Y_5 agonist bovine pancreatic polypeptide (bPP). At an oral dose of 40 mg/kg, **7** did not inhibit food intake in rats for a 3 h period following 24 h food deprivation, and a 30 mg/kg oral dose failed to inhibit spontaneous feeding, despite strong evidence that the compound occupies Y_5 receptors at these doses. It was further stated that **7** did not affect energy utilization in rats dosed at 30 mg/kg p.o. (60).



7 **8** **9**

Imidazole-based Y_5 receptor antagonists structurally related to **7** have independently been disclosed. Optimization of the weakly potent screening lead **8** resulted in identification of fused imidazole FR252384 (**9**) which displayed a K_i of 2.3 nM for recombinant human NPY Y_5 receptors (61). Compound **9** was stated to be orally bioavailable and brain penetrant but to be devoid of anorectic effects in Zucker rats.

The lack of anti-feeding effects of the imidazole **7** is consistent with studies reported for the structurally unrelated carbazole urea **10** (62,63). Compound **10** exhibited high affinity for rat and human NPY Y_5 receptors (IC_{50}=9 nM and 3 nM, respectively). Moreover, **10** did not display significant affinity for other NPY receptors or a diverse panel of unrelated receptors, enzymes or transporters. The compound was shown to be a functional antagonist of human Y_5 receptors in cell-based Ca^{2+} flux (IC_{50}=6 nM) and gene reporter assays (IC_{50}=37 nM). Key steps in the discovery of **10** from a previously reported thioether lead **11** (Y_5 IC_{50}=350 nM) were replacement of the thioether by a carbon linker and incorporation of the carbazole anilide to confer improved potency as in **12** (Y_5 IC_{50}=2 nM), improvement of pharmacokinetic properties by replacement of the pyridyl propionamide as a morpholine urea moiety to afford **13** (Y_5 IC_{50}=7 nM), and introduction of the 4-methyl substituent to eliminate the genotoxicity of the embedded aminocarbazole fragment (62).

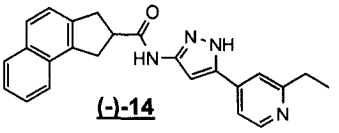


10 **11** **12** R = **13** R =

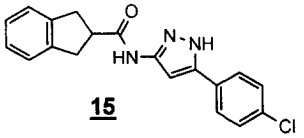
In rat, **10** possessed good oral bioavailability (F=76%) and a half-life of 3.7 h. The csf levels of **10** approximated the free plasma concentration, consistent with favorable CNS penetration. Oral administration of **10** (3 mg/kg) blocked food intake elicited by a selective Y_5 agonist, consistent with occupation of Y_5 receptors by **10**, but did not block NPY-stimulated food intake. Furthermore, **10** at a dose of 10 mg/kg p.o. did not attenuate feeding induced by fasting in lean Wistar rats, or spontaneous feeding in Wistar and obese Zucker rats. Compound **10** was also evaluated chronically in both normal Wistar rats, and diet-induced obese Wistar rats maintained on a high-energy diet following a dosing regimen consistent with chronic Y_5 receptor blockade (10 mg/kg p.o., b.i.d.). In these experiments, rats treated with **10** displayed caloric intake and body weight gain equivalent to that of vehicle-treated

control animals. In contrast, positive control rats that received the mixed norepinephrine/serotonin reuptake inhibitor sibutramine displayed reduced food intake and loss of body weight (63).

SAR studies on the development of the orally bioavailable, brain penetrant aryl pyrazole NPY Y_5 receptor antagonist **(-)-14** from the screening lead **15** (hY_5 K_i=59 nM) have recently been published (64). Critical steps in the optimization of **(-)-14** were modification of the indane carboxamide moiety to enhance affinity, and replacement of the 4-chlorophenyl moiety by 4-(2-ethylpyridinyl) to improve oral bioavailability. In a competitive binding assay employing ^{125}I-PYY as radioligand, compound **(-)-14** displayed high affinity for human NPY Y_5 receptors expressed in LMtk$^-$ cells (K_i=3.5 nM) and was about 7-fold more potent than its enantiomer. In addition, the compound did not show significant affinity for NPY Y_1, Y_2 or Y_4 receptors. Compound **(-)-14** blocked NPY-induced Ca^{2+} flux in CHO cells expressing human NPY Y_5 receptors (IC_{50}=14 nM), confirming that it is a functional antagonist. Oral administration of **(-)-14** to rats (10 and 30 mg/kg) resulted in high brain levels, but only moderate inhibition of feeding induced by the Y_5 agonist bPP. Central administration of **(-)-14** suppressed bPP-induced feeding, but not food intake elicited by NPY (64).

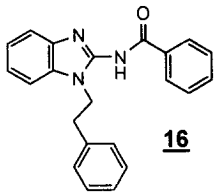


(-)-14



15

In contrast to the lack of activity of **7** and **10** in rodent models of spontaneous feeding, the benzimidazole GW438014A (**16**) displayed potent anorectic effects (65). In competition binding assays employing ^{125}I-PYY, **16** displayed moderate affinity for recombinant NPY Y_5 receptors expressed in CHO cells (K_i=211 nM), and did not bind significantly to NPY Y_1, Y_2 and Y_4 receptors (K_i>10,000 nM) or to a panel of unrelated receptors and transporters. Compound **16** reversed PYY-mediated inhibition of forskolin-induced cAMP production in HEC-1B cells expressing human NPY Y_5 receptors (IC_{50}=197 nM), confirming its antagonist properties. In rats, **16**, possessed poor oral bioavailability, however a 10 mg/kg dose of **16** administered i.p. afforded brain levels approximately two-fold above the K_i. In sated rats, **16** (3 mg/kg i.p.) significantly attenuated NPY-stimulated feeding, and had no effect on unstimulated feeding. The compound (10 mg/kg i.p.) caused profound and long-lasting reduction of food intake in food-deprived rats and in normal dark cycle feeding, and was stated to be non-aversive. In addition, repeated administration of **16** (10 mg/kg i.p.) to lean and obese Zucker rats for 6 days caused a reduction in food intake and fat mass (65).

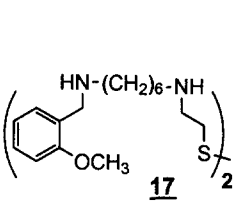


16

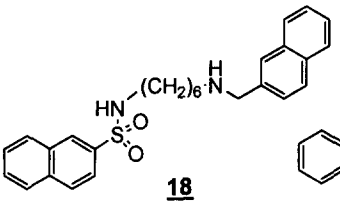
The in vivo profile of **16** is inconsistent with that of **7**, **10**, and **(-)-14** in that only **16** blocked feeding elicited by NPY, despite strong evidence that the latter compounds antagonized NPY Y_5 receptors at the doses tested. Furthermore, in contrast to **16**, compounds **7** and **10** were inactive in models of spontaneous feeding. Overall, the in vivo profile of **16** parallels that of the diaminoquinazoline **6**, thus a mechanism(s) for the anorectic effects of **16** unrelated to central NPY Y_5 receptor antagonism appears likely.

Finally, the design of potent, selective NPY Y_5 receptor antagonists from the weakly potent, non-selective lead benextramine **17** (hY_5 K_i=5 μM; hY_1 K_i=2 μM) has recently been published (66,67). Incorporation of an aryl sulfonamide connected to

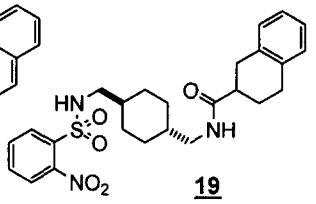
a benzylic amine through an alkyl linker as a GPCR privileged structure motif resulted in **18** (hY_5 K_i=123 nM) with enhanced Y_5 receptor affinity and selectivity. Rigidification of the alkyl linker as a *trans* bis-(1,4-diaminomethylene) linker, as in **19** (hY_5 K_i=8 nM), and application of high throughput synthesis techniques, resulted in an array of potent, selective Y_5 receptor antagonists (66, 67). This work is significant by virtue of its influence on the discovery of several diverse series of potent and selective Y_5 receptor antagonists represented by the quinazoline **6**, as well as a variety of other chemotypes (56, 68-71).



17



18



19

PERSPECTIVE

Much effort in the field has been devoted to addressing the potential for Y_1 and Y_5 antagonists to be developed for therapeutic intervention in obesity. Accumulated data supports a role for the NPY Y_1 receptor in modulating feeding after food deprivation and in genetically obese rodents in which NPY tone is elevated. These data suggest that centrally acting Y_1 receptor antagonists might be beneficial in obese patients who are dieting, formerly obese patients who have undergone substantial weight loss, or patients with complete or partial leptin deficiency. The potential for Y_5 antagonists as anti-obesity agents is less clear. The weight of evidence suggests that Y_5 antagonists do not significantly affect natural feeding in rodents, which is consistent with the phenotype observed for the Y_5 receptor knockout mouse. The well-documented stimulatory effects of selective Y_5 agonists on feeding may simply reflect an artificial situation that is not physiologically relevant. Some Y_5 antagonists have been reported to produce marked anorectic effects in vivo, however data supporting a Y_5 receptor mediated effect is generally lacking. However, the possibility remains that there is interplay between NPY Y_1, Y_2, and Y_5 receptor signaling in mediating the effects of NPY on energy homeostasis. Therefore Y_1 and Y_5 receptor antagonists in combination might have a more pronounced effect on energy homeostasis than antagonism of Y_1 or Y_5 receptors alone.

Key issues remain as to whether studies of NPY receptor antagonists in rodent obesity models are relevant to common obesity in the human population, whether patient subclasses that might benefit from treatment by NPY receptor blockade can be identified, and to what extent opposing effects of multiple complementary pathways in energy homeostasis will counteract changes in body weight. Given the myriad of physiological functions of NPY, there is also the potential for mechanism-based side effects associated with NPY receptor mediated pharmacotherapy.

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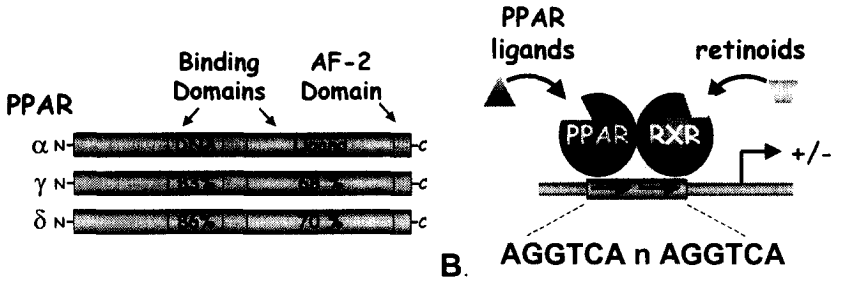
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Chapter 8. Modulators of Peroxisome Proliferator-Activated Receptors (PPARs)

Daniel D. Sternbach
GlaxoSmithKline
Five Moore Drive, Research Triangle Park, NC 27709

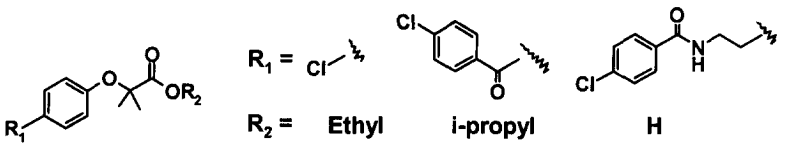
Introduction - The **P**eroxisome **P**roliferator-**A**ctivated **R**eceptors (**PPARs**) comprise a family of ligand-activated transcription factors belonging to the nuclear receptor gene superfamily (1-3). There are three PPAR subtypes that are products of separate genes and are commonly designated PPARα, PPARγ and PPARδ. In addition to a ligand-binding domain (LBD) these receptors also have an N-terminal domain and a DNA binding domain (Fig. A). The N-terminal domain is least well characterized and bears little homology among the subtypes. The ligand-binding domain has somewhat greater homology and the remaining variation allows these subtypes to be pharmacologically distinct. Upon binding a ligand the receptor forms a heterodimer with the 9-cis-retinoic acid receptor (RXR) (Fig. B). The highly conserved DNA-binding domains recognize, together with their RXR heterodimer partner, DNA sequences (response elements) containing direct repeats of the hexanucleotide AGGTCA separated by one nucleotide (e.g. AGGTCAnAGGTCA). The heterodimer interacts with the downstream transcription machinery and ultimately turns on the transcription of target genes.



Coactivators are linker proteins that recognize the activated PPAR receptor as well as the transcription machinery and thus are also required to turn on gene transcription (4-6). In some cases, negative regulation also occurs through the binding of corepressors (7). Thus gene transcription is regulated by a number of proteins and ligands in a very sensitive manner that might differ depending on the tissue.

PPARα, first cloned in the early 1990's, plays an important role in lipid metabolism. It acts as a dietary fat sensor by upregulating lipid metabolism (predominantly beta-oxidation) in the presence of fatty acids which are presumed to be the natural ligands for PPARα (8, 9). Although the PPARα DNA binding domains (DBDs) are identical across species, the ligand binding domains (LBDs) are not (human and murine receptors are 91% homologous), presumably reflecting evolutionary adaptation to different dietary ligands. This is particularly important when assessing the behavior of PPARα ligands in different animal models and how this behavior might extrapolate to humans. The fibrate drugs (e.g. clofibrate **1**, fenofibrate **2** and bezafibrate **3**) which are hypolipidemic agents, work through their agonist activity on PPARα. In rats, fibrates cause the formation of peroxisomes which in turn oxidatively metabolize fatty acids.

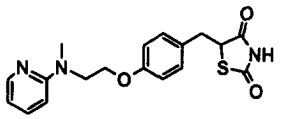
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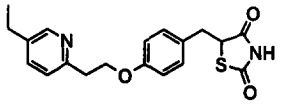
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In humans the fibrates are useful in the treatment of dyslipidemia by lowering serum triglycerides and raising HDL cholesterol (HDLc). They function by increasing clearance and decreasing the synthesis of very low density lipoproteins (VLDL) which are rich in triglycerides (10). Fibrates also lower serum levels of apolipoprotein CIII (apoCIII) which is a known inhibitor of VLDL clearance. PPARα is also implicated in atherosclerosis. Agonists have been shown to down-regulate the expression of VCAM-1, inhibition of NF-κB, inhibition of AP-1, and the reduction of plasma levels of interleukin-6, fibrinogen and C-reactive protein (11-14).

PPARγ has a surprisingly high degree of homology (95%) across all the species in which it has been cloned (15). Two isoforms of PPARγ have been detected in humans. Even though the isoforms have different tissue distributions, their functional differences are still unclear (16-18). PPARγ is central in the regulation of adipocyte differentiation (19, 20). This differentiation in turn leads to the expression of several other genes involved in glucose and lipid homeostasis (see ref 1 and references therein). The natural ligands for PPARγ known to date, eicosanoids and their metabolites such as prostaglandin J_2, are weak agonists with EC_{50}s in the micromolar range. PPARγ is an important regulator of target genes involved in glucose and lipid metabolism (21). The key finding that the thiazolidinedione (TZD) antidiabetic drugs Avandia (rosiglitazone, **4**) and Actos (pioglitazone, **5**) were PPARγ agonists led to an explosion of research in the area (22, 23).



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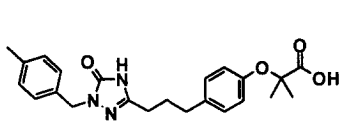
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The finding that PPARγ is expressed in macrophage foam cells has stimulated research in the potential use of PPARγ ligands as anti atherogenic agents since foam cells are precursors to atherosclerotic lesions (24-26).

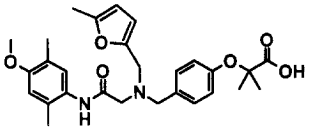
PPARδ was cloned from a number of species in the early 1990's (1). It has been known by several names such as NUC1 (27), FAAR (28) and PPARβ (29) but the generally accepted name today is PPARδ (27-29). The LBDs of the human and rodent receptors are about 90% homologous. The fact that PPARδ is ubiquitously expressed, and that there are few potent selective ligands available, has hampered research in determining its biological function (30,31). Unlike PPARα and PPARγ no drugs have been identified that target PPARδ. Recently, there have been hints that PPARδ agonist may be useful for treating dyslipidemia and certain dermatological conditions while an antagonist may be useful for osteoporosis and for colorectal cancer (32-35). PPARδ binds various long chain fatty acids at micromolar concentration making it a likely fatty acid sensor (36). Recently this interaction with fatty acids has been linked to VLDL particles which can activate PPARδ in macrophages, potentially implicating this nuclear receptor in atherosclerosis (37).

In vitro assays - Activity of synthetic PPAR ligands is usually measured through a binding assay (if a radioligand is available) or through a cellular transient transfection reporter gene assay. The latter assay makes use of agonist stimulated dimerization leading to expression of the reporter enzyme (e.g. secreted placental alkaline phosphatase or firefly luciferase) which can be assayed with a colorimetric or photometric plate reader (1 and references therein).

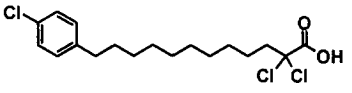
X-ray structures – The x-ray crystal structures of PPARδ with a fatty acid or a synthetic fibrate ligand have been determined (36). The crystal structure of PPARα with a small molecule ligand and the coactivator motif from the steroid receptor coactivator 1 (SRC-1) has recently been solved (38). The structure of PPARγ itself and as a PPARγ/RXR dimer with the SRC-1 coactivator fragment has also been determined (39, 40). These structures show that all the receptors have a Y-shaped binding pocket in common. However PPARγ and PPARα have an extra binding area that accommodate somewhat larger ligands. Most of the small molecule PPAR ligands have an acidic moiety (e.g.TZD, carboxylic acid etc.) that binds to Ser289, His323, His449 and Tyr473 using the PPARγ numbering. Since these receptors all bind fatty acids of different sizes, the binding pockets are large and accommodating to a variety of ligands. The volume of the binding pocket is largest for PPARγ followed by PPARα with PPARδ being the smallest.



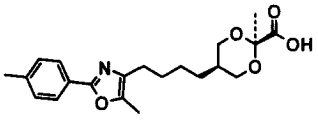
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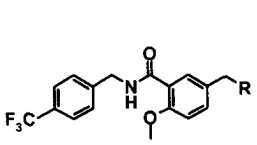
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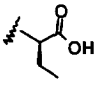
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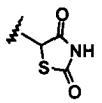
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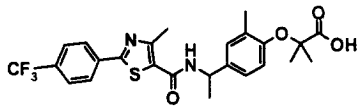


10 R =



11 R =

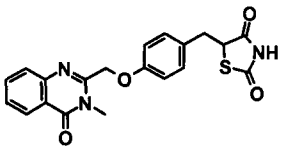




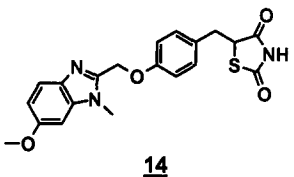
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PPARα agonists - Outside of the fibrates (see above) few new selective PPARα ligands have been published. LY 518674 (**6**) is one recent example targeted at diabetes and syndrome X (41). Compound **7** is described in a recent patent for potent PPARα activators that are claimed for arteriosclerosis, hypercholesterolemia and coronary heart disease (42). When db/db mice were treated with BM-17.0744 (**8**) glucose and trigycerides were normalized after four weeks without affecting cardiac function (43). Compound **9** (NS-220) is 3000 fold selective for PPARα over PPARγ and PPARδ, and lowers vLDLc by 50% while raising HDLc 34% in the KK-A mouse (44). A series of carboxylic acids related to compound **10** were found to be selective PPARα agonists. Interestingly the corresponding TZD **11** (KRP 297, MK 0767) was 10 fold selective for PPARγ while the (S)-**10** was 300 fold selective for PPARα (45-47). In a recent patent **12** was described as the preferred compound exhibiting 2nM potency against PPARα, 100 fold selective over PPARδ, and 500 fold selective over PPARγ in the cellular transfection assay.

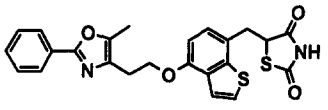
PPARγ agonists – With the initial success of Avandia and Actos many pharmaceutical companies have focussed research on PPARγ agonists resulting in several clinical candidates. Consistent with the binding motifs most of the PPARγ selective ligands have an acidic group, such as a carboxylic acid or a thiazolidinedione (TZD). Balaglitazone **13** (NN 2344, DRF 2593), a TZD currently in the clinic, was reported to lower glucose levels by 55% and lower serum trigycerides by 41% in the db/db mouse model (48). Rivoglitazone **14**, another TZD, was found to lower glucose by more than 50% in a spontaneously diabetic mouse model (49). TZD **15**, (BM13.1258, R-483) was reported to be a potent PPARγ agonist and insulin sensitizer in the obese rat. Paradoxically, mean glucose oxidation increased in an insulin-independent fashion leading the authors to conclude that muscle glucose metabolism might be affected *via* another biochemical pathway (50). Calyx has reported **16** as being effective in reducing glucose by 52% in the db/db mouse model without any weight increase compared to the controls (51). Tak 559 (**17**) is a PPARγ agonist in phase II clinical trials that was shown to lower plasma glucose levels and triglycerides with only a slight increase in weight in KKA mice (52). T131 (structure unknown) is a PPARγ agonist, currently in clinical trials, that does not cause cardiac hypertrophy or a decrease in hematocrit in animal models. It has a novel structure that interacts with the receptor differently from the approved PPARγ agonists (53).



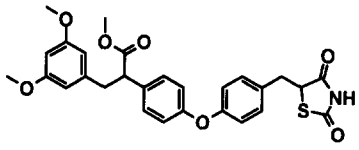
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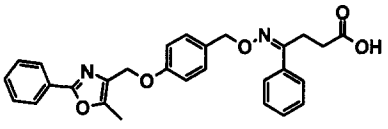
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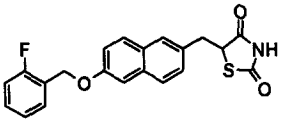
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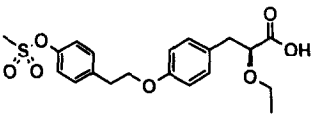
17

PPARα/γ agonists – A large number of PPARα/γ dual agonists have been made and put into the clinic. The improved therapeutic profile expected for these dual agonists is the added beneficial effects on serum lipids and diminished weight gain (54). A few TZDs fall in this class but most of the compounds are carboxylic acids. The dual agonist TZDs currently in clinical development are **12** (KRP 297) (55) and **18** (netoglitazone) (56). The alpha-ethoxycarboxylic acid headgroup has been used on many of the earlier PPARγ agonists and is also found in **19** (Galida, tesaglitazar, AZ242) currently in phase II clinical trials (57, 58). The crystal structure of this ligand with both the PPARγ and PPARα receptors suggests that the carboxylic acid allows binding to both receptors while maintaining the AF2 helix in its activating position, whereas a TZD headgroup could not (58). Compound, **20** (ragaglitazar, NN 622) also a PPARα/γ dual agonist with an alpha ethoxy headgroup (59, 60) was recently discontinued in phase III trials because of the incidence of bladder tumors in

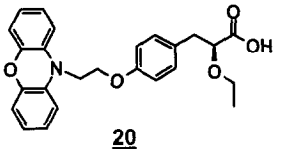
rodents (61). An extensive SAR study on **20** led to the more potent dual agonist **21** (EC_{50} PPARα =360nM, PPARγ =170nM) where the oxygen of the oxazine has been removed to form a carbazole (62). The tyrosine derivative GW409544 (**22**), is the most potent PPARα/γ described to date with a PPARα EC_{50} of 2nM and a PPARγ EC_{50} of 0.2nM (38). A clever modification of the tyrosine motif that incorporates the nitrogen into the sidechain thus eliminating the stereogenic center produced **23** (BMS 298585) which is twice as potent on PPARγ as PPARα (EC_{50} = 120 and 240nM respectively) in the cellular transfection assay. It is reported to lower glucose in the db/db mouse model with a beneficial effect on plasma lipids without weight gain (63-65). Compound **24** (LY 465608) (66, 67) is composed of a fibrate headgroup and oxazole tail that is similar to many of the PPARγ agonists [Note: This compound was previously incorrectly identified as LY 519818 (structure unknown)] (2). It is six times more potent on PPARα than on PPARγ (EC_{50} =150 and 880nM respectively). Treatment of apoE knockout mice with **24** resulted in a 2.5-fold reduction in aortic atherosclerotic lesion (68). The SAR on a series based on BVT.142 (**25**) showed that replacement of the R group could enhance the PPARγ selectivity form 6:1 for **25** to >300:1 for **27**. The x-ray structure of **26** showed that the key interactions with the AF2 helix found with most other PPARγ agonists was missing. This might explain the lower potency for this series (EC_{50}s PPARγ =300-1600nM and PPARα = 2500->10000nM) (69).



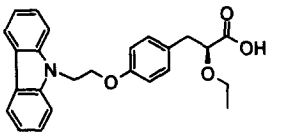
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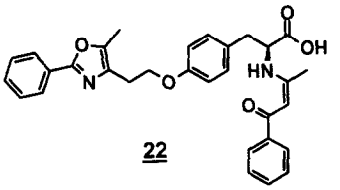
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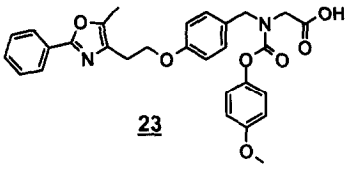
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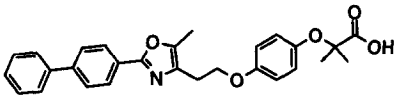
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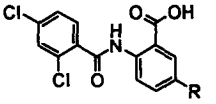
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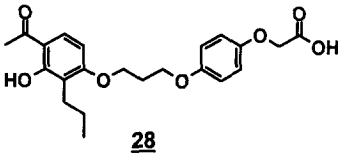
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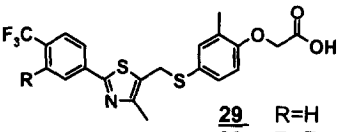
	R
25	**Me**
26	**2-thienylmethoxy**
27	**3-ethoxyphenyl**

PPARδ agonists – Only a few selective PPARδ agonists have been reported. The phenoxyacid **28** (L-165,041) which is about 600 fold selective for PPARδ binding over PPARγ, was found to raise HDLc in db/db mice in contrast to the effect of PPARα ligands (70). Another potent and selective PPARδ agonist **29** (GW501516) which is 1nM on PPARδ and 1000 fold selective over the other subtypes, was shown to increase HDLc in prediabetic obese rhesus monkey by up to 79% (32). The SAR

surrounding the discovery of **29** and the somewhat more selective **30** has recently been published (71).

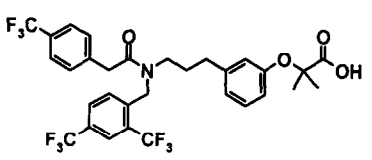


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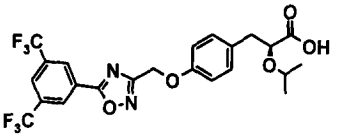


29 R=H
30 R=F

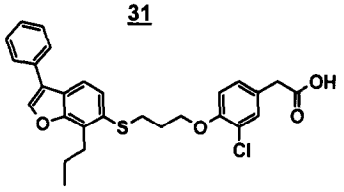
PPARδ/γ agonists – While there have been many reports of PPARα/γ dual agonists there have been few reports of selective PPARδ/γ dual agonists. Generally speaking, achieving PPARδ selectivity is usually more difficult because of the smaller size of the PPARδ pocket. One recent report describes the discovery of a PPARδ/γ dual agonist **31** from a 480 compound library (72). When dosed in the ZDF rat **31** lowered glucose and trigycerides by about 50% while raising HDLc by 24%.



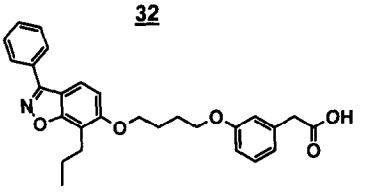
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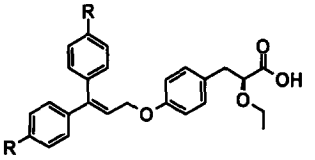
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33



34

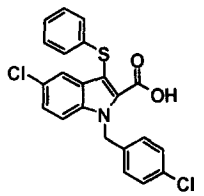


35 R = Br
36 R = Ph

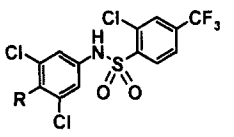
PPAR pan agonists – In recently published studies, selective PPAR agonists were dosed in combination to show that some synergy could be achieved with respect to glucose lowering and effects on lipids (73-75). This study demonstrated that non-selective or PPAR pan agonists might be useful therapeutics. The pan agonist oxadiazole **32** was found to be 320nM against PPARδ and 10-fold more potent on the PPARα and PPARγ receptors in the cellular transfection assay. Most of the PPARα and PPARγ activity resides in the (-) isomer, which is presumed to have the (S) configuration as depicted, while the PPARδ activity is similar for both enantiomers (76). The benzofuran **33**, also a pan agonist in the low nanomolar range, lowered glucose levels in a db/db mouse model by 77% but was less effective in the ZDF rat model (27% glucose lowering) (77). A structurally related series of benzisoxazoles derived from meta substituted phenyl acetic acids yielded the potent pan agonist **34**, which at a dose of 10mg/kg effectively lowered glucose in the db/db mouse. Since **34** did not bind the mouse PPARα receptor the *in vivo* results only reflect a PPARγ effect (78). A series of alpha ethoxy acids that were conceptually derived from **20** by removal of the oxygen in the oxazine moiety and exchanging the N along with its adjacent C with a double bond yielded a series of PPAR pan agonists the most potent depicted as **35**. Compound **36**, about 3-fold

more potent on PPARγ and 3-fold less potent on PPARα than **35**, was tested in the db/db mouse and found to be as effective as **20**, lowering the AUC in the OGTT by 52% (79).

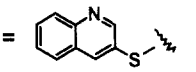
PPAR modulators – Some PPAR ligands bind to the receptor well but only elicit a partial response in the functional transfection assay. These compounds are called partial agonists, modulators or antagonists depending on how they are characterized. The partial PPARγ agonist **37** is potent against PPARγ (EC_{50}≈57nM) in the cellular transfection assay but only reaches about 25% of the maximum response of the standard rosiglitazone (80, 81). It is also a partial agonist in the adipocyte differentiation assay, inducing the expression of adipose fatty acid binding protein (aP2) mRNA to only 40% in 3T3-L1 cells. Interestingly, **37** ameliorated hyperglycemia and hyperinsulinemia in fat-fed c57BL/6J mice without weight gain and cardiac hypertrophy that is characteristic of the TZD full agonists. Sulphonamides **38** and **39** are among the preferred compounds in a recent Tularik patent covering PPARγ modulators (82). While **37**, **38** and **39** all have acidic groups it is difficult to see how they might bind in the receptor like TZDs. The different binding mode might well be responsible for the partial agonist activity. The three similar 2-chloro-5-nitro-*N*-benzamide derivatives **41** (GW9662) (83), **42** (T0070907) (84) and **43** (85) are all reported to be PPARγ antagonists as shown by binding and transfection experiments. These compounds also inhibit adipocyte differentiation *in vitro*. Both 41 and 42 have been shown to irreversibly covalently modify Cys^{285} in helix 3 of the PPARγ LBD. While this cysteine is conserved in PPARα and PPARδ the irreversible binding with these subtypes is not significant on the time scale of most of the experiments.



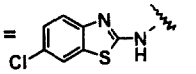
37

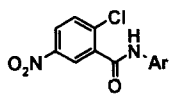


38 R =

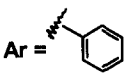


39 R =





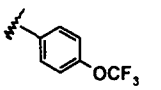
41 Ar =



42 Ar =



43 Ar =



Conclusion – The research on PPARs has grown dramatically in past years. There were more than 800 references mentioning PPAR in 2002. The initial correlation of PPARs with the efficacy of certain drugs (fibrates, glitazones) stimulated much of this research. The search for improved drugs has led to the exploration of mixed PPAR agonists. Researchers have found that the initial correlations may have been too simplistic and that subtleties relating to partial agonism and differential gene expression patterns may be important in designing better follow-up drugs.

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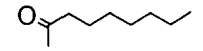
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Chapter 9 . Ghrelin Receptor Modulators

Jeffrey A. Dodge and Mark L. Heiman
Lilly Research Laboratories
Indianapolis, IN 46285

Introduction - Ghrelin (**1**) is a 28 amino acid peptide produced predominantly by the stomach and intestines that has recently been identified as the endogenous ligand for the growth hormone secretagogue receptor 1a (GHS-R1a). It is uniquely characterized by an *n*-octanoyl moiety on the Serine-3 residue that is derived from a post-translational modification and is essential for biological activity. Ghrelin has been shown to induce adiposity in rodents by increasing food intake and decreasing the utilization of fat tissue). In humans, ghrelin stimulates appetite and induces food intake. Other physiological effects of this hormone include the stimulation of GH secretion in rats, dogs, and humans. While ghrelin was only recently discovered in 1999 (1), the GH secretory properties of compounds acting at the GHS-R1a, *i.e.*, GHSs, have been known for over 20 years and have been reviewed. This report focuses on the advances in the understanding of how ghrelin and other ligands that interact at the GHS-R1a receptor affect food intake, energy utilization, and adiposity.



GSSFLSPEHQRVQQRKESKKPPAKLQPR

1

GHS-R1a: The Ghrelin Receptor - A single gene found at chromosomal location 3q2 expresses the GHS receptor. Two types of GHS receptor complementary DNAs (cDNA), that are presumably the result of alternate processing of a pre-mRNA, have been identified and designated receptor 1a and 1b. Their closest relatives are the neurotensin receptor and the motilin receptor type 1A, with 59% and 52% similarity, respectively. cDNA 1a encodes the GHS1a receptor, which is a 366 amino acids protein with seven-transmembrane regions. The 1b cDNA encodes a shorter form, GHS-R1b receptor, which consists of 289 amino acids with only five–transmembrane regions. The human GHS-R1a receptor shares 96 and 93% identity with the rat and pig GHS-R1a receptor.

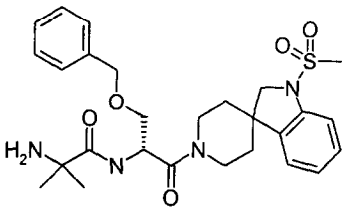
The binding of ghrelin and synthetic GHS's, such GHRP-6 (**2**) and ibutamoren (MK677, **3**), to the GHS-R1a receptor activates the phospholipase C signaling pathway, leading to increased inositol phosphate turnover and protein kinase C activation, followed by the release of Ca^{2+} from intracellular store. GHS receptor activation also leads to an inhibition of K^{+} channels, allowing the entry of Ca^{2+} through voltage-gated L- and T-type channels. Differently from the GHS-R1a receptor, the GHS-R1b receptor fails to bind and respond to ghrelin agonists and its functional role remains to be defined. Ghrelin binds with high affinity to the GHS-R1a receptor displacing [^{35}S]ibutamoren or [^{125}I][Tyr^{4}]ghrelin.

Expression of the GHS-R1a receptor was shown in the hypothalamus and anterior pituitary gland, locations that are consistent with its role in regulating GH release (8-10). The GHS-R1a receptor is largely confined to somatotroph pituitary cells and to the arcuate nucleus, a hypothalamic area that is important for

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His-D-2-CH_3-Trp-Ala-Trp-D-Phe-Lys-NH_2

2



3

the appetite-stimulating activities of ghrelin and GHSs (11-14). This is supported by the demonstration that ghrelin, as well as GHSs, effectively stimulates the expression markers of neural activity in the arcuate nucleus neurons (15, 16). The activated hypothalamic cells include GHRH-containing neurons, but also cells expressing the appetite stimulating neuropeptide Y, and the endogenous melanocortin receptor antagonist, Agouti-related protein (AGRP) (17-18). Less abundant, but detectable, levels of GHS-R1a receptor mRNA were also demonstrated in various extra-hypothalamic areas (19-21). More recent localization studies have demonstrated that GHS-R1a is also expressed in multiple peripheral organs including the stomach and intestine, pancreas, kidney, heart, and aorta, as well as in different human pituitary adenomas and various endocrine neoplasms of lung, stomach, and pancreas (20, 22-32).

Effects of Ghrelin on Food Intake - Ghrelin administration to rodents causes weight gain (2,33). Changes in body weight induced by ghrelin administration become significant after 48 h and are self-evident at the end of two weeks. Ghrelin-induced weight gain is based on accretion of fat mass without changes in longitudinal skeletal growth and with a decrease in lean mass (2). The positive energy balance produced by ghrelin may be mediated *via* leptin-responsive neurons in specific regions of the hypothalamus (34). However, the possibility of direct effects of ghrelin on adipose tissue, as well as effects on the hypothalamus-pituitary-adrenal axis, cannot be ruled out as contributing factors to ghrelin-induced adiposity.

Ghrelin has been shown to stimulate food intake in rodents (2). This effect is dose-dependent and occurs more powerfully after central than after peripheral administration suggesting a central mechanism of action. The increase in food intake after ghrelin injection in rodents occurs in less than 60 minutes (35). Ghrelin's orexigenic action when administered centrally is comparable to that of the brain derived neuropeptide Y and is more potent than that of any other orexant (35). In fact, ghrelin is the first orexigenic signal that is derived from an organ outside of the brain, *i.e.*, the stomach (36). Unlike other comparably potent orexigenic agents (neuropeptide Y, AGRP, melanin-concentrating hormone), which are solely active when injected in the brain, peripherally administered ghrelin exhibits orexigenic and adipogenic effects (37). The absolute need for ghrelin to penetrate the blood brain barrier is not clear, at present, although recent work indicates that the *n*-octanoyl side-chain may a play a role in the direction and extent of passage of ghrelin across the blood-brain barrier (38).

Two major hypothalamic pathways are presumably the predominant mediators of ghrelin's influence on energy balance (14, 33). One involves the neuropeptide Y neurons and the other one involves AGRP (39). Ghrelin increases AGRP and neuropeptide Y after acute and chronic administration, and hypothalamic AGRP-mRNA expression levels are found to be up-regulated after chronic activation of the GHS receptor for several weeks (18, 33, 39, 40). Complete absence of

neuropeptide Y in neuropeptide Y-gene disrupted mice does not influence ghrelin action, while studies show a prevention of orexigenic effects when co-administering a neuropeptide Y receptor antagonist with ghrelin (40). Apart from increased food intake, other mechanisms may also contribute to an increase in fat mass, such as a decrease in energy expenditure or reduced cellular fat oxidation (41). Recently, evidence that ghrelin action might be mediated in part by afferent activity of the vagus nerve has been documented (42).

GHRELIN AGONISTS

Ghrelin Structure-Function Studies - The discovery of ghrelin has prompted structure-function studies of this novel peptide. Much of this work has focused on understanding two key features of this peptide: the nature of the *n*-octanoyl group and the minimal sequence requirements for the 28 amino acid peptide backbone. The *n*-octanoyl group is required for ghrelin's biological activity. In fact, the non-acylated ghrelin circulates *in vivo* in amount far greater than the acylated form and does not displace radiolabelled ghrelin from its hypothalamic or pituitary binding sites (43, 44). In addition, it has no GH-releasing or other known endocrine activities (1,37). Structure-function studies on ghrelin have found that aliphatic esters have comparable, or improved, potency and functional activity relative to the native *n*-octanoyl group (45, 46). As shown in Table 1, these include analogs with unsaturated (**4**) or branched octanoyl (**5**) groups and longer chain aliphatic groups (**6**). Less hydrophobic groups, such as acetyl (**7**), have significantly diminished activity while benzoyl ester **8** shows more modest decreases in binding and functional activity. Polar components in the side chain, such as the amine

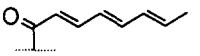
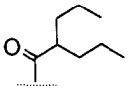
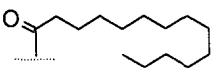
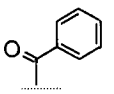
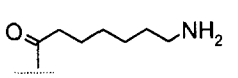
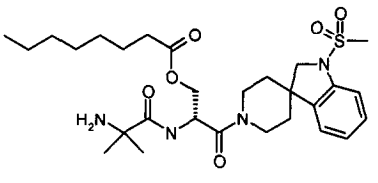
		GHS-R1a	
		Binding IC_{50}	Functional EC_{50}
GSS(**X**)FLSPEHQRVQQRKESKKPPAKLQPR		(nM)	(nM)
1	hGhrelin	0.25	32
	X		
4		0.98	39
5		0.96	38
6		0.87	8.3
7		>2000	>2000
8		11	53
9		>2000	>2000

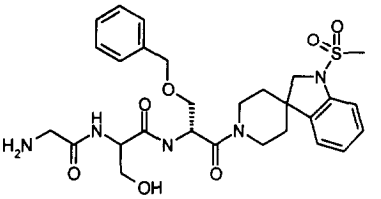
Table 1. *n*-Octanoyl Ser-3 ester derivatives of ghrelin.

functionality in **9**, diminish activity as well, further emphasizing the necessity of lipophilicity in the ester moiety of ghrelin. Octanoylation at Ser-2 versus Ser-3 does not significantly alter functional activity while movement to Ser-6 or Ser-18 resulted in a loss of activity. Replacement of the serine ester moiety with an amide group shows comparable activity to ghrelin as does ether or thioether replacements. The L-configuration Ser-3 is important for ghrelin activity.

The *n*-octanoyl side-chain has also been incorporated into peptidomimetic GHS analogs creating hybrid analogs such as **10**, **11**, and **12**. Replacement of the benzyloxy side chain of **3** with the octanoyl ester moiety results in compound **10** which has functional potency similar to ghrelin. Modifications of the aminoisobutyric moiety of **3** to mimic ghrelin, such as found in **11**, also results in potent ghrelin agonist (47).



10



11

Peptide Minimization - Truncation of ghrelin peptide sequence has resulted in the identification of analogs with weaker affinity to the receptor than ghrelin but similar functional activity (45, 46). As shown in Table 2, systematic minimization of the peptide backbone indicates that only the first five residues (**13**) are required for full activation of the receptor. Further minimization to tetrapeptide **14** results in diminished activity while the tripeptide is inactive. Other studies have shown that **13** and **14** retain some functional activity *in vitro* while lacking receptor affinity and *in vivo* efficacy for GH release (48). The tetrapeptide **14** has been evaluated by 1H-NMR analysis and compared to **2** (49). While some structural similarities were found when superimposing these two peptides, the analysis suggests that both the binding affinities and the requirement for the *n*-octanoyl group cannot be readily rationalized in their model.

		Binding IC_{50} (nM)	Functional EC_{50} (nM)
12	$CO(CH_2)_6CH_3$ GSSFLSPEHQRVQQRNH_2	9.6	17
13	$CO(CH_2)_6CH_3$ GSSFLNH_2	55	11
14	$CO(CH_2)_6CH_3$ GSSFNH_2	889	72

Table 2. Truncated peptide analog of ghrelin.

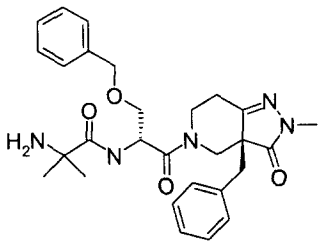
Pharmacological Effects of Ghrelin Agonists on Food Intake, and Energy Balance - While extensive efforts have been directed towards optimizing molecules that stimulate pituitary GH release (4), the effects of GHSs on appetite regulation and energy homeostasis have remained considerably less characterized, particularly with respect to orally active ghrelin mimetics. In fact, only peptide ligands such as ghrelin, **2**, **3**, GHRP-2 (**15**), and ipamorelin (**16**), which are all distinguished by poor oral activity, have been prospectively evaluated for their adipogenic effects (2).

15 Ala-D-2-CH_3-Trp-Ala-Trp-D-Phe-Lys-NH_2

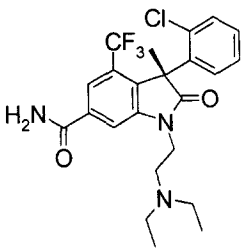
16 Aib-His-D-3-Nal-D-Phe-Lys-NH_2

The earliest report of the orexigenic effects of GHSs predates the discovery of ghrelin by over 4 years (50). In this study, increases in food intake are observed with **2** after intracerebroventricular administration to rats. Similar effects have been shown for other GHSs including **15** (and analogs) and **16** (51-54). We have recently shown that GHRP-2 increases fat mass in mice lacking NPY (40). Compound **15** also increases 6-hour food intake in NPY knockout mice while not affecting 24 hour food intake. The adipogenic effects of **2** and **16** have been studied recently (55). Two weeks of treatment of **16** to GH-intact mice causes an increase in food consumption as well as fat mass, outcomes that appear to be independent of the compounds' GH releasing properties. Other work supports the hypothesis that the feeding effects observed with ghrelin and GHSs are likely independent of GH (40).

Capromorelin (**17**) has been shown to increase body weight in rats after extended treatment (28 days) without concomitant changes in body fat or lean mass (4, 56). The orexigenic effects of **3** have not been reported although increases in growth velocity have been observed in rats and humans (57). In humans **3** increases energy expenditure and fat free mass in obese male patients after two months of treatment (58). Total body fat was not affected in this study. The effects of SM-130686 (**18**) on food intake and body weight have been recently reported (59, 60). The pharmacological profile for this compound differs from previous GHS analogs in that it is a partial agonist at the GHS-R1a receptor. Unlike **15** and **16**, treatment of rats for 9 days with **18** causes an increase in fat free mass. The increase in body weight observed with **18** is not accompanied with an increase in food intake.



17



18

Other ligands - Des-Gln14-ghrelin, the result of an alternative splicing of the ghrelin gene, is also a natural ligand for the GHS-R1a receptor and possess the same endocrine activities (61). Other known ligands include adenosine which is not able to activate the receptor (62). More recently it has been demonstrated that cortistatin (CST) binds with high affinity the GHS-R1a in human hypothalamus and pituitary tissues (63). Cortistatin is a recently described neuropeptide, which binds to all somatostatin receptors subtypes with good affinity. Somatostatin analogues such as octreotide, lanreotide and vapreotide bind the GHS-R1a with an affinity lower than that of CST (64).

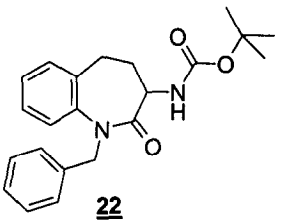
GHRELIN ANTAGONISTS

The identification of antagonists to block ghrelin-induced food intake has received attention as a potential treatment for obesity and related conditions such as Prader-Willi Syndrome. GHS-R1a receptor antagonists have been reported in the literature for many years having been originally identified as inhibitors of GHS-induced secretion of GH in pituitary cell culture (65-67). Peptide antagonists include substance P derivative **19** (65) and GHRP-6 analog **20** (67). More recently, peptide antagonists that are based on the structure of ghrelin, such as **21**, have been reported (66). A non-peptidic compound (**22**) that suppresses the GHS-induced GH release has also been reported (8). While these antagonists have proven useful in blocking the GH response *in vitro* and/or *in vivo*, only **20** has been shown to suppress ghrelin-induced food intake (33). Proof-of-principle studies have shown that food intake can be decreased by administration of antisera for ghrelin to rats that have been food restricted (33).

19 D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2

20 His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2

21 GSS($CO(CH_2)_6CH_3$)FLSPR



22

Conclusions - Administration of ghrelin causes a positive energy balance and increases adiposity in rodents *via* multiple mechanisms. Peptidic ghrelin agonists have also been shown to demonstrate ghrelin-like effects on feeding, energy utilization, and adiposity. Several ghrelin antagonists have been identified, to date, although more potent and selective chemical tools will be required in order to better understand whether blocking the effects of ghrelin at the receptor level is a viable method for regulating food intake.

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Chapter 10. Current and future gastrointestinal prokinetic agents

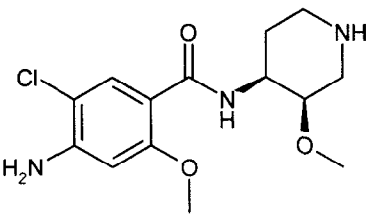
M. Chovet and L. Buéno*
Pfizer Global R & D, Fresnes, France
* Department of Neurogastroenterology, INRA, Toulouse, France

Introduction - Gut dysmotility involves both myogenic and neuronal mechanisms corresponding to modifications of smooth muscle contractility (phasic or tonic contractions) or motor coordination (altered motor pattern). Prokinetic agents are drugs able to stimulate smooth muscle contractility or to modulate gastroduodenal motor coordination. These actions are of particular interest in the treatment of 1) dyspesia, a major gastroduodenal disorder centered in the upper abdomen where gastrointestinal hypomotility is often associated with symptoms such as discomfort or pain and 2) constipation which is defined by a lower gastrointestinal (bowel) transit impairment ending in altered stools or defecations.

TREATMENT OF UPPER GASTROINTESTINAL SYMPTOMS

Rationale for dyspepsia - Drug treatment for patients with functional dyspepsia is still controversial even if antisecretory agents such as histamine antagonists and proton pump inhibitors are the most used agents. For patients with symptoms suggesting upper gastrointestinal (GI) dysmotility, prokinetics such as cisapride have been largely used and current meta-analyses indicate that they exhibit a potent efficacy (1).

Serotonergic agents - The most widely known serotonergic agent used for the treatment of dyspepsia is a mixed 5-HT_3 antagonist and 5-HT_4 agonist, cisapride (1). Both 5-HT_3 and 5-HT_4 receptors are involved in the control of gut motility. They are located on afferent nerves pre- or post-synaptically, enhancing the release of acetylcholine (Ach) and substance P (SP) in response to afferent nerve stimulation (2).



1

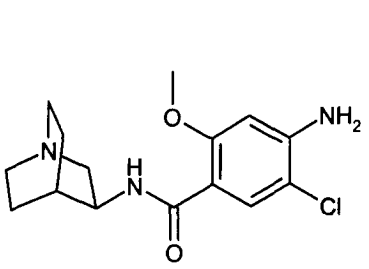
Cisapride has been reported to accelerate gastric emptying in both normal patients and those with gastric motor abnormalities (3). Its role in stimulating gastric emptying and improving symptoms in patients with gastroparesis has been studied extensively. The motility effect is most likely due to its 5-HT_4 agonist effects facilitating acetylcholine release. Along with its ability to increase antral contractility, cisapride has many other physiological effects in the stomach. It has been shown to relieve gastric outlet obstruction in patients with diabetic autonomic dysfunction, possibly through its ability to affect inhibitory nitrergic transmission (4). It can also affect fundic relaxation and accomodation in response to meals, and may counteract enteric negative feedback in response to intraduodenal lipids (4).

Cisapride has been reported to accelerate gastric emptying in several gastroparetic syndromes (5). Its efficacy in controlling symptoms, however, is controversial. The effect of cisapride on gastric emptying in 22 patients with

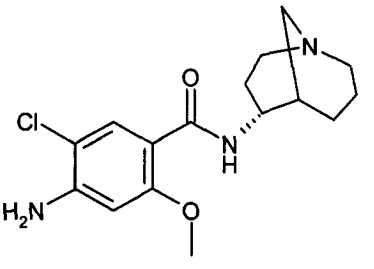
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gastroparesis in a randomized, double blind trial was studied. After 6 weeks of therapy, there was significant symptomatic improvement (5). Another study compared cisapride and metoclopramide (6). The results indicated that cisapride was significantly better than equivalent doses of metoclopramide in accelerating or normalizing gastric emptying, and more patients reported symptomatic improvement on cisapride compared with metoclopramide and placebo. Other studies have demonstrated no significant symptomatic improvement. Cisapride was compared to placebo over a 6-week period study and authors documented an improvement after cisapride treatment in solid-phase gastric emptying and pain, but no change in the other symptoms related to gastroparesis (7). Similarly, a randomized, double blind, placebo-controlled study reported that cisapride accelerated gastric emptying but failed to show any significant symptomatic improvement (8). However, cisapride was withdrawn from the market in the United States in 2000, as it produces severe adverse cardiac effects, leaving the market open to novel, safe and efficacious treatments.

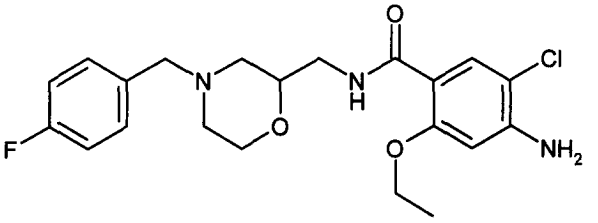
Several new and more selective 5-HT_4 agonists are in development or under preclinical investigation as prokinetics for the treatment of functional dyspepsia. As other 5-HT_4 agonists, most of them are still substituted benzamides like zacropride (**2**), renzapride (**3**) or mosapride (**4**). These compounds often have some dopamine D_2 and 5-HT_3 receptor antagonist properties.



2

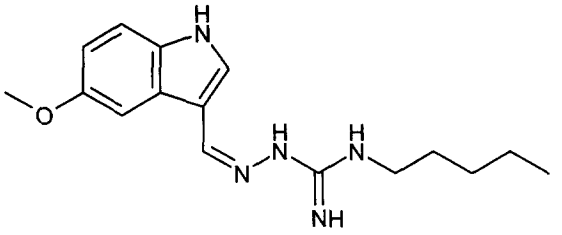


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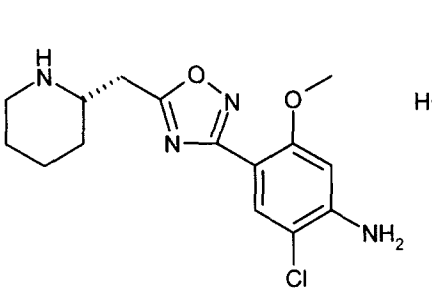
The recent cloning of the human 5-HT_4 receptor has given rise to a series of more selective and attractive agents. Tegaserod (**5**), a 5-HT_4 partial agonist, already approved for the treatment of Irritable Bowel Syndrome (IBS), is under evaluation for dyspepsia. In animals and healthy volunteers, it has been shown to improve gastric emptying (9).



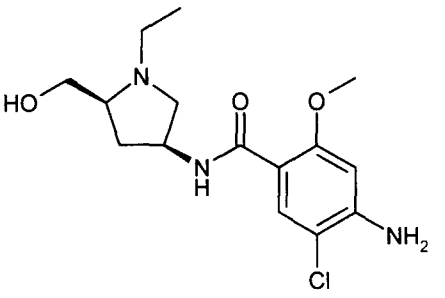
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Similarly YM-53389 (**6**), a very selective 5-HT_4 receptor agonist, improves upper gastrointestinal propulsion in animals and is under consideration for clinical trials in dyspeptic patients (10).

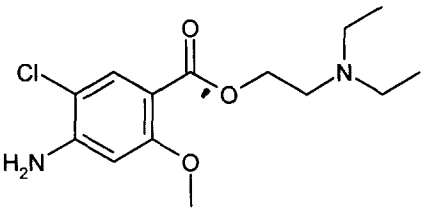
TKS-159 (**7**) is also a novel selective 5-HT_4 selective agonist (11). It stimulates both antral and duodenal contractions and acccelerates gastric emptying, these effects being blocked by a 5-HT_4 antagonist, SDZ-205-557 (**8**).



6



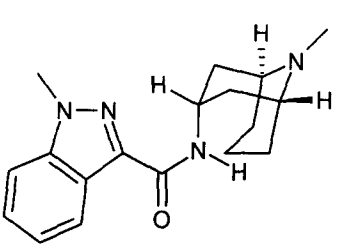
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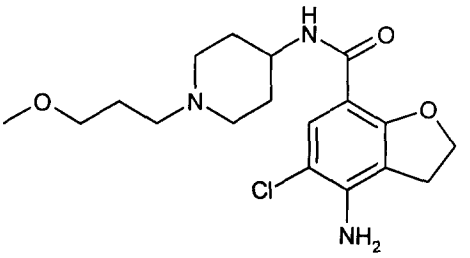
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In contrast the antral prokinetic effects of cisapride are partially reduced by granisetron (**9**) (a 5-HT_3 receptor antagonist) suggesting a possible prokinetic component due to activation of 5-HT_3 receptors.

Among the other 5-HT_4 selective agonists, prucalopride (**10**) has also been shown to stimulate gastric emptying in humans (12). In patients with functional constipation associated with upper GI symptoms, prucalopride at a daily dose of 4 mg during 7 days accelerates gastric emptying by 22% with improvement of the corresponding symptoms (12). Similarly renzapride consistently shorthens the oro-cecal transit time in patients with upper and lower GI symptoms (13).



9



10

In agreement with the hypothesis that the prokinetic effect of cisapride is linked in part to activation of 5-HT_3 receptors, selective 5-HT_3 agonists have been proposed as new prokinetic agents. Indeed MKC-733, a selective 5-HT_3 agonist, dose dependently accelerates both gastric emptying rate and oro-cecal transit time in healthy volunteers (14). This compound also relaxes gastric fundus and stimulates fasting human antral motility.

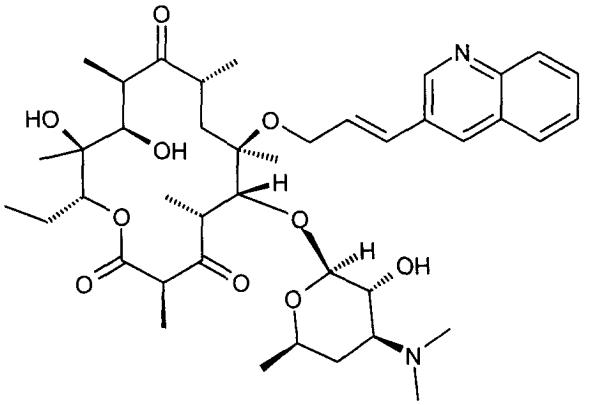
Cholecystokinin antagonists - Cholecystokinin (CCK) is a logical target for pharmacotherapy of proximal gastrointestinal tract motility. CCK is released from intestinal cells in response to specific food components including fasty acids and amino acids. Its major effects are the stimulation of pancreatic enzyme secretion and gallbladder contraction. CCK slows gastric emptying by negative feedback and stimulates the perception of satiety. Conversely, the CCK_A-receptor antagonist, loxiglumide, increases gastric emptying (15). CCK_A antagonists have also been shown to block the effect of sham feeding on delaying the onset of phase III contractions (16). The role of CCK_A antagonists in gastroparesis is currently being investigated. Moreover, it is known that selective CCK_A receptor antagonists such as devazepide and loxiglumide reduce transient lower esophageal sphincter relaxating ($TLESR_s$) in both dogs (17) and healthy volunteers (18). These $TLESR_s$ are not only responsible of gastro-esophageal reflux but also generate upper GI symptoms associated with dyspepsia. CCK_A antagonists such as dexloxiglumide and itriglumide, which is a selective CCK_B antagonist, do not consistently affect gallbladder emptying and are not associated with an enhancement of gallstone formation. Clinical efficacy of CCK_A antagonist in the treatment of functional dyspepsia has been demonstrated (19) as well as the role of fat and CCK in the genesis of symptoms (20).

Motilides - Motilin, a 22-amino acid peptide, triggers phase III migrating myoelectric complex (MMC) activity in the stomach. During gastric phase III activity, strong antral contractions empty all residual (indigestible) chyme from the stomach. The motilin receptor has been identified as a guanosine triphosphate-binding protein (G protein) (21). It is located throughout the enteric nervous system, with decreasing density from the stomach to the lower intestinal tract. Motilin also stimulates cholinergic activity consistent with the observation that atropine reduces the motilin effect. The stimulus for motilin release is still unknown.

Erythromycin (**11**) is a highly potent motilin agonist that increases the amplitude and frequency of antral contractions and initiates gastric phase III contractions (22, 23). This effect is attributable to the 14-member ring structure of a family of compounds referred to as macrolides. Radionuclide studies verify that erythromycin accelerates gastric emptying. A brisk acceleration of solid-phase gastric emptying after intravenous erythromycin was reported (24). Patients with diabetic gastroparesis given erythromycin intravenously (200 mg) cleared approximately 95% of the test meal at 120 minutes compared with only 40% in the placebo-treated patients. Gastric emptying also improved after 4 weeks of continuous oral therapy with erythromycin at

250 mg three times a day. These findings have been consistently confirmed by other studies (25).

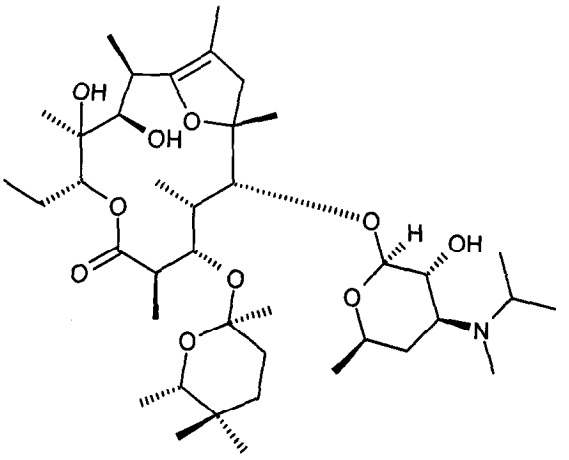
Research focusing on the optimal delivery and dose of erythromycin is continuing. Studies have shown that intravenous preparations are more effective, although the risk of cutaneous infection may ourweigh the benefits when compared with oral therapy (26). The optimal dose for the desired therapeutic effect is under debate. Although uncertain, there is a dose-dependent effect of erythromycin dependent on different subtypes of motilin receptors. In humans, erythromycin at a low dose (40 mg) generates a premature antral contractile front dependent on a cholinergic pathway, whereas a higher dose (200 mg) produces a more sustained contraction via a non-cholinergic pathway. Current recommendations are to begin at 250 mg/day and increase to 3 times per day as needed (5). Whether or not there is a down-regulation or tachyphylaxis, it still must be determined.



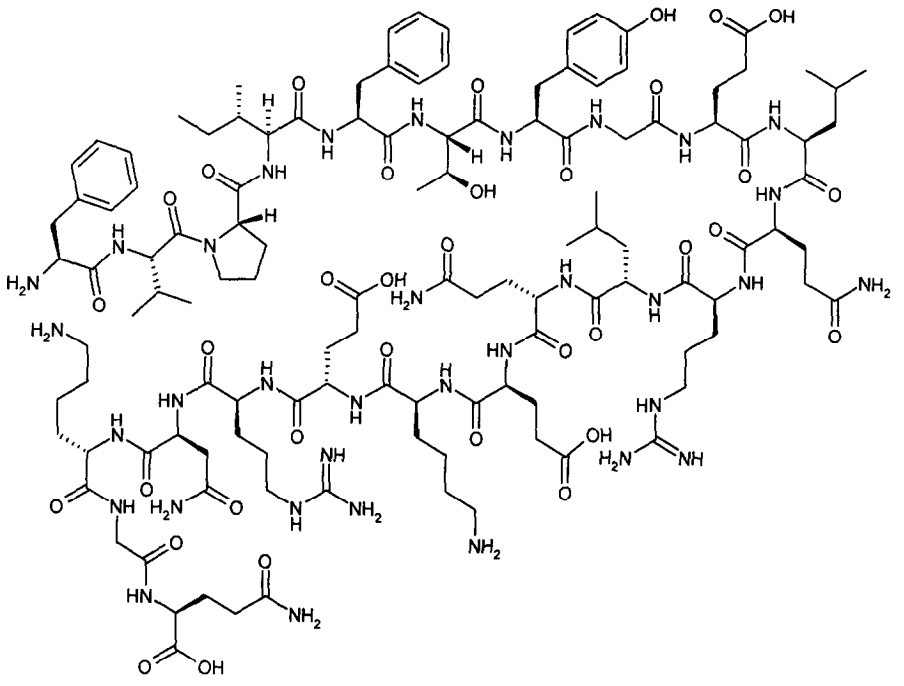
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A retrospective analysis was conducted on patients receiving erythromycin for 1-19 months and concluded that it may remain effective for at least this period (27).

Motilides and motilactides are macrolide derivatives being developed to promote motility in the gut. These new motilin agonists are intended to be devoid of antimicrobial activity and, thus, anticiotic-induced complications. Motilin agonists currently in development are: EM574 (**12**), KC-11458 and KW-5139 (**13**). EM574 has effects comparable with cisapride in improving gastric emptying in dogs (28). This agent is currently undergoing clinical trials. KW5139 is a peptide analogue of motilin in which there is a 1-amino acid substitution (leucine for methionine at position 13) (29). This agent has increased gastric motility in patients with early gastric stasis after pylorus-preserving pancreatoduodenectomy.



12



13

TREATMENT OF CONSTIPATION

Rationale for IBS and non-IBS constipation - According to ROME II criteria, Irritable Bowel Syndrome (IBS) is subdivided into 3 major subsets of patients exhibiting either diarrhea (IBS-D), constipation (IBS-C) or alternating constipation and diarrhea (IBS-A) representing 30, 50 and 20% of all IBS patients respectively. However chronic constipation may occur in patients not exhibiting abdominal pain and consequently not included in IBS-C patients.

Chronic constipation as IBS-C is often associated with slow colonic transit or rectal retention with impared rectal sensitivity and/or motility reflex.

Chronic constipation is a common complaint for which pharmacological therapy has not had a dramatic impact, evidenced by the absence of any good controlled trials demonstrating efficacy. Metoclopramide appears to be ineffective in the treatment of chronic constipation (30) and bulking agents have some demonstrated efficacy but many they generate abdominal bloating. Classical laxatives stimulating water secretion may be used in acute situation and osmotic agents such as polyethylene glycol have a demonstrated efficacy with limited side effects, but their prolonged use cannot be recommended.

Serotonergic agents: 5-HT_4 agonists - Cisapride has shown some promise in the treatment of idiopathic constipation even though its effect on colonic motility is not clear (31). Regardless, there have been several controlled trials in which cisapride increased the number of bowel movements in patients with constipation (32). The most significant results were obtained in patients with idiopathic constipation and children with refractory constipation (33). However, 2 recent studies show no effect in constipation-dominant IBS (34, 35).

Tegaserod (**5**) (HTF 919) is a partial 5-HT_4 receptor agonist in development. In constipation-predominant IBS, oral tegaserod, 2 mg twice daily for 1 week, accelerates small intestinal transit and increases proximal colonic emptying (36). Tegaserod, 2 mg or 6 mg twice daily, was superior to placebo in relieving abdominal discomfort, bloating and constipation. In vitro studies suggest that tegaserod, in a range of concentrations likely to occur during clinical use, does not delay cardiac repolarization or prolong the QT interval of the electrocardiogram, as does cisapride (37). Accordingly, administration of up to 100 mg to humans has not been associated with any detectable change in QT intervals. Tegaserod has gained the FDA approval for IBS-C treatment in females and is presently marketed in the US.

Prucalopride (**10**) (R093877), the first of a new chemical class of benzofurans, has specific agonist activity at 5-HT_4 receptors. In vitro studies suggest it is less potent than cisapride, and approximately equivalent to tegaserod, in producing relaxation in isolated canine smooth muscle strips (38). In a rat model of postoperative ileus, prucalopride (1 and 5 mg/kg) had a modest effect in promoting intestinal transit. The combination of prucalopride (1 mg/kg) and the 5-HT_3 antagonist, granisetron (**9**) (50 µg/kg), significantly improved intestinal transit after laparotomy with or without mechanical stimulation of the intestine (39). In 50 healthy human volunteers, 0.5-4 mg prucalopride daily for 7 days accelerated colonic transit and increased proximal colonic emptying, but had to effect on gastric emptying or small intestinal transit (12).

Serotonergic agents: mixed 5-HT_4 agonist / 5-HT_3 antagonist - The assumed advantage of such association is limited to a predominant 5-HT_4 agonist effect on colonic transit associated with an antinociceptive activity reducing abdominal pain linked to the 5-HT_3 receptor antagonism.

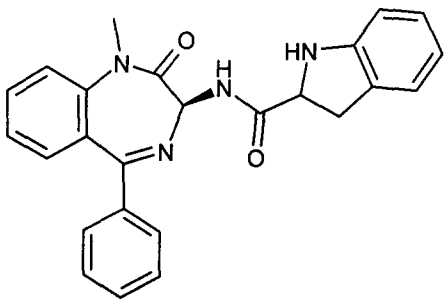
Renzapride (**3**), a mixed compound with 5-HT_4 agonist and 5-HT_3 antagonist activities, has recently demonstrated improvement of symptoms in IBS patients. In this pilot study, IBS-C patients were daily treated with renzapride (2 mg bid) during 28 days associated with an increased number of stools and looseming of stool consistently but also a 30-64% decrease in abdominal pain/discomfort without any cardiac effects on QTs interval (13).

CCK antagonists - CCK has been shown to stimulate colonic motility, and despite the existence of CCK receptors on colonic smooth muscle cells, the colonic motor response is indirectly mediated. In dogs and humans, this response involves an opioid component, which also participates in the colonic motor response to eating (40). In dogs, CCK-induced colonic motor stimulation involves opioid receptors in the central nervous system, while postprandial colonic stimulation involves central CCK-A receptors, which have been localized to the ventromedial hypothalamus in the rat (41).

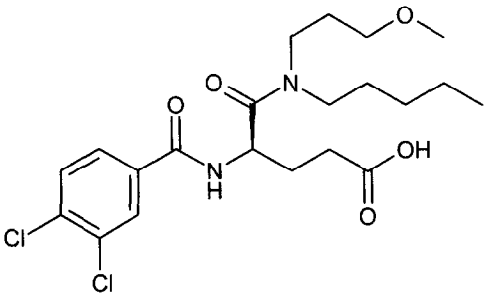
Clinical evidence suggests that CCK plays a part in IBS. An elevated level of plasma CCK in IBS patients with diarrhea and a diminished level of the peptide in IBS patients with slow transit constipation have been demonstrated. A one-week treatment with the CCK-receptor antagonist loxiglumide (800 mg three times daily) strongly accelerated colonic transit in healthy volunteers (42). Since CCK is known to stimulate colonic motility, it seems paradoxical that a CCK antagonist should also stimulate colonic transit. Other data provide further evidence that CCK is involved in the regulation of colonic motility (43). These authors have shown that loxiglumide selectively delayed transit of the ascending colon in IBS patients. Loxiglumide did not affect the more distal parts of the colon, nor did it exert any effect in healthy controls, whatever the colonic site considered. The selectivity of loxiglumide action in IBS patients correlates with the hypersensitivity to CCK observed in this group. Also, the action of loxiglumide was confined to the proximal colon, which is highly innervated; this supports the hypothesis that CCK acts through the afferent fibers of the vagus nerve.

Several CCK antagonists are under consideration for the treatment of IBS. Only proglumide and benzotript have been approved in Europe for use in humans for other indications. Both asperlicin and L-364718 (**14**) (devazepide) are more potent than the approved CCK antagonists, but only L-364718 is orally effective in animal models. However, no studies were reported with these compounds in constipated patients.

The results of a pilot study in IBS patients with dexloxiglumide (**15**) have already been published (44). This study has shown a proportion of responders greater than placebo particularly in non D-IBS (dexloxiglumide: 60% vs placebo: 43% of responders). More recently a Phase II clinical trial has been performed in IBS constipated patients showing similar global improvement in terms of percentage of responders (dexloxiglumide: 61% vs placebo: 46%) with significant effects on both pain and bloating and with the increased frequency of stools (unpublished data).



14



15

Conclusion - Prokinetic agents are promizing agents for the treatment of functional Bowel Disorders but until now their use was limited related to a poor prokinetic activity and side effects. Moreover, they are dedicated to selected patients according to a recognized classification such as ROME II classification for Functional Dyspepsia and Irritable Bowel Syndrome. For both syndromes about 30 to 50 % of patients may exhibit a motor defect that can be treatted by prokinetic agents. Among them, the agents targetting 5-HT receptors and particularly selective 5-HT4 receptor agonists are just entering the market with encouraging results. However, abdominal pain remains the major symptom in Functional Bowel Disorders and the efficacy of prokinetic agents on this symptom remains to be documented.

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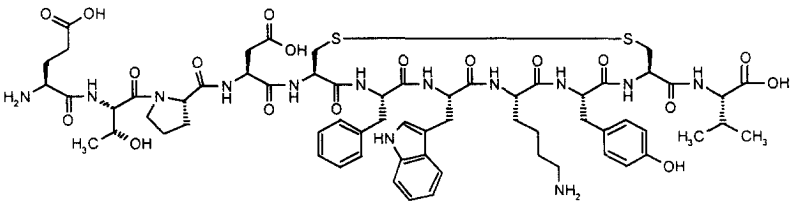
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Chapter 11. Urotensin-II Receptor Modulators

Dashyant Dhanak, Michael J. Neeb and Stephen A. Douglas
GlaxoSmithKline Pharmaceuticals
King of Prussia, PA 19406-0939

Introduction - Human urotensin-II, **1**, a cyclic undecapeptide was first isolated as the corresponding piscine analog in the 1960's from a vestigial organ in teleost fish (1) where it has been proposed to be involved primarily in osmoregulation (2 - 4). Subsequent efforts have led to the identification of urotensin-II (U-II) isoforms from a variety of species including frog, mouse, rat, pig, monkey and human (5 - 10).



1

Across these species, a remarkably high degree of evolutionary conservation in the amino acid sequence of U-II is evident (Table 1) and has led to the suggestion of some commonality in the physiological regulation of major organ systems including, but not limited to, the cardiovasculature. In support of such a role, recent studies have associated elevations in plasma/urinary urotensin-II levels with a variety of human cardiorenal disease states including essential and portal hypertension/cirrhosis, end-stage heart failure, renal disease and most recently, type-II diabetes (11 - 14). Nevertheless, the limited functional data currently available in human isolated vascular tissue and in healthy human volunteers are the subject of debate and the role(s) and clinical significance of urotensin-II in human (patho)physiology remain to be determined. The identification of human U-II, its specific G-protein-coupled receptor UT (formerly the orphan G-protein-coupled receptor SENR and GPR14) and the demonstration of dramatic *in vivo* hemodynamic effects in the non-human primate have led to a resurgence of interest in U-II research (9). In this article we aim to provide an overview of the research in the area of urotensin receptor modulators as well as the progress in understanding the role of the U-II/UT in human physiology.

Species	Sequence
Human	E T P D **C F W K Y C** V
Porcine A	G P T S E **C F W K Y C** V
Porcine B	G P P S E **C F W K Y C** V
Rat	T A P E **C F W K Y C** I
Mouse	N A A P E **C F W K Y C** I
Frog	A G N L S E **C F W K Y C** V
Goby	A G T A D **C F W K Y C** V
Sucker α	G S G A E **C F W K Y C** V
Sucker β	G S G T E **C F W K Y C** V
Carp α	G G G A D **C F W K Y C** V
Carp δ	G G G A D **C F W K Y C** I
Trout	G G N S E **C F W K Y C** V

Table 1. Amino acid sequence alignment of urotensin-II isoforms from several fish and mammalian species. The area shown in bold indicates the conserved C-terminal cysteine disulfide bridged cyclic hexapeptide sequence.

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The vasoconstrictor activity of urotensin-II has been demonstrated in mammalian tissues including those from rats, rabbits, dogs, marmosets, cynomolgus monkeys and, most importantly, man (15). *In vitro*, U-II isopeptides are characterized by their potent, efficacious and sustained vasoconstrictor activity (EC_{50} typically ~0.1 to 3.0 nM) making it the most potent mammalian vasoconstrictor identified to date. However, although reactivity is seen in a diverse range of species, significant anatomical and species differences exist, and the vasoconstrictor activity of U-II appears to be dependent upon both the anatomical origin of the vessel studied and the species from which it is isolated. In addition, a low receptor density coupled with the relatively high plasma levels of U-II as may also be important factors affecting the pattern of *in vivo* reactivity observed with U-II (16, 11).

UROTENSIN-II RECEPTOR (UT)

The presence of a FWK tripeptide motif within the cyclized region of all known U-II isoforms originally led to this peptide family being described as "somatostatin-like". Indeed, it was originally proposed that U-II and somatostatin shared a "common binding site" (17). However, both radioligand binding and functional studies demonstrated that U-II mediated its biological action(s) via a unique "U-II-specific" receptor (18, 19). Using a "reverse pharmacology" approach, the identity of such a molecular entity was unequivocally demonstrated (9, 20). This allowed for the designation of U-II as the cognate ligand for a novel rat orphan G-protein-coupled receptor (GPCR) originally designated GPR14 or SENR (sensory epithelial neuropeptide-like receptor; 21, 22). This receptor is now termed "UT" by the NC-IUPHAR Receptor Nomenclature Sub-committee on Urotensin Receptors (23). The homologous human receptor was subsequently identified by conventional genomic library screening and the novel 389 residue intronless receptor was found to exhibit 75% identity with the 386-residue rat homolog (9). Phylogenetically, UT is most closely related (~25% amino acid identity) to somatostatin ($SST_{2/4}$), opioid (κ, δ and μ) and galanin receptors. However, compared to U-II, in an intracellular Ca^{2+} mobilization assay, somatostatin-14 (SST-14) and other cysteine disulfide bridged peptides (MCH, cortistatin-14) had much lower, if any, agonist activity (EC_{50} U-II = 0.1 nM, EC_{50} SST-14 = 3,670 nM) in a UT-expressing cell line. Conversely, under similar assay conditions, U-II was unable to activate the somatostatin 2A receptor (EC_{50} U-II >2,000 nM, EC_{50} SST-14 = 0.8 nM) confirming each peptide's selectivity for its cognate receptor (24). The functional activity of several other somatostatin analogs was also investigated at UT homologs stably expressed in HEK293 cells by measuring either intracellular calcium mobilization using a FLIPR-based functional assay or inositol phosphate (IP) formation (25). In this system, Lanreotide, a somatostatin receptor agonist used clinically to treat growth hormone-secreting neurodendocrine tumors (*e.g.* acromegaly), and hU-II were potent (EC_{50}s = 79 nM and 2 nM, respectively), full agonists for promoting IP formation at hUT, whereas SST-14 was again inactive as an agonist up to 1 uM. In contrast, while hU-II was a potent agonist (EC_{50} = 3 nM) at rat UT, neither lanreotide nor SST-14 had significant agonist activity up to 1 uM. These results were proposed to indicate that a class of somatostatin analogs were UT ligands and that furthermore, these peptides exhibit differential functional activities across UT orthologs.

PEPTIDIC UT LIGANDS

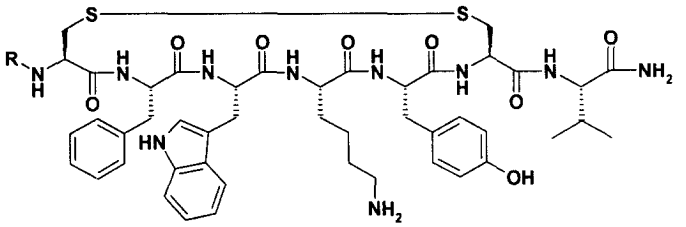
Agonists - Even before the ligand-receptor pairing of U-II and UT was discovered, two groups independently demonstrated that goby U-II caused marked concentration-dependent contractions of helical strips from major arteries in the rat (19, 26). The former further showed that less than full length peptide was sufficient to elicit arterial contractions. For instance, while the *C*-terminal residue is required for full activity, several of the *N*-terminal residues could be deleted such that goby U-

II[6-12] retains high potency (ED_{50} = 3 nM) and full agonist activity compared to the full-length peptide (Table 2).

	Peptide	EC_{50} (nM) rat aorta ring contraction	K_i (nM) hUT	K_i (nM) rUT
2	hU-II	0.73 ± 0.13	1.68 ± 0.31	2.34 ± 0.85
3	U-II[4-11]	0.16 ± 0.10	1.20 ± 0.30	1.33 ± 0.29
4	U-II[4-11]NH_2	0.37 ± 0.05	1.63 ± 0.10	14.1 ± 1.71
5	U-II[5-11]NH_2	30.2 ± 2.30	18.1 ± 6.71	183 ± 48

Table 2. Activity of truncated and amidated hU-II octapeptides

A recent report on the structural requirements of the N-terminus of amidated hU-II octapeptides [4-11] has shown some interesting requirements for the Asp^4 residue (Table 3). Removal of this residue altogether (U-II[5-11]NH_2) results in a substantial reduction in potency and affinity (27). The amino group on Asp was not required as evidenced by the high potency observed with the succinyl analog **6**. Asp replacements in which the carboxylic acid functionality was differently displayed were also high affinity and potency compounds. Interestingly, however, whilst the fumaric acid derived amide **8** was equipotent at both human and rat receptors, the corresponding maleic acid amide **9** was able to distinguish between UT orthologs showing a much lower affinity for the rat receptor and ultimately resulting in a reduction in potency in the functional rat aorta ring contraction assay. Conversion of the Asp^4 residue to an Asn resulted in retention of affinity and potency indicating that acidity *per se* was not necessary. Lastly, the hydrocarbon derivative **12** had only modest activity suggesting that a group with the capability of being a H-bond acceptor was preferred.



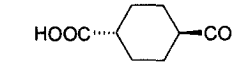
	R	EC_{50} (nM) rat aorta ring contraction	K_i (nM) hUT	K_i (nM) rUT
6	$HOOCCH_2CH_2CO$	0.12 ± 0.03	1.14 ± 0.01	1.65 ± 0.04
7	$HOOCCH_2CH_2CH_2CH_2CO$	0.16 ± 0.04	1.78 ± 0.11	2.08 ± 0.12
8	trans-HOOCCHCHCO	0.05 ± 0.01	1.43 ± 0.06	0.89 ± 0.09
9	cis-HOOCCHCHCO	10.0 ± 2.7	3.13 ± 1.38	19.5 ± 5.36
10		0.16 ± 0.02	n.d.	n.d.
11	$NH_2COCH_2CH(NH_2)CO$ (Asn)	0.54 ± 0.12	1.89 ± 0.88	1.50 ± 0.38
12	$CH_3CH_2CH_2CH(NH_2)CO$	61.8 ± 17.7	15.4 ± 0.74	17.7 ± 1.09

Table 3. N-terminus structural requirements of amidated hU-II octapeptides

U-II peptide agonists have been utilized somewhat successfully to help identify the ligand-binding site of rUT (28). Three photoreactive U-II analogs containing a benzoylphenylalanine residue (Bpa) were prepared and tested for their affinity in competition binding experiments with ^{125}I-hU-II (Table 4). All three induced inositol phosphate generation upon incubation with COS-7 cells expressing the rUT indicating agonist activity and had efficacies in the same rank order as their binding affinities. Photoaffinity labelling experiments using the most potent of the three, ^{125}I-[Bz-Phe^6]U-II, followed by digestion of the protein suggested that residue 6 of U-II

would interact with Met^{184} and/or Met^{185} of the fourth trans-membrane domain of the receptor. Photoaffinity labelling of rUT double mutants, M184L/M185L and M184A/M185A resulted in a reduction of incorporated label demonstrating that Met^{184} and Met^{185} are indeed critical for covalent insertion of the photoligand.

Sequence	IC_{50} (nM) hUT
Glu-Thr-Pro-Asp-c[Cys-**Bpa**-Trp-Lys-Tyr-Cys]-Val	3.2 ± 0.5
Glu-Thr-Pro-Asp-c[Cys-Phe-**Bpa**-Lys-Tyr-Cys]-Val	266 ± 58
Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-**Bpa**	466 ± 150

Table 4. Photoaffinity labeled U-II analogs

Modified hU-II[4-11] peptides synthesized by replacing the cysteine residues, independently or together, with penicillamine (β,β-dimethylcysteine) have also been investigated (29). These studies found that the Cys5Pen peptide modification (P5U) resulted in a peptide of high potency comparable to wild type U-II in a rat isolated aorta contractility assay (EC_{50} = 0.25 nM). Conformational analysis, using NMR and molecular modeling techniques, indicated that the putative biologically active conformation of the new peptide was stabilized by the introduction of the penicillamine unit.

Modified truncated hU-II analogs have been prepared in which the Cys residues which form the disulfide bridge are replaced by amino acids allowing the formation of more chemically stable lactams (30). Conformational analysis was performed on two of these synthetic cyclic peptides as well as hU-II. The results of this study showed a correlation between activity in an isolated rat aorta ring contraction assay and conformational similarity to U-II. Peptide **13**, which by solution phase NMR analysis overlaps well with U-II, had modest agonist activity, while the two carbon smaller cyclic peptide **14** overlaps U-II poorly and has no measurable activity in the assay.

	Sequence	EC_{50} (nM) rat aorta rings
	hU-II(4-11)	4.0
13	H-Asp-cyclo[Orn-Phe-Trp-Lys-Tyr-Asp]-Val-OH	500
14	H-Asp-cyclo[Dap-Phe-Trp-Lys-Tyr-Asp]-Val-OH	no activity

Table 5. Cysteine bridge modified U-II analogs (Dap = 2,3-diaminopropionic acid)

A comprehensive analysis of the structural requirements for the binding and functional potency of U-II peptide analogs has been carried out and reported recently (31). The studies confirmed earlier work by demonstrating the agonist activity of full length and truncated goby U-II in a FLIPR-based assay utilizing rat UT expressed in CHO cells (19). As expected, the shorter peptides maintained high potency; even the *C*-terminus truncated peptide goby U-II[6-11] retains good efficacy in the FLIPR assay. A further Ala scan of the peptide showed that the residues in the cyclic region were critical for agonist activity (Table 6).

Sequence	Ca^{2+} mobilization EC_{50} (nM)
Ala-Gly-Thr-Ala-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val (goby U-II)	0.17 ± 0.05
Gly-Thr-Ala-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.29 ± 0.18
Thr-Ala-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.11 ± 0.05
Ala-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.16 ± 0.05
Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.10 ± 0.04
c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.76 ± 0.65
c[Cys-Phe-Trp-Lys-Tyr-Cys]	1.6 ± 0.9
Ala-Ala-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val	0.6 ± 0.10
Ala-Asp-c[Cys- Ala-Trp-Lys-Tyr-Cys]-Val	6.5 ± 1.5
Ala-Asp-c[Cys-Phe- Ala-Lys-Tyr-Cys]-Val	>1000
Ala-Asp-c[Cys-Phe-Trp- Ala-Tyr-Cys]-Val	>1000
Ala-Asp-c[Cys-Phe-Trp-Lys- Ala-Cys]-Val	67%@1000
Ala-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Ala	0.40 ± 0.20

Table 6. Structural requirements for functional activity of U-II analogs

In an extension of these studies, this group went on to develop and examine a rhodopsin based docking model of rat UT (32). The docking procedure was based on the assumption that the lysine residue within the cyclic hexapeptide core of U-II interacts with Asp 130 on transmembrane (TM) helix 3 of the GPCR and also that the ligand receptor interactions would mutually restrict each other's conformation. This model proved useful in predicting modifications for the docked peptides leading to analogs of increased activity. Interestingly, in the full length goby U-II peptide, introduction of unnatural amino acid, Nal, (2-naphthylalanine) in place of the Tyr residue resulted in a compound which had a K_i = 40 pM (measured against ^{125}I-rU-II) and retained full potency and efficacy (Table 7).

Sequence	Ca^{2+} mobilization EC_{50} (nM)	K_i(nM) rUT
Ala-Gly-Thr-Ala-Asp-c[Cys-Phe-Trp-Lys-**Nal**-Cys]-Val	0.34 ± 0.10	0.04 ± 0.02

Table 7. Activity of non-natural amino acid containing U-II derivatives

In response to the lack of selective receptor ligands and knockout or transgenic animals, [Orn^8]U-II was identified as a full agonist at both the human and rat receptors in a FLIPR based calcium mobilization assay (33). The selection of the Lys residue for modification was based on earlier work suggesting that this residue was critical for biological activity (34). In contrast to the FLIPR results, the peptide behaved as a selective, competitive antagonist in the rat aorta bioassay and retained a small, residual agonist activity. This apparent discrepancy between the assay results was explained by the relative level of receptor expression in the two systems. In the FLIPR assay, UT is highly expressed leading to an increase in the efficiency of the stimulus response coupling. If this is the case, maximal effects can be elicited by even low efficiency agonists. Since the rat aorta bioassay more closely reflects the natural system, the authors concluded that the peptide was actually a partial agonist.

Antagonists - As mentioned above, the sequence similarity between the cyclic regions of somatostatin and U-II has suggested that their analogs could have the potential to modulate each other's receptors. Based on such a hypothesis, a series of somatostatin analogs were tested and some, at micromolar concentrations, were found to be able to block the hU-II induced rat aorta ring tone (35). In addition, the authors reported that the peptides did not distinguish between rodent and mammalian UT orthologs having similar affinity for rat and human UT (Table 8). A patent application describing these efforts has also been filed (36).

Sequence	K_i (nM) rUT	K_i (nM) hUT	EC_{50} (nM) rat aorta rings
4Fpa-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Nal-NH_2	n.d.	n.d.	3100
4Fpa-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2	310 ± 8	555 ± 54	3300
4Cpa-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2	293 ± 1	562 ± 27	8700

Table 8. Somatostatin analogs with UT activity (4Fpa = 4-fluorophenylalanine, Pal = 3-pyridylalanine, Tle = *tert*-leucine, 4Cpa = 4-chlorophenylalanine)

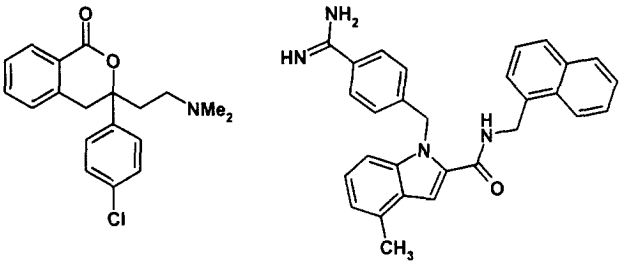
The peptide Cpa-c[D-Cys-Pal-D-Trp-Lys-Cys]-Cpa-NH_2, (SB-710411) previously reported to be a somatostatin antagonist (37) has been shown to inhibit the vasoconstrictive action of U-II in the rat isolated aorta in a competitive and surmountable manner with a pK_b of 6.38 (38). The study also showed that the contractile potency of other spasmogens (angiotensin-II, phenylephrine and KCl) were unaltered in the presence of 10 uM SB-710411. At this concentration of peptide, however, the contractile response to endothelin was affected but since the effect was also observed with other SST receptor ligands, it was attributed to an inherent property of the SST receptor ligand(s) rather than U-II dependent activity. Further complicating the profile of this compound is that while SB-710411 is a competitive antagonist at the rat UT in HEK293 cells it behaves as an efficacious agonist toward the monkey and human receptors. In a related study, in cells expressing either the hUT or rUT, the neuromedin B receptor antagonist BIM-23127 (D-Nal-cyclo(Cys-Tyr-D-Trp-Orn-Val-Cys)Nal-NH_2) alone had no effect on the intracellular calcium mobilization responses, while hU-II was a sub-nanomolar agonist (39). While BIM-23127 was not an agonist in either cell line, it potently antagonized the hU-II-induced intracellular calcium mobilization responses in hUT-HEK293 cells (K_b =30 nM) and rUT-HEK293 cells (K_b =20 nM). An additional and structurally related neuromedin B receptor antagonist, BIM-23042 (D-Nal-cyclo(Cys-Tyr-D-Trp-Lys-Val-Cys)Nal-NH_2) displayed different functional activities at several UT orthologs, where it was either a full agonist (human & monkey UT), a partial agonist (mouse UT), or a competitive antagonist (rat UT).

NON-PEPTIDE UT LIGANDS

Although still a relatively immature area, the identification and design of low molecular weight (<500 g mol^{-1}), non-peptidic UT ligands is a rapidly emerging field of research. Undoubtedly, one major driver for the intense study has been the recognized need to develop potent, selective and bioavailable tool molecules to help to delineate the rich mammalian biology of U-II. Additionally, both rational structure-based approaches as well as more classical high throughput screening based methodologies have recently yielded promising leads for initiating drug discovery programs. Even so, a confounding issue that has been faced by researchers in the area is that many of the reported small molecule ligands are specific for human (or primate) UT and show little or no affinity for the corresponding rodent (mouse, rat) UT. This often severely limits their utility, particularly in an *in vivo* setting. Nonetheless, significant advances have been made and a number of the more advanced compounds are just beginning to enter human clinical trials.

Agonists - A single report of a small molecule UT agonist has appeared describing the identification of **15** **(AC-7954)** from a functional, cell based screen of 180,000 drug-like compounds (40). (±)**15** had affinity for both human UT (hUT pEC_{50} = 6.5) and rat UT (rUT pEC_{50} = 6.7) and the interaction with hUT was shown to be stereospecific with only the (+)-enantiomer having significant receptor affinity (pEC_{50} = 6.6) . By comparison, in the same assay, human U-II had over 5-orders of

magnitude greater UT affinity (pEC_{50} = 11.3 and 11.0 at the human and rat UT respectively). **15** was UT selective and did not bind to a number of other dopaminergic, adrenergic and serotonergic GPCRs or peptide GPCRs. Interestingly, **15** was also selective for UT over opioid (κ) and somatostatin (sst-2, sst-2 and sst-5) receptors which are the most closely related to UT having some, albeit relatively limited, sequence similarity to UT.



(±)**15** **16**

Antagonists - Several reports have now appeared in both the scientific and patent literature describing non-peptide UT antagonists. In general, most of the compounds reported contain features typical of GPCR binding small molecules such as a basic amino group believed to form a charged interaction with an Asp acid (D130 in hUT) within TM3 of the receptor and multiple aromatic moieties.

In an elegant study, a combination of structure-based design and high throughput virtual screening has been used to discover potent, small molecule UT antagonists (34). Using a classical and systematic investigation of the SAR of U-II, the identity of the key residues in the cyclic hexapeptide core of the peptide was established and the analysis was used to generate a solution ^{1}H-NMR structure for the binding conformation U-II.

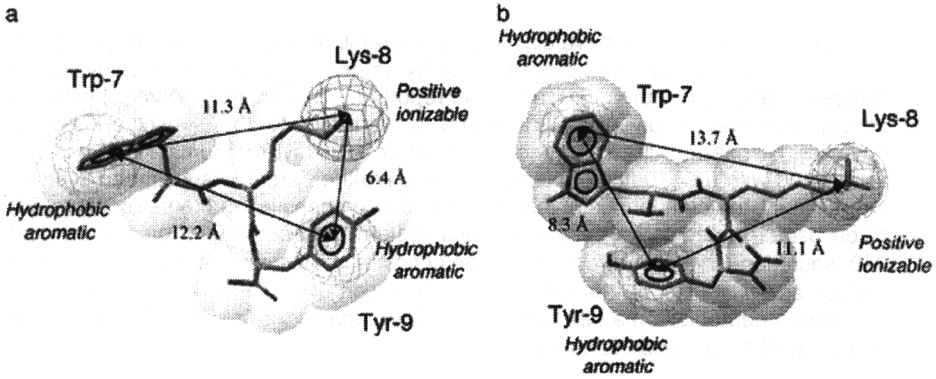
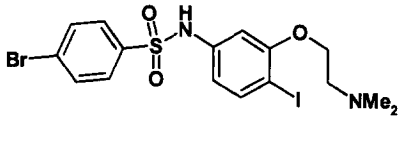


Figure 1. Pharmacophore query used for virtual screening mapped onto bioactive fragment of NMR structure of (a) U-II and (b) Ac-CFwKYC-NH_2. The light blue spheres represent two hydrophobic aromatic features; the red sphere represents positive ionizable features of the three point 3D pharmacophore. The gray spheres display the shape requirements linked to the pharmacophore query (Reprinted with permission from 34. Copyright 2003 American Chemical Society)

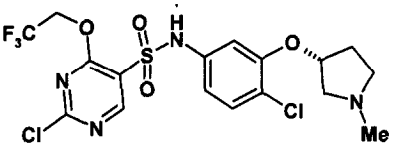
The resulting 3-point pharmacophore for UT binding was then used as the basis for computational searching of a small molecule library to identify a number of templates which antagonized the U-II-induced intracellular Ca^{2+} mobilization (FLIPR) with IC_{50} values in the 0.4 - 7 uM range. The most potent class was substituted

indoles such as **S6716 (16)** which had an EC_{50} in the functional assay of 400 nM. The authors recognized that these benzamidine containing compounds are likely to suffer from poor oral absorption based on earlier work on related factor Xa inhibitor compounds.

To date, however, the patent literature has documented a much larger number of UT antagonists. This no doubt is a reflection of the increased level of interest in the potential therapeutic application of such compounds. In a series of published patent applications, diarylsulfonamides with a variety of substitution patterns have been claimed for diverse therapeutic indications including hypertension, CHF, ischemic heart disease and stroke. The initial reports in this series of compounds center around ortho substituted (dialkylaminoalkoxy)arylsulfonamides in which the basic amine center is linked to the biarylsulfonamide by means of a linear three atom chain (41). More elaborate variation of the sulfonamide to incorporate, for example, biaryethers and heterocycles such as pyrimidines, are claimed in later applications from the same group (42 - 44). The biological activity of UT antagonists was determined in a radioligand competition binding assay using [^{125}I]-hU-II and cell membranes containing stable, cloned hUT and typical compounds in the series such as the analog **17**, had a K_i in this assay of 1,300 nM. In the most recent patent applications covering this series, the early compounds have been further investigated and constraint of the amine side chain by incorporation into a heterocyclic ring together with additional manipulation of the aryl groups is reported(45 - 48). This has resulted in the identification of compounds of higher UT affinity such as the pyrrolidine sulfonamide, **18** (K_i = 590 nM).

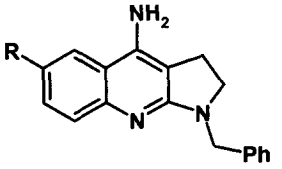


17



18

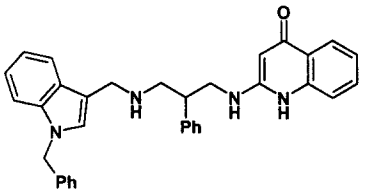
Japanese researchers have claimed fused 4-aminoquinolines such as **19** - **21** as having UT antagonism and hence of utility in the treatment of cardiovascular diseases (49). Only human UT affinity is reported. The reported *in vitro* biological activity, however, shows that UT affinity is sensitive to the substituent at the quinoline 6-position with the presence of either a methyl or bromine group being associated with an ~10-fold higher activity relative the parent unsubstituted system. Interestingly, the compounds of this class have also been claimed more recently (50) to be useful remedies for central nervous system diseases based on the amyloid β40 secretion-inhibitory effect of UT antagonism. This was demonstrated in a cellular assay measuring the U-II induced secretion of amyloid β-protein (Aβ) from the human neuroblastoma cell line IMR32 where analog **20** (3 - 10 uM) was shown to reduce Aβ40 secretion to control levels.



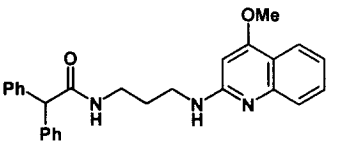
Compound	R	IC_{50}/ nM
19	H	33
20	CH_3	4.3
21	Br	2.4

Other non-peptidic UT antagonists based on the quinoline template include the 2-aminoalkyl quinolin-4-ones **22** (51, 52) disclosed in two patent applications that were published in 2002. A range of substituents in the quinolone benzo ring as well as the 2-position are claimed in these applications and the biological activity at the hUT reported is in the low nanomolar to micromolar range. Structurally related to

the quinolin-4-ones, the corresponding quinoline diamines **23** (hUT K_i = 90 nM) have also been investigated for UT antagonism (53). Furthermore, the similarity in some of the quinoline 2-substituents claimed in these applications indicate that the two series are indeed related and suggests that perhaps they bind to the receptor in a similar manner.

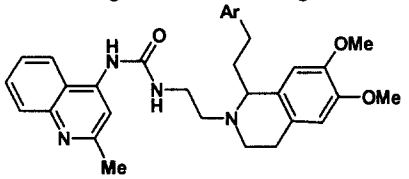


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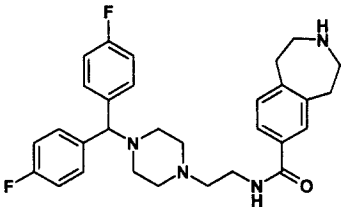
23

Further evidence that the bicyclic quinoline nucleus appears to provide a particularly permissive template for UT antagonism is provided by yet another patent application from a Swiss group claiming 1,2,3,4-tetrahydroisoquinolinyl and analogs, primarily as antihypertensives (54). Using a rhabdomyosarcoma cell line known to express hUT endogenously (54), the binding affinity of compounds in this class in a radiolabeled competition binding assay ([^{125}I]-hU-II) was determined to be in the 67 - 550 nM range. Moreover, some of the compounds (e.g. **24**) were effective in blocking the U-II induced contractions of an isolated rat aortic arch indicating that they possess at least some binding affinity for the rat UT ortholog. However, the nature of the antagonism (competitive or insurmountable) in the tissue based assay was not defined and was in the high micromolar range.

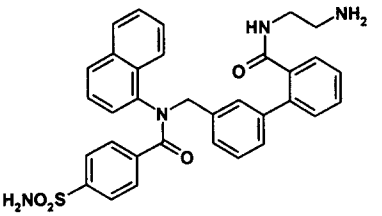


Compound	Ar	hUT Binding $_{Ki}$	Rat aortic arch contraction pD2'
24	Ph	125	5.23
25	3,4-$(MeO)_2$-Ph	63	
26	2,5-di-F-Ph	67	

A structurally distinct chemotype of UT antagonists is represented by the tetrahydrobenzazepines **27** also reported in a patent application by Japanese workers (56). The compounds disclosed are among the most potent U-II antagonists known (e.g. **27**, IC_{50} = 1.7 nM for the inhibition of U-II binding to hUT) and are alleged to be inhibitors of vasoconstriction and hence likely to be useful for the treatment of cardiovasular disorders. Similarly, the biphenyldicarboxamide scaffold represented by the general structure **28**, has also provided highly potent UT antagonists (IC_{50} ~10 nM) that additionally incorporate high somatostatin (SST_5 IC_{50} ~5 nM) receptor affinity (57).

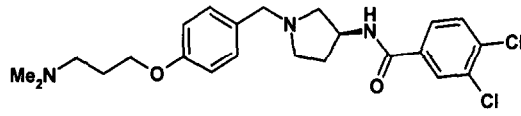


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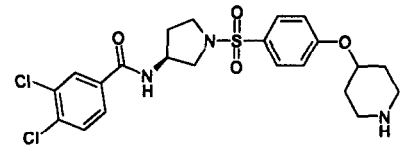
As mentioned above, U-II and UT share some primary sequence similarity at both the ligand and receptor level to somatostatin and the SST receptors. Importantly however, neither somatostatin nor U-II show any affinity for UT or the SST receptors respectively. By virtue of combining UT and SST5 antagonism, amongst other indications, these compounds are claimed to be useful as combined antihypertensives and antidiabetic agents.



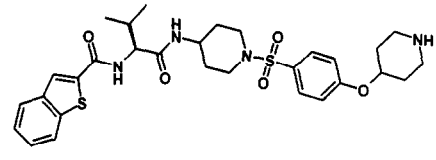
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Simple pyrrolidinyl and piperdinyl amide derivatives **29** have been prepared using a solid phase combinatorial library approach and show affinity for UT (58, 59). Two broad sub-series of compounds are evident that differ by either the presence or absence of an amino acid moiety bridging the pyrrolidine ring and a terminal amide group. Additionally, replacement of the alkyl group linking the (substituted)phenyl moiety to the pyrrolidinyl ring with a sulfonamide group as in **30** - **33** is clearly tolerated in the series (60 - 63) based on more recent disclosures but the presence of an alternate basic center elsewhere in the molecule appears to be necessary.

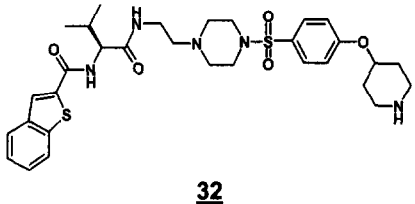
Remarkably, UT activity is insensitive to considerable structural change in the series as evidenced by the introduction of relatively large groups on the periphery of the molecule (**29**) or change of the core from a 5-membered pyrrolidine (**30**) to a 6-membered piperidine or piperazine (**31** and **32**) ring.



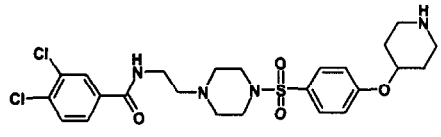
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Conclusion - The impressive *in vitro* and *in vivo* pharmacological activity of urotensin-II, particularly in monkeys, has stimulated a great deal of interest in the potential (patho)physiological role of the peptide and its cognate receptor in human disease. Although the early studies suggest an important role in peripheral vascular and cardiac function, the published data remain, to date, equivocal. The availability of small molecule urotensin-II receptor ligands (agonists and antagonists), coupled with advances in molecular approaches (e.g. UT or U-II knock out mice) will undoubtedly help to define better the clinical significance of the U-II/UT axis but the interspecies variability in the response to U-II further complicates the interpretation of *in vivo* studies of such antagonists in pre-clinical animal models of disease. Ultimately, any clinical utility of urotensin receptor modulators may require the evaluation of suitable compounds in man and it is encouraging that the first compounds are beginning to enter into human clinical trials.

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SECTION III. INFLAMMATORY, PULMONARY, AND GASTROINTESTINAL DISEASES

Editor: William K. Hagmann
Merck Research Laboratories, Rahway, NJ 07065

Chapter 12. Bradykinin-1 Receptor Antagonists

Mark G. Bock, J. Fred Hess and Douglas J. Pettibone
Merck Research Laboratories, West Point, PA 19486

Introduction - Beginning with the first pharmacological evidence for the existence of the bradykinin B1 receptor in 1978 (1), its cloning in 1994 (2), and the early demonstration of its role in inflammation and pain (3-7), there has been considerable speculation that blockade of this receptor could provide a novel way to treat chronic inflammatory pain. While peptide-based B1 antagonists have been available over the last decade and have been critical in defining the physiology of this receptor, the discovery of nonpeptide B1 antagonists, and their prospect of a more favorable pharmacokinetic profile and oral bioavailability, has been elusive until recently. With the advent of these prototypical nonpeptide antagonists, we are closer to testing clinically whether this approach will produce important new therapeutics. In addition, the recent demonstration of an active B1 mechanism in the CNS has broadened the analgesic potential of blocking this receptor. In this review, we provide a status report of these recent developments.

KININS AND THEIR RECEPTORS

The kallikrein-kinin system is comprised of kininogen, the precursor of the kinin peptides, kallikrein, a serine protease that acts on kininogen to liberate the kinin peptides, and the two receptor subtypes, the bradykinin B1 and B2 receptors, that mediate the biological response to the kinin peptides (8-10). The kinin peptides exhibit a wide variety of biological activities including vasodilation, pain, inflammation, lymphocyte diapedesis, smooth muscle contraction, natriuresis and diuresis. The role of this system in pain and inflammation has prompted interest in the development of therapeutic agents to modify its activity. Though some drug discovery effort has been directed toward the inhibition of the enzyme(s) producing bradykinin, kallikrein (11), most of this effort has focused on the bradykinin receptors. Earlier drug discovery endeavors targeted the bradykinin B2 receptor, whereas recent findings indicate that the bradykinin B1 receptor may provide a promising therapeutic target.

A single gene in humans, kininogen (KNG), encodes the multifunctional precursor for the kinin peptides (12). Alternative splicing of this gene results in either high molecular weight kininogen (HK) or low molecular weight kininogen (LK) (13) (Figure 1). HK and LK are secreted proteins that contain three cysteine protease inhibitor (cystatin) domains, along with the region encoding the kinin peptides. HK contains two additional domains involved in the intrinsic blood coagulation cascade that are eliminated by the alternative splicing event that produces LK. Rare mutations in the KNG gene result in Flaujeac deficiency, an asymptomatic condition (Online Mammalian Inheritance in Man, OMIM #228960). The disorder is detected in individuals exhibiting a prolonged activated partial thromboplastin time and either low or undetectable levels of kininogen. One KNG mutation that has been defined results in a truncated protein, with the individual having a complete loss of ability to produce any kinin peptides (14). Additionally, a kininogen deficiency that arose spontaneously in a strain of rats does not produce

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an overt phenotype (15). Thus, it is apparent that the production of kinin peptides is not essential for either the development or the general health of mammalian species. It is therefore reasonable to assume that the kallikrein-kinin system will be amenable to the development of therapeutic agents with acceptable safety profiles.

Kinin peptides are released by the proteolytic cleavage of kininogen by either plasma kallikrein or tissue kallikrein (8-9). Kallikreins are inactive zymogens until activated either by autoproteolysis or the action of a number of other proteases. Mutations in plasma kallikrein, encoded by the KLKB1 gene, result in Fletcher factor deficiency that has a similar phenotype to Flaujeac deficiency, a prolonged activated partial thromboplastin time, with no overt symptoms (OMIM #22900). Until recently, there were only three kallikrein genes identified in humans. However, sequencing of the region on human chromosome 19 that contains the tissue kallikrein gene revealed that it is part of a gene cluster that contains at least 15 members; a similar cluster containing 25 kallikrein genes is present on mouse chromosome 7. Since the substrate specificity of the recently identified members of the kallikrein family are not established and it is possible that other proteases act on kininogen, the spectrum of kinin generating enzymes is not fully defined. Kallikrein inhibitors, such as the peptide P8720, have shown some promise as anti-inflammatory agents in preclinical models (16-17); however, their specificity has not been evaluated in light of the recent data indicating an expanded kallikrein gene family.

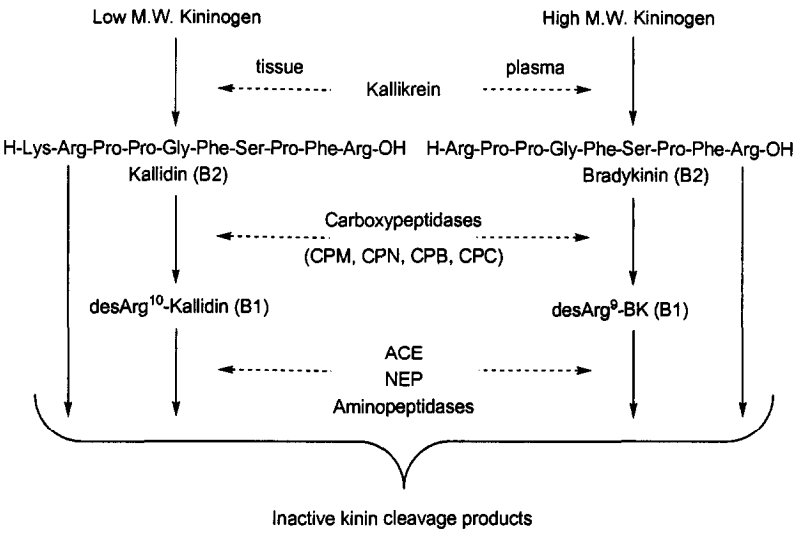


Figure 1. Kinin catabolism pathway.

The bioactive kinins include bradykinin (BK) and kallidin (Lys-BK), and the carboxypeptidase metabolites desArg^9-BK (DABK) and desArg^{10}-kallidin (DAK). The processing of kinin peptides by amino- and carboxypeptidases is illustrated in Figure 1. The active kinin peptides are substrates for a number of proteases and are rapidly degraded to inactive peptides, principally by the angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP), but also by aminopeptidases (18). A body of evidence indicates that some of the cardioprotective and some of the side effect liability of ACEi can be attributed to elevated bradykinin (18-21).

The bradykinin receptor B1 and B2 subtypes are G-protein coupled receptors that are encoded by adjacent genes, BDKRB1 and BDKRB2, on human

chromosome 14. The bradykinin receptor subtypes are not highly related and are only slightly more similar to each other than to angiotensin receptors (2). BK and kallidin are equipotent in activating the bradykinin receptor B2 subtype, whereas DABK and DAK are inactive on this receptor (22-23). Conversely, DABK and DAK activate the bradykinin B1 receptor, with BK and kallidin being less potent (2,24-25). Targeted disruption of the genes encoding the bradykinin B1 and B2 receptors in mice indicate that they are the only receptor subtypes that mediate the biological activity of the kinin peptides (26-27). Species differences with respect to the kinin peptides are apparent for the bradykinin B1 receptor as the affinity for the desArg-kinin peptides differs significantly across species (28-30).

The bradykinin B2 receptor is constitutively expressed in a wide variety of cell types and tissues and its role in mediating the physiological effects of the prototypical kinin, bradykinin, has been extensively studied. Both agonist and antagonists of the B2 receptor have been evaluated for their therapeutic potential. The peptide agonist Cereport was developed in an attempt to exploit the ability of the B2 receptor to transiently increase the permeability of the blood brain barrier and improve the efficacy of chemotherapeutic agents, such as carboplatin, in treating brain tumors (31). The established role of bradykinin as a mediator of pain and inflammation led to the discovery of B2 receptor peptide antagonists. The peptide antagonist Icatibant showed some promise in small clinical trials for asthma and allergic rhinitis, however development of this compound has not been pursued (32-33). Similarly, the B2 receptor peptide antagonist CP-0127 showed possible benefit in initial clinical trials for brain trauma and sepsis but has not been developed further (34,35). Non-peptide antagonists of the bradykinin B2 receptor have also been reported and reviewed (6,36,37). The identification of highly selective B2 receptor antagonists, such as Icatibant, provided excellent tools for evaluating B2 receptor antagonists in preclinical animal models (18,19,38). These studies, coupled with findings using B2 receptor knockout mice suggest a cardioprotective role for this receptor (39-41). The modest effects of B2 receptor peptide antagonists in clinical trials along with potential cardiovascular side effect liability have dampened enthusiasm for further development of B2 receptor antagonists.

The B1 receptor was initially defined as a pharmacological entity that was synthesized *de novo* in an *in vitro* tissue preparation (1). Subsequent work reviewed by Marceau (10, 42) revealed that a cytokine network induces the expression of the bradykinin B1 receptor following tissue injury. Thus, the view of the B1 receptor as an inducible receptor was established. However, as discussed in more detail below, recent data is expanding this classical view to include a number of cells and tissues that constitutively express the B1 receptor (27, 43-45).

ROLE OF BRADYKININ B1 RECEPTORS IN PAIN

A large body of evidence has been developed over the past decade supporting a role of B1 receptors in mediating various pain responses. The inducible nature of the B1 receptor has led to the belief that B1 receptors are most relevant for pain during longer term inflammatory conditions, while B2 receptors are important mediators of acute pain and the early inflammatory response (3-6). This hypothesis was initially proposed based on studies comparing effects of the peripheral administration of peptide kinin agonists and antagonists in a variety of pain models with or without overt inflammation (7, 46-49). The results from these initial studies strongly implicated a peripheral site both for the origination of the painful stimulus and for the analgesic effects of B1 antagonists. Inflammatory trauma elicits a cascade of events including local generation of bradykinin, kallidin, and their des-arginine metabolites (see 3,4,10,61). In addition, the local generation of inflammatory mediators, particularly IL-1β and TNFα, enhances the expression of B1

receptors on a variety of cell types (e.g. endothelial cells, macrophages and neutrophils). This induction occurs via a complex pathway involving a number of mediators including various cyclooxygenase products, kinases, and the transcription factor NFκB (50,51). Local B1 activation of inflammatory cells in turn causes the release of a variety of substances that contribute to the overall response. Therefore, a peripheral mechanism for B1-supported pain during inflammation may involve sensitization of primary pain afferents through release of inflammatory mediators (such as prostaglandins and cytokines) from various cell types, but does not appear to involve direct activation of these sensory afferents (4,52).

Recently, however, studies with B1 antagonists and B1 knockout ($Bk1R^{-/-}$) mice have generated a broader view of the potential role of B1 mechanisms in both non-inflammatory and inflammatory pain and have implicated CNS involvement (27,53). In these studies, $Bk1R^{-/-}$ mice appear outwardly normal but they have blunted responses to thermal, chemical and mechanical nociceptive stimuli under non-inflamed conditions. In addition, the thermal hyperalgesic responses to carrageenan and complete Freund's adjuvant (CFA) challenges are markedly reduced in the $Bk1R^{-/-}$ mice, as are lipopolysaccharide (LPS)-induced hypotension and polymorphonuclear leukocyte accumulation in a carrageenan-induced pleurisy model. Significantly, a role for a central B1 component in pain perception is suggested by *in vitro* studies showing that in the absence of inflammation, the ventral root potential evoked by electrical stimulation of the dorsal root is enhanced by application of the B1 agonist DABK to a wild-type spinal cord preparation, but not a preparation from $Bk1R^{-/-}$ mice. The phenomenon of 'wind-up' to repeated dorsal root stimulation *in vitro* is also reduced by about 50% in the B1 knockout mice (27). Consistent with a B1 mechanism that is active in spinal cord neuronal plasticity, peptide B1 antagonists have recently been shown to be analgesic in rodent models of neuropathic pain, such as pain arising from chronic constrictive nerve injury (CCI) and diabetic neuropathy (54, 55). At least in the case of CCI, the analgesic activity of a B1 antagonist is only apparent after the receptor has been induced on primary afferents (54). An active central B1 pain mechanism is more directly demonstrated by the thermal hyperalgesic response observed after intrathecal injection of DABK to wild-type mice, an effect that is absent in $Bk1R^{-/-}$ mice and blocked by a B1 antagonist (56). Similarly, intrathecal injection of a B1 antagonist blocks the hyperalgesic response to intraplantar administration of CFA (56,57).

Peripheral nerve injury and inflammatory damage is known to produce inflammatory responses in the spinal cord, as well as at the site of injury, that are conducive for B1 receptor induction (58-60). Activation of B1 receptors either on central terminals of primary afferents and/or second order neurons of the dorsal horn may subserve a CNS mechanism. All of the components of the kinin system have been localized in the CNS (61). Intrinsic kininergic neurons have been detected in the spinal cord dorsal and ventral horns (62). B1 receptors have been immunolocalized to A-delta and C-fibers as well as intrinsic neurons of the dorsal horn (43,45,63). They have also been localized to neurons in various human brain regions (64).

PEPTIDE BRADYKININ B1 RECEPTOR ANTAGONISTS

Peptide antagonists for the B1 receptor were discovered over two decades ago (65). The initial prototype compounds were derived by replacing the aromatic residue Phe^8 in the endogenous bradykinin agonist peptide fragments, $desArg^9$-BK and Lys-$desArg^9$-BK, with Leu (66). While these first generation antagonists, [Leu^8]$desArg^9$-BK (**1**) and Lys[Leu^8]$desArg^9$-BK (**2**) (Figure 2), are specific for the bradykinin B1 receptor versus a variety of other receptors (e.g. AT_1, NK-1), they are susceptible to enzymatic degradation and can act as partial agonists thereby limiting

their utility (67). Thus in the intervening years, an objective has been to identify analogs with enhanced potency, selectivity, specificity, and prolonged duration of action *in vivo*. Numerous enzymes are suspected to be involved in proteolytic deactivation of these peptides, including angiotensin-converting enzyme (ACE), the neutral endopeptidase 24.11 (NEP 24.11), and the aminopeptidases M and P (AmM, AmP) (Figure 1) (68-70). Strategies to enhance the potency and enzyme stability of the original peptide bradykinin antagonists have relied on the incorporation of non-proteinogenic amino acids, D-configured amino acids, uncommon peptide bonds, and other measures (70-75). Progress in this direction has been made with the development of AcLys[D-βNal^7, Ile^8]desArg^9-BK (R 715, **3**) and LysLys[Hyp^3, Cpg^5, D-Tic^7, Cpg^8]desArg^9-BK (B9958, **4**), two compounds which show resistance to degradation by peptidases and are pure antagonists on human B1 receptors (71, 72, 76). More recently, the two newly produced compounds, AcOrn[Oic^2, (αMe)Phe^5, D-βNal^7, Ile^8]desArg^9-BK (R 954, **5**) and AcLys[Oic^2, (αMe)Phe^5, D-βNal^7, Ile^8]desArg^9-BK (R 955, **6**) also have demonstrated stability against AmM, AmP, and ACE (70). This profile will allow researchers to test the relative importance of AmP in the cessation of B1 receptor antagonist effects for peptide antagonists *in vivo*.

	Peptide Antagonists	human B_1 receptor
1	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH	6.37
2	H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH	8.01
3	Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-D-(βNal)-Ile-OH	8.49
4	H-Lys-Lys-Hyp-Pro-Cpg-Phe-D-Tic-Cpg-OH	8.65
5	Ac-Orn-Oic-Pro-Gly-(αMe)Phe-Ser-D-(βNal)-Ile-OH	8.46
6	Ac-Lys-Oic-Pro-Gly-(αMe)Phe-Ser-D-(βNal)-Ile-OH	8.43

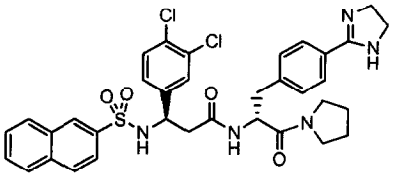
Abbreviations: **Ac-Lys** = N$^\alpha$-acetyl lysine; **β-Nal** = 3-(2-naphthyl)alanine; **Hyp** = 4-hydroxyproline; **Cpg** = cyclopentylglycine; **D-Tic** = *D*-2,3,4-tetrahydroisiquinoline-3-carboxylic acid; **Oic** = L-(3a*S*,7a*S*)-octahydroindole-2-carboxylic acid; **Ac-Orn** = N$^\alpha$-acetyl ornithine.

Figure 2. Peptide antagonist potencies (pA_2) measured on kinin receptor bioassay systems.

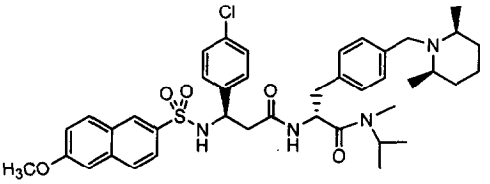
NON-PEPTIDE BRADYKININ B1 RECEPTOR ANTAGONISTS

Interest in the development of non-peptide bradykinin B1 antagonists is slowly producing results. While the availability of these compounds has been anticipated for some time, reports describing non-peptide B1 antagonists have only recently appeared. The first small molecule B1 antagonists were disclosed in a 1997 patent application and as they retain considerable peptide character are more properly termed dipeptidomimetics (77). One of the prototypical compounds (**7**) is comprised of an N-(arylsulfonyl)-β-amino acid and a phenyl alanine amide with *R* the preferred configuration at both asymmetric centers. The affinity of **7** for B1 receptors was found to be in the 10^{-10} M range as measured on a suspension of MRC5 cell membranes. The affinity for the B2 receptors was between 10^{-7} to 10^{-6} M. A more

recent patent application claims close molecular analogs of **7**. A preferred structure covered in this application is **8** (78). Based on the exquisite B1 receptor binding affinity of **7**, it can be assumed that modifications to this structure (cf. **8**) were made only to fine tune pharmacokinetic and/or physical properties. No supporting data for **8** or its analogs have been disclosed.

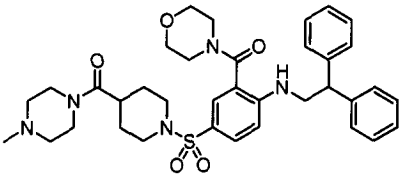


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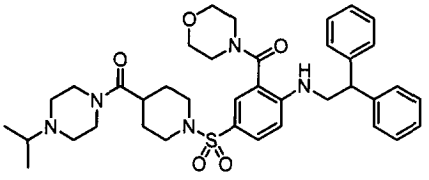


8

The arylsulfonamides **9** and **10** were described in the patent literature a few years ago and displayed potency in a receptor binding assay employing membranes derived from HEK 293 cells expressing the cloned human B1 receptor (79). K_i values ranging to as low as 0.5 nM were reported. The functional activity of these compounds was demonstrated in a primate model of thermal antinociception. In this test, the exemplified compounds were effective in preventing or reversing carrageenan-induced hyperalgesia at a dosage of 10 nmol/kg.

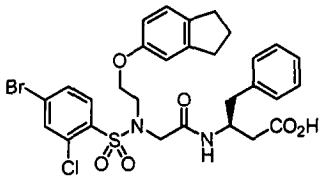


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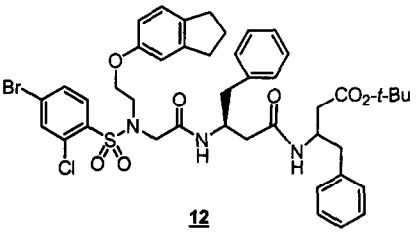


10

A more recent disclosure in the patent literature exemplifies benzenesulfonamides (**11** and **12**) (80). The acid **11** and its derivatives were claimed to displace the agonist desArg^{10}-kallidin at the human B1 receptor with a K_i value in the range of 0.5 nM to 2000 nM. Activity as anti-hyperalgesic agents was demonstrated in a thermal antinociception test in rhesus monkeys wherein the latencies to tail withdrawal from a warm water bath were measured. The exemplified compounds were efficient in preventing or reversing carrageenan-induced hyperalgesia at doses as low as 10 nmol/kg.



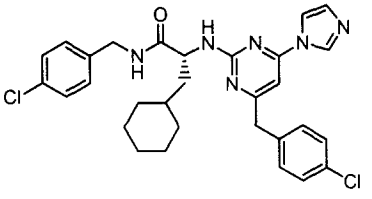
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12

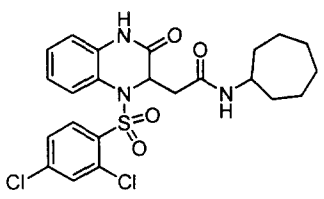
High throughput screening (HTS) of encoded combinatorial libraries led to the identification of the diaminopyrimidine **13** (PS-020990) (81,82). It is a compound readily dissected into four domains (corresponding to the combinatorial synthetic steps from which it was derived), the central element of which is the heterocyclic pyrimidine core. Compound **13** represents an optimized member of a library and inhibits the binding of [^{3}H]-desArg^{10}-kallidin to IMR-90 cells expressing an

endogenous human B1 receptor with a K_i value of 6 nM. It also inhibits agonist-induced (desArg^{10}-kallidin) phosphatidyl inositol turnover (K_{Bapp} = 0.4 ± 0.2 nM) and calcium mobilization (K_{Bapp} = 17 ± 2 nM) in IMR-90 cells. In addition, compound **13** is at least 1000-fold selective for the B1 receptor relative to the B2 receptor.

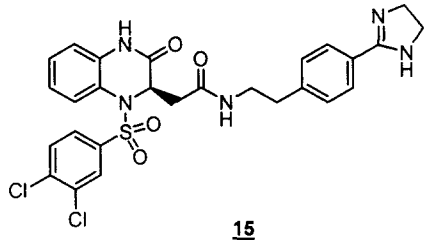


13

A patent issued earlier this year claims tetrahydroquinoxalines as B1 receptor antagonists (83). While the number of compounds in this disclosure is large, details relating to their biological activity are sparse. In a functional *in vitro* assay measuring intracellular calcium mobilization in stabilized CHO cells, compound **14** had an IC_{50} value of 17 nM. *In vivo* functional activity is also claimed but not reported. Coincidentally, the discovery of closely related dihydroquinoxalinones was disclosed in a separate report (84). Optimization of a HTS lead employing molecular modeling and following classical SAR trends yielded the potent (human B1, K_i = 0.034 nM) B1 receptor antagonist **15**. Compound **15** is selective for the B1 versus the B2 receptor (human B2, K_i > 10 μM), as well as a number of human opioid receptor subtypes (IC_{50}: 7.6 μM (μ), 3.2 μM (δ), 7.3 μM (κ)). In a panel of assays representing 170 enzymes, receptors, and transporters, **15** exhibited over 5000-fold selectivity for the human B1 receptor. Compound **15** also showed potent antinociceptive activity in a rabbit assay of inflammatory hyperalgesia with ID_{50} values of 3.5 μg/kg and 16.4 μg/kg for low and high intensity stimuli, respectively.

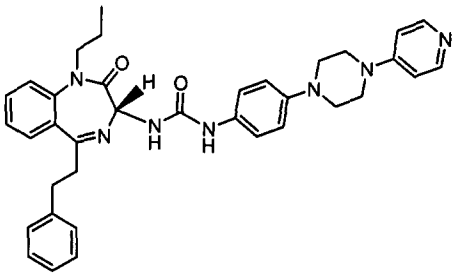


14



15

Receptor based screening also identified a 3-ureidobenzodiazepine structure as an antagonist of the human B1 receptor (85). Systematic modifications of the appendages at the 3- and 5-positions of the diazepine ring in the screening lead afforded the optimal compound (**16**, B1, K_i = 0.59 nM (human), 0.92 nM (rat); B2, K_i > 10 μM (rat)). In a rodent hyperalgesia assay, at a dose of 1 and 3 mg/kg (IP), carrageenan-induced hyperalgesia was suppressed 30% and 90%, respectively. By comparison, morphine dosed (IP) at 1 and 3 mg/kg resulted in 75% and 105% inhibition, respectively. That the efficacy of **16** and morphine are comparable in this rodent pain model despite the different target receptors, underscores the potential of selective B1 antagonists as efficacious agents in the amelioration of pain without the deleterious adverse effects associated with the opiates.



16

Conclusion - The picture that has emerged over the last several years suggests that the therapeutic potential of B1 antagonists to treat pain is broader than initially envisaged and could now include certain types of acute pain and neuropathic pain, in addition to chronic inflammatory pain. As such, B1 antagonists exhibit an analgesic profile that overlaps significantly with the opiates, but are unlikely to exhibit the unwanted side effects of morphine-like drugs such as sedation, respiratory depression and abuse potential. In addition, targeting both peripheral and central (e.g. spinal cord) sites of action may be important for optimizing the efficacy of this novel class of compounds.

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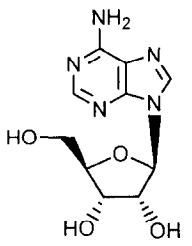
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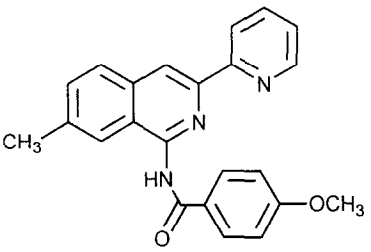
Chapter 13. A_3 Adenosine Receptors

Kenneth A. Jacobson, Susanna Tchilibon, Bhalchandra V. Joshi and Zhan-Guo Gao
Molecular Recognition Section, Laboratory of Bioorganic Chemistry,
National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD 20892

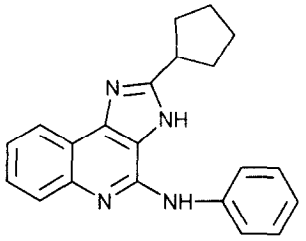
Introduction – Extracellular adenosine (**1**) is involved in many cytoprotective functions of the body, including conditioning the heart against ischemia, counteracting the damaging effects of excitotoxicity and seizure activity in the brain, and suppressing an excessive immune and inflammatory response (1). The possibilities of therapeutic intervention based on modulation of adenosine receptors and adenosine levels are numerous. There are four subtypes of adenosine receptors; of which the A_3 receptor was most recently identified as a result of its cloning from various species (2). The A_3 receptor is activated endogenously by higher concentrations of adenosine than are required for activation of the A_1 /A_{2A} receptors. The effector mechanisms are inhibition of adenylate cyclase and stimulation of phospholipase C (1,3). Both protective (usually at nM concentrations) and damaging effects (usually at μM concentrations) of A_3 agonists have been studied (3). The receptor is distributed at low, diffuse levels in the brain and in the human periphery where it is present in lungs, liver, heart, and immune cells such as eosinophils (2). Therapeutic interests related to A_3 receptors are: anti-inflammatory (possibly through depression of TNF-α levels) (4), cardioprotective (5), cerebroprotective (6), anticancer (7), and antiglaucoma (8). A mouse line lacking the A_3 receptor demonstrated that this is a non-lethal mutation that has inflammatory, cardiovascular, and behavioral consequences (9-11). The A_3-modulated release of histamine from mast cells may be specific to rodent species (12).



1



2



3

A_3 RECEPTOR STRUCTURE: LIGAND BINDING SITE, ALLOSTERIC MODULATION

Mutagenesis was recently carried out on the human A_3 receptor, a 7TM receptor, to locate a putative ligand binding site (13). In some cases, different amino acid residues are associated with agonist or with antagonist binding. Movement of a conserved Trp in TM6 has been proposed as an important step in receptor activation. In a rhodopsin-based docking model of the human A_3 receptor, this residue rotates characteristically upon *in silico* binding of agonists but not antagonists. This may be linked to a required rotation of TM6 during the receptor activation process (13).

Allosteric Modulators – Pyridinylisoquinolines (VUF5455, **2**) and imidazoquinolines (DU124183, **3**) were shown to positively modulate A_3 binding and/or action of agonists, although both substances retain antagonistic properties as well (14,15). These derivatives had no effects at the A_1/ A_{2A} receptor subtypes. Compound **3** augmented

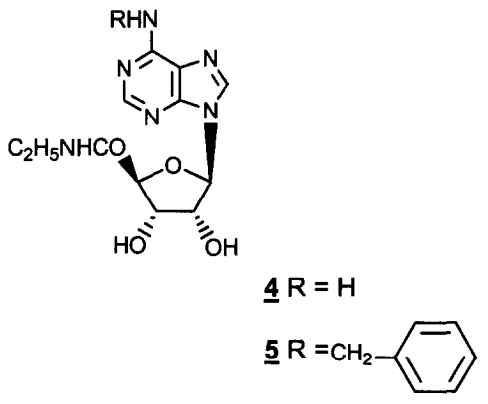
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the maximal efficacy of an A_3-agonist, in addition to decreasing the agonist dissociation rate from the receptor.

A_3 RECEPTOR AGONISTS

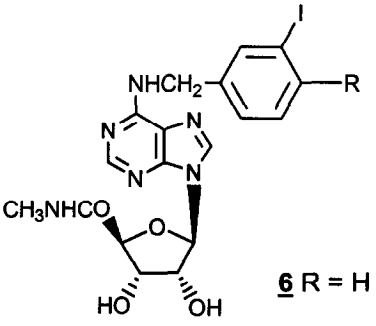
Nearly all of the known adenosine A_3-agonists are derivatives of adenosine (3). NECA (**4**) is a nonselective, but highly potent agonist, which was used for the initial characterization of the A_3 receptor and has been used as a less-than-optimal radioligand for the A_3 receptor (2).

Adenosine 5',N^6-Substitutions – Certain substitution at the N^6-position (such as the 3-iodobenzyl group) provides A_3 selectivity, although in some cases N^6-substitution of adenosine 5'-OH derivatives with large groups (e.g., substituted benzyl groups or large cycloalkyl rings) may reduce maximal efficacy to produce partial agonist activity at the A_3-receptor. This reduction of efficacy may be overcome by combining the N^6-substitution with 5'-uronamide substitution, as in NECA. For example, benzyl-NECA (**5**, slightly selective) and IB-MECA (**6**, ~50-fold selective versus A_1/A_{2A} receptors) are full agonists, while the corresponding 5'-OH nucleosides display roughly half of the maximal efficacy (13,16). IB-MECA (**6**) is currently in clinical trials for cancer treatment, due to its reported cytostatic effect on tumors (7). The radioiodinated ligand I-AB-MECA (**7**) is used widely for screening of new ligands of the A_3 receptor at which it is potent (K_d = 0.59 nM) but not highly selective (3). The efficacy at A_3 receptors is more easily diminished upon structural modification than at the other subtypes. Rigidification of the ribose ring moiety (5'-uronamides) also reduces A_3 efficacy. A small N^6 substitution (e.g., methyl, methoxy, or ethyl) provides high affinity at the human but not the rat A_3 receptor (17). Several A_3 selective agonists, NNC21-0238 (**8**, K_i = 20 nM) and NNC53-0055 (**9**, K_i = 4.6 nM) containing N^6-methoxy groups and various substitutions at the 5'-position have been reported (18). Acylation of the N^6-position (urea derivative **10**) has also been shown to enhance agonist potency at the A_3 receptor (19). The efficacy-promoting effects of the 5'-uronamide group are sufficiently strong to allow substitution of the adenine moiety with a 1,3-dibutylxanthine group, and thus DBXRM (**11**) is a selective and moderately potent, full A_3-agonist (3).



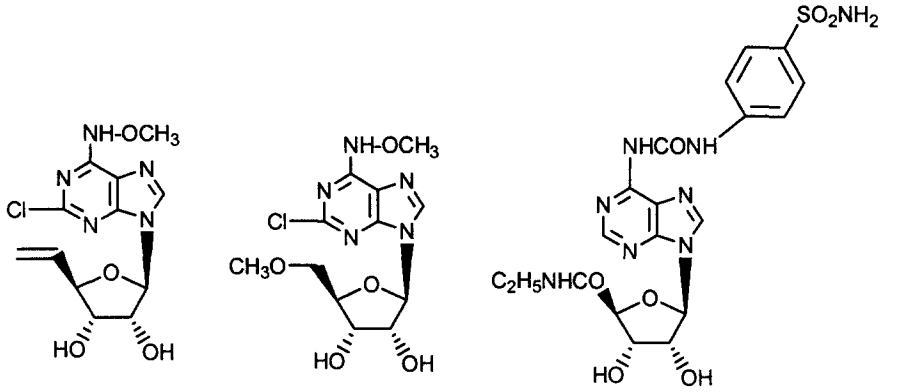
4 R = H

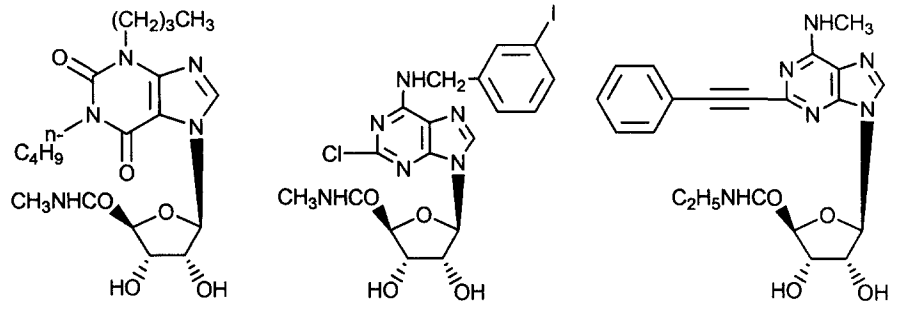
5 R = CH_2–



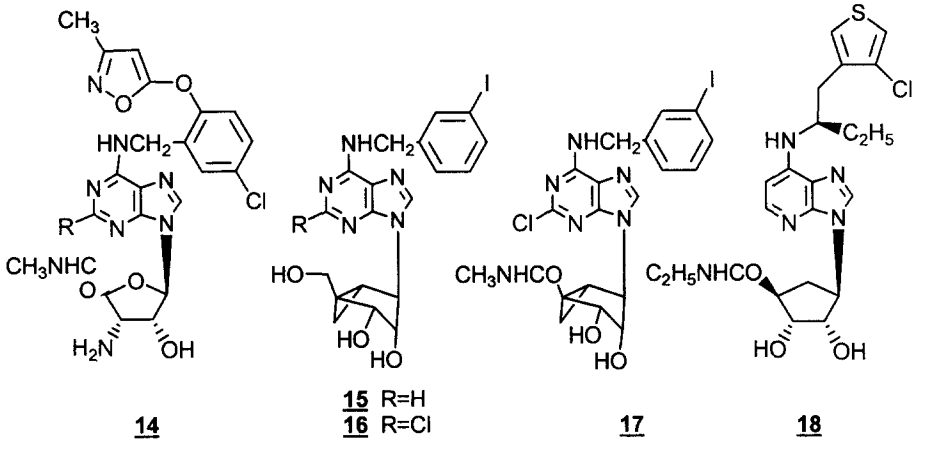
6 R = H

7 R = NH_2





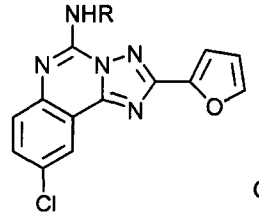
Adenosine 2-Substitutions – A 2-chloro substitution generally improves the potency at A_3 receptors (20). For example, Cl-IB-MECA (**12**) is a highly selective, full agonist with K_i value = 0.33 nM at the rat A_3 receptor. In some cases, chain elongation at the 2-position has enhanced A_3 receptor affinity, such as N^6-Me-PE-NECA (**13**) (21).



Ribose Substitutions of Adenosine – While the 5'-hydroxyl group of the ribose ring may be replaced, the 2' and 3'-hydroxyl groups are generally required for affinity and/or the ability to fully activate the receptor; i.e., achieve full efficacy (22). The 3'-hydroxyl may be replaced with 3'-amino which will maintain agonist affinity only in combination with the appropriate N^6-substitution. For example, CP608039 (**14**) is intended for use in peri-operative cardioprotection (23). The 3'-amino group, which is mainly charged at physiological pH, also enhances water-solubility. 3'-Amino-3'-deoxy analogues are useful for the design of neoligands in relation to the concept of neoceptors which is a means of engineering the binding site of a 7TM receptor for unique recognition of synthetic ligands (24). A ribose substitution thathas enhanced A_3 selectivity in a general manner is the replacement with the (N)-methanocarba ring system (**15** – **17**) which is superior to simple carbocyclics , such as the nonselective agonist AMP579 (**18**) (25, 26). The (N)-methanocarba, 5'-uronamides (e.g., MRS 1898, **17**) are full agonists, while the 5'-OH analogues (MRS 1743 (**15**) and MRS 1760 (**16**)) are low efficacy partial agonists (27). Another ring substitution that has been utilized in A_3 selective agonists is the 4'-methyl group (28).

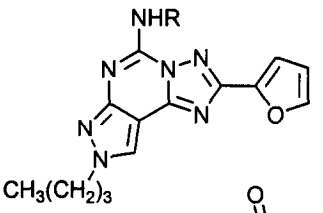
A_3 RECEPTOR ANTAGONISTS

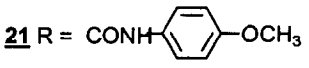
Nonpurines – Leads for novel A_3 antagonists have been obtained from compounds known to inhibit adenosine receptors and from library screening. The triazoloquinazoline derivative CGS15943 (**19**), previously known as a nonselective adenosine antagonist at the human subtypes, served as template for related nonpurine heterocyclic antagonists of the human A_3 receptor, due to the observation of its high affinity (K_i ~ 10 nM). Acylation of the N^6-amino group of **19** led to MRS 1220 (**20**) which is potent (K_i = 0.65 nM) and selective for human, but not rat A_3 receptors (29). In a related series of heterocyclic derivatives, the pyrazolotriazolopyrimidine derivative MRE3008-F20 (**21**) was found to be a highly selective antagonist at the human A_3 receptor and was radiolabeled to provide a hydrophobic, but useful radiotracer with a K_d = 0.80 nM (30). Another analogue (**22**) in the same series displayed a K_i = 0.16 nM at the A_3 receptor (31). A pyridinium moiety (**23**) was introduced to enhance water-solubility in this series of A_3 antagonists (32).

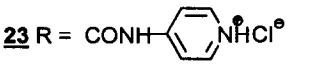


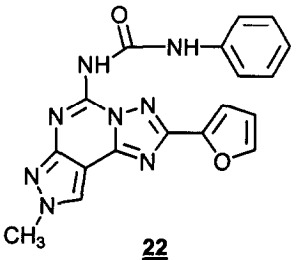
19 R = H

20 R = $COCH_2$

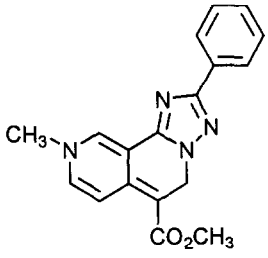




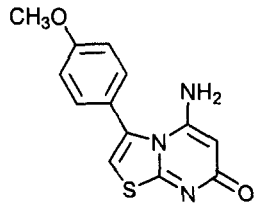




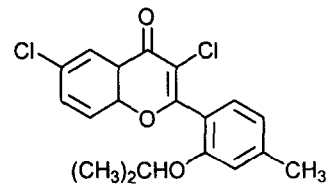
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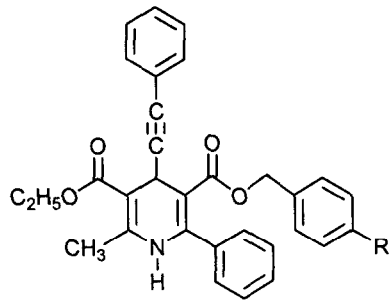
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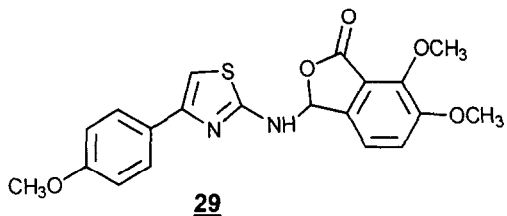


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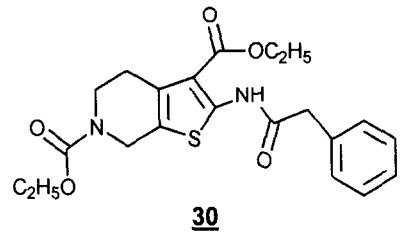


27, R = H
28, R = NO_2

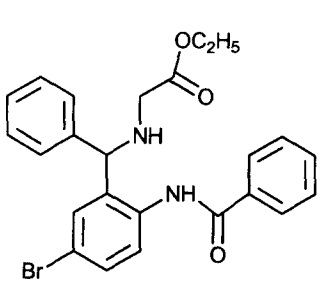
The earliest reported A_3 antagonists were the result of library screening in which novel heterocyclic antagonists derivatives were identified (3,33,34): L-249313 (**24**, K_i = 13 nM) and L-268605 (**25**, K_i = 18 nM), flavonoids (MRS1067, **26**, K_i = 560 nM), and dihydropyridines (**27**, **28**). The dihydropyridines are typically antagonists of L-type Ca^{2+} channels; however, the addition of extended (arylalkene or arylalkyne) groups at the 4-position and phenyl substituents at the 6-position combined to eliminate recognition at the Ca^{2+} channels. Chiral resolution of MRS1191 (**27**) was carried out to identify the more active isomer, although the racemic 4-nitro derivative MRS1334 (**28**) was the most potent in this series (K_i = 2.7 nM).



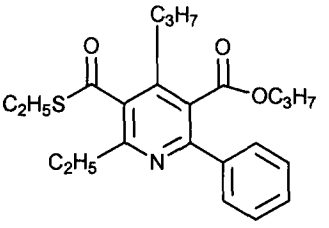
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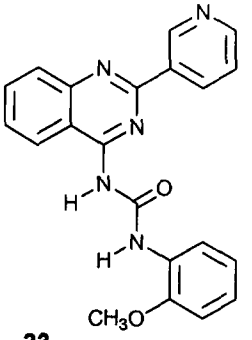
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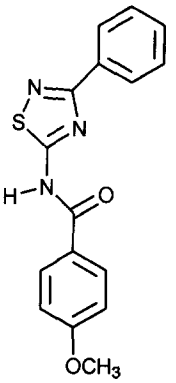
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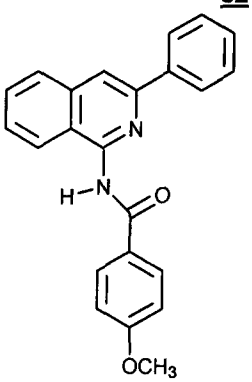
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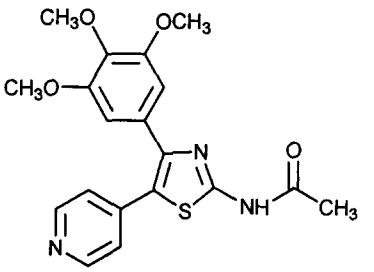
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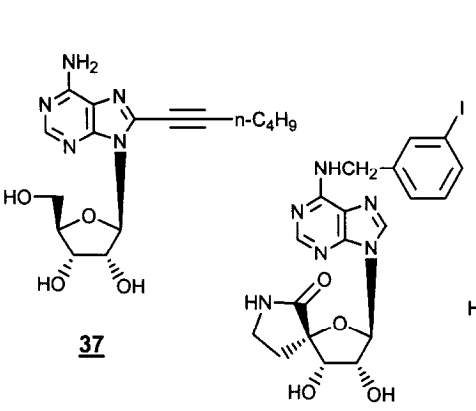
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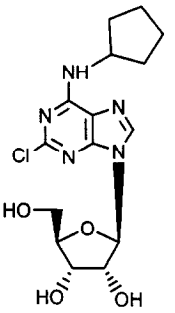
A diverse group of A_3 antagonists that has not yet been explored through SAR analysis was discovered by *in silico* methodology leading to structurally diverse hits (**29** - **31**) (35). The finding that dihydropyridines could be crafted into A_3-selective antagonists provided the lead for a series of pyridine derivatives that selectively blocked A_3 receptors (36). MRS 1523 (**32**) is noteworthy as a moderately potent A_3 antagonist at both human and rat receptors (K_i = 18.9 and 113 nM, respectively). Other novel heterocyclic, nonpurine-derived A_3 antagonists include a quinazoline VUF5574 (**33**, K_i = 4 nM), thiazole LUF5417 (**34**, K_i = 4 nM, nonselective), and an isoquinoline VUF8507 (**35**, K_i = 17 nM) (37,38). A series of N-[4-arylthiazol-2-yl]acetamides (**36**) was discovered to be A_3 antagonists through serendipity and then optimized structurally (39).

Purines and their derivatives – Adenine derivatives, including 9-ribosides (**37**, K_i = 650 nM), have been shown to be selective A_3 antagonists (40). Other nucleoside-derived antagonists include the spiro lactam derivative MRS1292 (**38**), CCPA (**39**, also a potent A_1 agonist), and DPMA (**40**, also a moderately selective A_{2A} agonist) (13, 17).

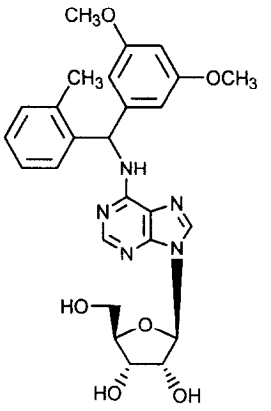


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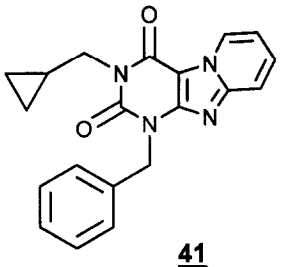
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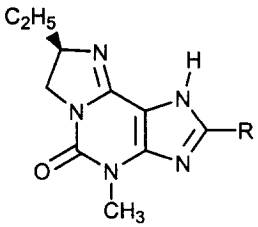
40

Xanthines, such as caffeine and theophylline, are the classical adenosine antagonists, however, they are typically of much lower affinity at the A_3 receptors than at the A_1 / A_{2A} / A_{2B} subtypes (1-3). This is a species-dependent phenomenon with affinity at the human A_3 receptor exceeding that at the rat A_3 receptor. Nevertheless, a recent report indicated the feasibility of designing A_3-selective xanthines through cyclization between the 7- and 8-positions leading to pyridopurine-2,4-dione derivatives (**41**) (41).

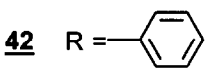
The SAR of 2-phenylimidazolepurin-5-ones as water-soluble derivatives of xanthines has been explored (42, 43). PSB-11 (**42**, (K_i = 2.3 nM) is highly potent and has been used as a radioligand with low nonspecific binding. The corresponding 2,3,5-trichloro derivative PSB-10 (**43**) is of even higher affinity and selectivity (K_i = 0.43 nM). Several classes of extended xanthine structures (**44**, **45**) were reported as A_3 antagonists (44,45). Triazolopurines, triazoloquinazolines, and pyrazolotriazolo-pyrimidine derivatives (**46**, **47**) have been reported as A_3 antagonists that are bioavailable *in vivo* when applied topically to the eye for use in the treatment of glaucoma (46,47). OT-7999 (**47**) displayed a K_i = 0.95 nM at the human A_3 receptor and >10,000-fold selectivity in comparison to the three other subtypes.



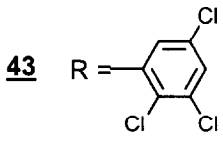
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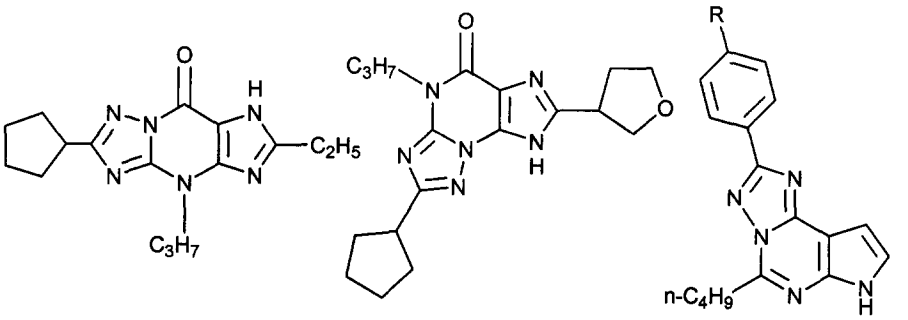


42 R =



43 R =





44 **45** **46** R = OCH_3 **47** R = CF_3

It has been suggested that in *in vivo* studies A_3 receptors are involved in a number of disorders of the central nervous and the cardiovascular systems, as well as in the inflammatory processes. The selective A_3 receptor agonist IB-MECA (**6**) has been demonstrated to induce behavioral depressant effects in mice (48). Chronic treatment with IB-MECA resulted in improved postischemic cerebral blood circulation, survival, and neuronal preservation, while the opposite effects were observed when IB-MECA was given acutely (49). A_3 receptor activation induced late preconditioning against infarction in conscious rabbits (50). In rats, Cl-IB-MECA (**12**) resulted in a short-lasting hypotension as a result of histamine release (51). The A_3 receptors are also involved in vasoconstriction *in vivo* (52). A_3 receptor activation may be involved both pro- and anti-inflammatory responses in mice (2). Stimulation of A_3 receptors both enhanced degranulation *in vitro* and directly produced degranulation of rat mast cells *in vivo* (53,54). A_3 receptor agonists inhibited murine macrophage tumor necrosis factor-α production both *in vitro* and *in vivo* (55,56). Mucociliary transport in rabbit trachea in vivo was enhanced by IB-MECA but not by an A_1 or A_{2A} agonist. Also, the effect of IB-MECA was blocked by the A_3 antagonist MRS1220 (**20**), but not by an A_1 or A_{2A} antagonist (57). Selective A_3 receptor antagonists, including OT-7999 (**47**), have been reported to reduce intraocular pressure in both mice and monkeys (58,59).

Conclusions - Highly selective agonists and antagonists of the A_3 receptor have now been reported. The adenosine receptors are now a mature field within medicinal chemistry. The further structural refinement of these selective agents and the discovery of novel agents are anticipated to provide new substances for therapeutic use.

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Chapter 14. CCR3 Antagonists for the Treatment of Respiratory Diseases

Kathleen M. Rupprecht, Bruce Daugherty, John Mudgett and William H. Parsons
Merck Research Laboratories
P.O. Box 2000, Rahway, NJ 07065

Introduction – Bronchial asthma and allergic rhinitis are chronic inflammatory diseases characterized by the selective migration of leukocyte subtypes from the vasculature into the bronchial epithelium and nasal mucosa, respectively. One of the most striking aspects of these diseases is the accumulation of the eosinophil leukocyte (1). It was this observation that led to the search for, and subsequent identification of the molecular entities responsible for this phenomenon. One such molecule, designated eotaxin, was isolated from the bronchoalveolar lavage fluid from allergen-challenged guinea pigs, and early results demonstrated that this protein was an eosinophil-selective β-chemokine (2). Chemokines, or chemotactic cytokines, are members of a large family (>50) of small molecular weight proteins (~8-10 kD). These proteins were initially characterized by their ability to stimulate migration of distinct subsets of leukocytes in the direction of a molecular gradient (chemotaxis). Chemokines can be classified into two major subfamilies based on the arrangement of the first two conserved cysteines in the amino-terminal domain of the protein (3,4). In the α- (or C-X-C) family, these two cysteines are separated by any amino acid, whereas in the β- (or C-C) family, the first two cysteines in the protein are adjacent.

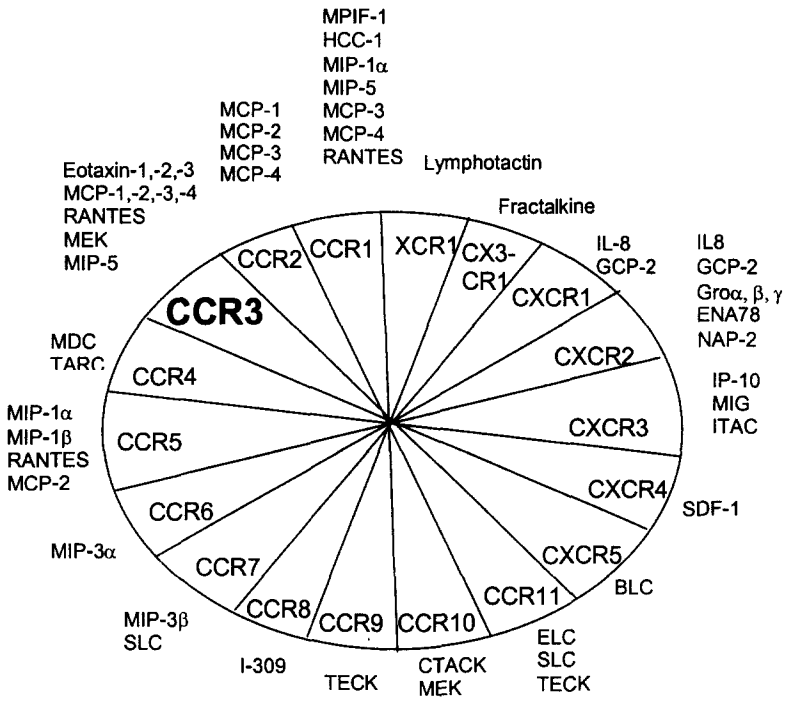


Figure 1. The Chemokine / Chemokine Receptor Family
(adapted from B.A. Premack and T.J. Schall, Nature Medicine, 2, 1174 (1996))

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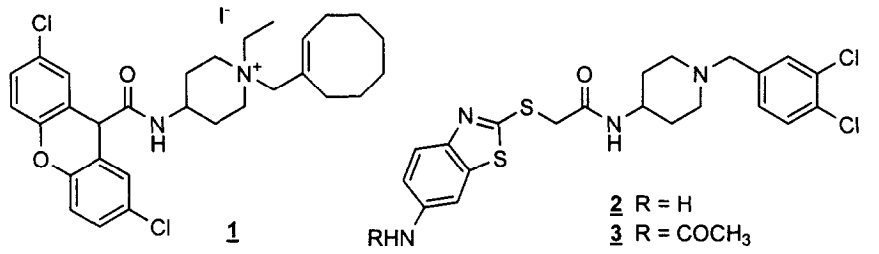
The discovery of eotaxin led to the rapid identification of its receptor on eosinophils. This receptor, designated CCR3, is a member of the G-protein coupled receptor superfamily whose members contain seven transmembrane α-helical domains (5,6). CCR3 was the third β-chemokine receptor cloned and characterized in a family of close to 20 members (3). As one of the most promiscuous chemokine receptors identified to date, CCR3 can be activated by as many as 11 chemokine agonists (7). Three of these CCR3 β-chemokine agonists, eotaxin, eotaxin-2 and eotaxin-3 are CCR3-selective while the remaining CCR3 β-chemokine agonists bind to and activate other chemokine receptors. Although the expression of CCR3 was first thought to be restricted to eosinophils, CCR3 has recently been shown to be expressed on a much wider diversity of cells involved in airway inflammation. These cells include basophils, mast cells, airway epithelial cells, and TH_2 T-lymphocytes (8-11). In addition to chemotaxis, the triggering of CCR3 on target cells with β-chemokines leads to a series of complex biochemical and physiological processes which include chemokine secretion, intracellular calcium mobilization, integrin up-regulation, mitogen-activated protein kinase activation, oxidative burst, actin polymerization, rapid shape change, and exocytosis of granule contents. These cellular activation events, coupled to their effects on target airway tissues, have been implicated in the chronic inflammation and tissue destruction observed in patients with asthma and allergic rhinitis.

Target validation for CCR3 and its β-chemokine ligands for asthma and allergic rhinitis stems from results gleaned from human studies, as well as from animal models. In human asthmatics, CCR3-active β-chemokines are elevated and markedly up-regulated upon allergen challenge (12-19). Additionally, serum levels in humans of the CCR3 prototypical chemokine eotaxin are associated with the diagnosis of asthma and clinical disease severity (20). A polymorphism in the eotaxin gene resulting in a reduction of circulating eotaxin levels has been shown to be directly correlated with an increase in lung function among asthmatics (21). Recently, in clinical trials, a monoclonal antibody directed against human eotaxin (CAT-213) administered intranasally to patients with allergic rhinitis resulted in positive effects on nasal patency and reductions in tissue eosinophils and mast cells (22, 23). In animal models, CCR3-active β-chemokines are increased upon allergen challenge (2, 24-28). In a mouse model of allergen induced airway inflammation, elimination of pulmonary eosinophils using a rat anti-mouse CCR3 monoclonal antibody resulted in an attenuation in mucus accumulation in the airways and abolished airway hyperresponsiveness to methacholine (29). Moreover, the airways of mice in which the CCR3 gene has been deleted by homologous recombination, are virtually devoid of eosinophils and the mice fail to develop airway hyperresponsiveness post allergen challenge (30). Taken together, these studies demonstrate substantial evidence for the β-chemokine/CCR3 axis playing a significant role in the clinical pathophysiology of allergic airway diseases. For this reason, CCR3 has recently become an attractive target for the development of small molecule therapeutics.

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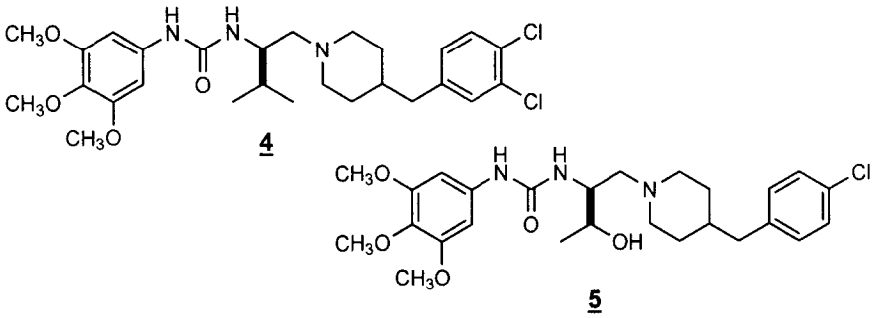
Through screening of compound collections, selective CCR3 antagonist designs containing arylpiperidine motifs linked by a tether of three to six atoms to a second aromatic group have been identified. Xanthenecarboxamide UCB35625 **1**, potently inhibits binding of ^{125}I-eotaxin to human CCR3 (IC_{50} = 0.58 nM) and ^{125}I-MIP-1α binding to human CCR1 (IC_{50} = 0.9 nM) (31). In functional studies, compound **1** inhibits eotaxin-induced chemotaxis of murine pre-B 4DEA4 cells transfected with CCR3 (IC_{50} = 93 nM) and MIP-1α-induced chemotaxis with 4DEA4 cells transfected with CCR1 (32). To improve selectivity for CCR3 and to eliminate the quaternary

ammonium group of **1**, a library designed using clustering techniques to optimize diversity identified benzothiazolethioactamides **2** and **3** (33).



Compound **2** is 800-fold selective for CCR3 (IC_{50} = 2.3 nM) *vs.* CCR1 (IC_{50} = 1900 nM). In functional studies with human peripheral blood eosinophils, compound **2** inhibits eotaxin-induced (10 nM) increases in intracellular Ca^{2+} (IC_{50} = 27 nM) and eotaxin-induced chemotaxis of human eosinophils (IC_{50} = 21 nM) (34). The acetamide **3** is more selective for CCR3 (IC_{50} = 1.5 nM) *vs.* CCR1 (IC_{50} = 5400 nM) (35).

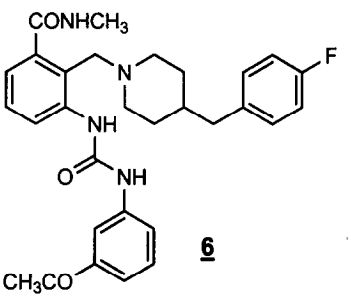
In a second variation of the arylpiperidine design, a urea group comprises a portion of the tether. RO1164875/608 **4** and RO3202947/001 **5** inhibit ^{125}I-eotaxin binding (IC_{50} = 59 nM and 1.4 nM, respectively), and eotaxin-induced eosinophil chemotaxis IC_{50} = 4.9 nM and 1.8 nM, respectively) (36-38).



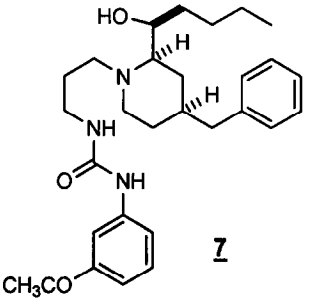
Compounds **4** and **5** have been evaluated in animal models of respiratory disease (39). In ovalbumin-sensitized mice, compound **4** (30-300mg/kg, q.d., p.o.) produced a dose-dependent reduction in bronchial aveolar lavage (BAL) eosinophils and IL-13 collected 24-72 hr after challenge. When compound **4** was administered orally (300 mg/kg) to allergen-challenged mice, there was a reduction in bronchial hyperresponsiveness to inhaled methacholine and an approximate 50% reduction in BAL eosinophils. When Cynomolgus monkeys naturally sensitive to inhaled Ascaris were treated with compound **5** (40 mg/kg, s.c.), they produced a 50% reduction in BAL eosinophils compared to vehicle-treated animals. This report demonstrated that CCR3 blockade partially inhibits eosinophil accumulation in the lung after allergen challenge.

An evaluation of the conformational constraints of the urea-containing tether of compounds **4** and **5** led to a family of compounds exemplified by **6** (IC_{50} = 1 nM) (40-45) . It exhibits functional antagonist activity in blocking eotaxin-induced eosinophil chemotaxis (IC_{50} = 1.4 nM) and is selective for CCR3 *vs.* a panel of chemokine receptors. The SAR studies demonstrated that the benzyl group spacing is important for CCR3 specificity *vs.* CCR1. Optimal conformational/tether-spacing requires ortho phenyl substitution, followed in order of potency by meta and para

spacing. The 4-fluoro substituent provides a 3 to 10-fold increase in potency. Interestingly, when some of these compounds are converted to quaternary piperidinium salts, they are transformed from functional antagonists to functional agonists, thus inducing mobilization of Ca^{2+} and chemotaxis.

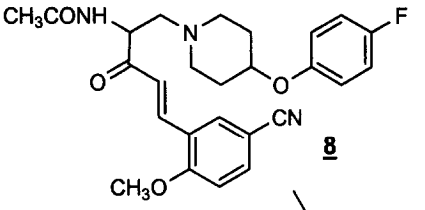


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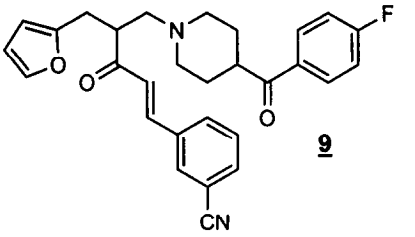


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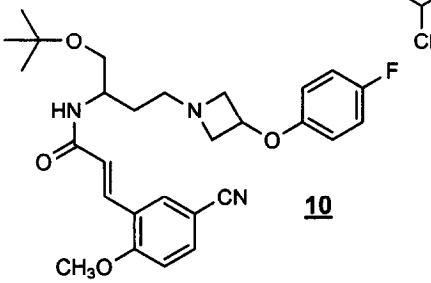
Investigation of the substitution patterns at the 2-position of the piperidine group led to the identification of compound 7 which contains a 2-pentanol substituent (CCR3 binding (IC_{50} = 1 nM) (46). The *erythro* diastereomer, the hydroxy group, and the chain length are each critical for optimal potency.



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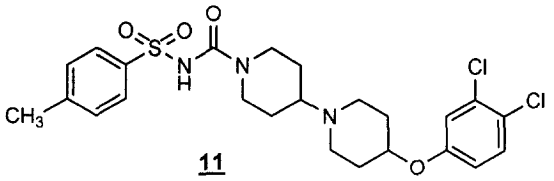


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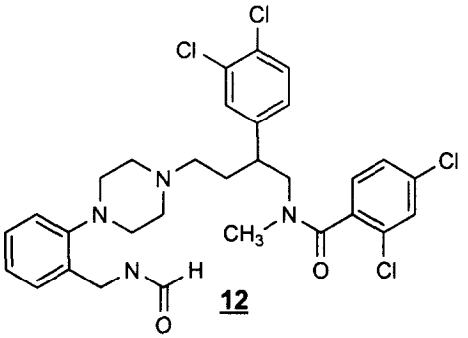
In compounds 8 and 9, conformational rigidity is obtained via a trans α, β-unsaturated (cinnamoyl) ketone. In this design, the usual methylene link between the phenyl group to the 4-position of the piperidine can be replaced by an ether linker as in compound 8 (CCR3 binding IC_{50} = 2.8 nM) or a carbonyl linker as in compound 9 (CCR3 binding IC_{50} = 2 nM) (47-49). Compound 10 offers a novel twist to this design (CCR3 binding IC_{50} = 1 nM) (50). The use of an azetidine ring in place of a piperidine ring suggests that the latter is not required. To compensate for the smaller ring, the backbone is extended and the cinnamoyl group is switched from a ketone to an amide connection.



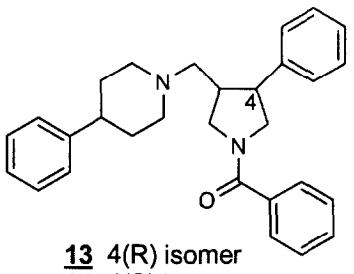
11

Compound **11** represents a combination of several designs incorporating a sulfonylurea group and a tether constrained in a second piperidine ring (51-54). Analogs from this series are functional antagonists as measured by their effects on eotaxin-induced Ca^{2+} flux in human eosinophils and their ability to antagonize the eotaxin-induced chemotaxis of human eosinophils (55).

Piperazine analog **12** was identified as an inhibitor of eotaxin binding in AML-14 cells transfected with human CCR3 (56). Both enantiomers were similarly potent *vs.* CCR3 (S-isomer IC_{50} = 60 nM, R-isomer IC_{50} = 20 nM), but were not selective *vs.* other targets, nor did they exhibit pharmacokinetic profiles suitable for in vivo evaluation. Conformational rigidification by linking the N-methyl group with various carbons of the alkyl tether identified a trans pyrrolidine design in which 4(R) isomer **13** is selective for CCR3 (IC_{50} = 710 nM, CCR5 IC_{50} = >10,000 nM) and 4(S) isomer **14** is selective for CCR5 (IC_{50} = 14nM, CCR3 IC_{50} = >10,000 nM) (57).

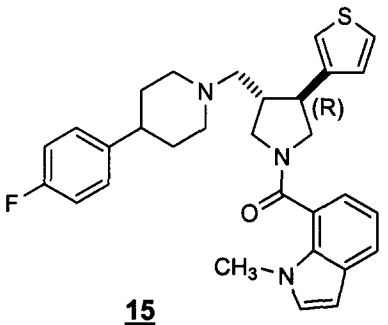


12

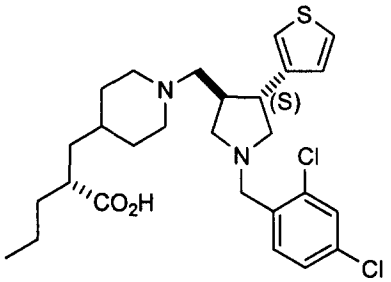


13 4(R) isomer
14 4(S) isomer

Systematic modification at the N1, 3, and 4 positions of this design led to the potent and chemokine-selective analog **15** (CCR3 IC_{50} = 0.7 nM) (57-61). To improve potency, and PK properties and to reduce off-target activity, three further modifications were made. Replacement of the C4 phenyl group with a thiophene group and the amide carbonyl linker with a methylene linker led to a significant enhancement in CCR3 potency. Interestingly, when the latter switch is made the stereochemistry at C4 required for CCR3 selectivity switched from (R) to (S). Replacement of the phenyl group on the piperidine ring with a 2-carboxypentane group led to significant reduction of off-target activity (62). Compound **16** exemplifies this improved design. Compound 1**6** is a potent selective CCR3 antagonist (CCR3 IC_{50} = 2.1 nM, CCR5 IC_{50} = >10,000 nM) and antagonist of human eosinophil chemotaxis (IC_{50} = 28 nM). It exhibits a suitable PK profile for *in vivo* studies (oral bioavailability in rats = 40%). In preliminary studies, compound **16** demonstrated efficacy in a model of eotaxin-induced dermal eosinophil accumulation in Rhesus monkeys (57).

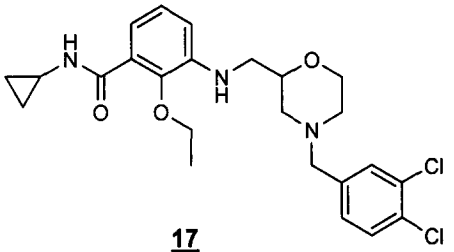


15

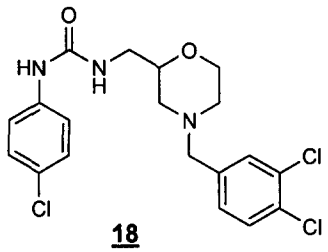


16

CCR3 antagonists where the piperidine ring is replaced by a morpholine are exemplified by compounds **17** and **18** (63-64). Compound **17** Is a potent inhibitor in the CCR3 binding assay (IC_{50} = 4.1 nM). In ovalbumin-sensitized guinea pigs, compound **18** (CCR3 binding IC_{50} = 660 nM; 0.2-20 mg/kg, p.o.) produced inhibition of lung eosinophilia and bronchial hyperreactivitiy (64).

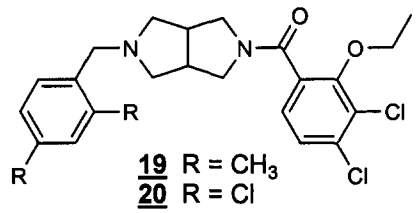


17



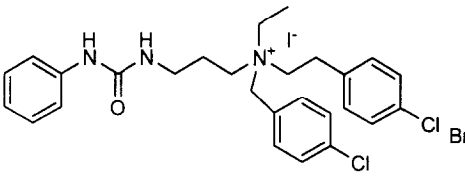
18

A novel structural class comprising a pyrrolo-[3,4-c]pyrrolidine tether/linker is illustrated by compounds **19** and **20** (65). The selectivity of this series was evaluated for inhibition of eotaxin-induced chemotaxis in murine pre-B cells transfected with CCR3 and in MCP 1-induced chemotaxis in THP-1 monocytes. In general, compounds where R = CH_3 were selective for the CCR2 receptor and compounds where R = Cl were selective for the CCR3 receptor. Analogs of **20** completely blocked the binding of eotaxin to the CCR3 receptor in murine pre-B cells transfected with CCR3 at 1 uM.

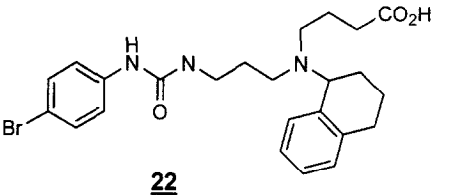


19 R = CH_3
20 R = Cl

Urea analogs **21** and **22** that completely block eotaxin-induced chemotaxis at 10 uM concentrations, suppress type II collagen-induced arthritis in mice at 50 mg/kg s.c. and 20 mg/kg *s.c.*, respectively (66). In ovalbumin-sensitized mice, compound **21** (50 mg/kg, *i.p.*) produced a reduction in bronchial hyperresponsiveness to injected acetyl choline and an approximate 50% reduction in BAL eosinophils.

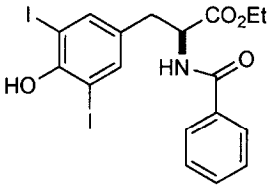


21

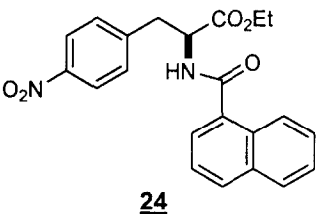


22

Tyrosine analog SK&F 45523 **23**, was identified in a FLIPR-based screen measuring effects on eotaxin-induced Ca^{2+} flux in RBL-2H2 cells transfected with human CCR3 (67). Compound **23** inhibited binding of ^{125}I eotaxin to human eosinophils with an IC_{50} = 800 nM. Elaboration of the design led to SB-328437 **24** which is a potent antagonist in the eosinophil binding assay (IC_{50} = 4.5 nM), and is a functional CCR3 antagonist since it inhibits eotaxin–induced Ca^{2+} flux in human eosinophils (IC_{50} = 35 nM) (68). Moreover, compound **24** is at least 250-fold selective over a panel of other chemokine receptors. More recent patent applications claim compounds where the ester group of **24** is replaced by less metabolically labile dihydrooxazole amide and hydrazino groups (69-71).



23



24

Conclusion – There is strong evidence linking eosinophil recruitment and activation in the lung with bronchial asthma and allergic rhinitis. More recently, the chemokine receptor CCR3 has been identified as a potentially critical receptor in respiratory migration of eosinophils (5,6). Since then, significant progress has been made to identify potent, small molecule antagonists of CCR3. Interestingly, most CCR3 antagonist designs possess a basic nitrogen. This phenomenon is also found in many compounds which bind to other receptors in the chemokine family. Based on mutagenesis studies of CCR2 and CCR5, it was postulated that the basic nitrogen present in these designs interacts with a glutamic acid in transmembrane helix 7 (72, 73). This amino acid is present in CCR3 in this region as well. In the new era of targeted therapy for respiratory diseases, it remains to be determined in anticipated clinical studies whether CCR3 antagonists will fulfill their therapeutic potential.

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Chapter 15. Emerging Opportunities for the Treatment of Inflammatory Bowel Disease

Jeremy D. Gale[1], Kim F. McClure[2] and Nick Pullen[1]
[1] Pfizer Global Research and Development, Sandwich, Kent, CT13 9NJ UK
[2] Pfizer Global Research and Development, Groton, CT 06340, USA

Introduction - Inflammatory Bowel Disease (IBD), in particular Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and relapsing condition, characterised by an uncontrolled inflammatory response of the gastrointestinal tract. This leads to compromises in epithelial integrity, transmural inflammation, bleeding, diarrhoea and pain. However, in their clinical presentation, immunological bias (Th1/Th2) and genetic pre-disposition, CD and UC are distinct, requiring different medical and surgical management (1, 2). Whilst CD can present in any location between mouth and anus, UC is restricted to the large bowel, often initially confined to the most distal regions and advancing proximally. CD lesions are focal, penetrating the full thickness of the bowel and leading to perforation and fistulae, whereas UC lesions are generally more continuous and superficial in nature.

Many hypotheses have been developed to explain the underlying disease pathophysiology of IBD. The observation that patients have increased mucosal permeability which may lead to chronically-enhanced immune responses to enteric bacterial antigens and abrogated self-tolerance is common amongst them. This commensal relationship between gut pathogens and IBD is also reflected by the transient improvement observed in CD patients when given antibiotic/probiotic therapy. Mutations of one gene in particular, *NOD2*, appear to be responsible for the exacerbated inflammatory responses to enteric bacteria in some individuals (3, 4). *NOD2* serves as an intracellular receptor for bacterial lipopolysaccharide and is linked to the activation of the transcription factor, NF-κB. However, only approximately one fifth of CD cases can be attributed to the *NOD2* mutation and there does not appear to be any linkage between mutations in the *NOD2* gene and UC. As a target cluster, the NF-κB pathway has long been centre stage in the development of anti-inflammatory or immunosuppressive approaches for the treatment of IBD. NF-κB activation and nuclear translocation is increased in lesions from IBD patients, as are the numerous inflammatory cytokines (including TNFα, IL-1β, IL-6, IL-8), adhesion molecules (ICAM-1, VCAM-1 and MAdCAM-1) and enzymes involved in inflammation, such as inducible nitric oxide synthase and cyclooxygenase-2, whose transcription is mediated by NF-κB. Dysregulated expression of a number of NF-κB target genes, especially TNFα, has been implicated in the pathogenesis of IBD, leading to the development of several therapeutic agents that target this axis (see Table 1).

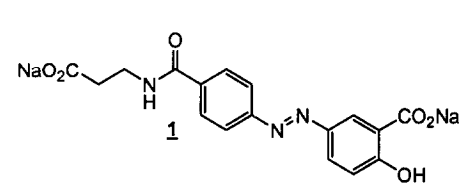
The goal of therapeutic management of IBD is to treat the active symptomatic disease of all patients and to restore mucosal integrity and function. To delay the recurrence of acute exacerbations, it is essential to provide a well-tolerated and safe maintenance therapy. A significant proportion of CD and UC patients become refractory to standard treatment as the disease progresses, necessitating the use of powerful immunosuppressant drugs, such as azathioprine, methotrexate or 6-mercaptopurine. Bowel resection may become necessary. There is a need to identify superior agents that are both more clinically efficacious and have a safer side-effect profile than current mainstay therapies. This review will outline recent progress on the characterisation of new chemical entities and delivery systems that have the potential to favourably impact on the management of IBD.

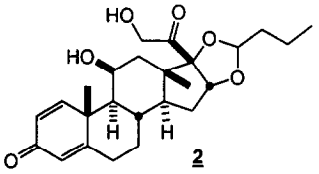
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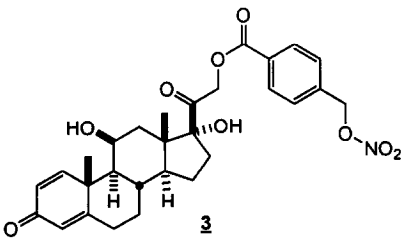
FIRST-LINE PHARMACOTHERAPY

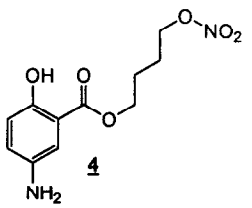
The founding member of the 5-aminosalicylate drugs, 5-ASA's, was sulfasalazine, a chemical combination of sulfapyridine and 5-ASA. These agents (*eg.* mesalazine and olsalazine) are first-line therapy for the treatment of patients with mild to moderate UC and CD, and as a maintenance therapy to prevent disease relapse in UC. For disease in the distal bowel, multiple delayed- and sustained-release formulations, as well as pro-drugs such as olsalazine, have been designed to release the majority of an oral dose directly in the distal ileum/colon, thus preventing topical exposure in the proximal small intestine. The most recently introduced pro-drug, balsalazide (1) contains azo bonds that are cleaved by colonic bacterial azo-reductases and release the active 5-ASA, mesalazine, locally. For rectal disease, rectal foams are also administered. The mechanism by which the 5-ASA's exert their anti-inflammatory activity appears to be, in part, by inhibiting the activation of NF-κB (5).

Mild disease can be treated with 5-ASA's, but many patients eventually require corticosteroids to control symptoms. Glucocorticoids (*eg.* prednisolone) are some of the most effective therapies for inducing clinical remission in patients with active CD and UC when used for short periods of treatment. However, adverse effects (incl. obesity, osteoporosis, hypertension and adrenal suppression) as well as the fact that chronic dosing invariably culminates in development of clinically-challenging, steroid-refractory disease, limits their use. Moreover, significant numbers of patients are unable to discontinue glucocorticoid therapy without disease exacerbation. Budesonide (2) is a second-generation corticosteroid and is available as a controlled-release oral formulation for distal CD and as an enema for topical treatment in UC. Budesonide has an extensive first-pass metabolism and lower systemic bioavailability (~11%), enabling it to achieve similar efficacy to prednisolone, but with significantly fewer glucocorticoid-related side effects. Given the higher receptor affinity of budesonide and that as a CYP3A4 substrate, plasma levels can be substantially elevated in the presence of CYP3A4 inhibitors (*eg.* ketoconazole), the outcomes of long-term safety studies are awaited to confirm budesonide's safety profile compared with other corticosteroid therapies. Budesonide is a good illustration of how engineering features into a molecule can limit the potential for systemic side effects.









Further attempts have been made to chemically-modify these agents to increase their efficacy or reduce adverse events associated with their use. Specifically, an NO donor-containing derivative of prednisolone, NCX-1015 (3), has been shown to have superior efficacy, compared to the parent molecule in the mouse TNBS colitis model (6). Similarly, an NO-releasing form of mesalazine, NCX-456 (4), has superior efficacy to the parent molecule in the rat TNBS colitis model (7). Notwithstanding these recent developments, there appears to be limited scope to extend existing approaches to overcome their limitations. Thus, this article will focus on emerging mechanisms for which there is rationale for activity in IBD.

HARNESSING GENERIC IMMUNOMODULATORY MECHANISMS

Numerous animal studies have underscored the role of NF-κB-reporter genes in contributing to the inflammatory responses that result in tissue destruction. A number of pioneering biological approaches have emerged from this characterisation (Table 1). However, for the majority of these approaches, there is currently limited substrate for medicinal chemistry intervention.

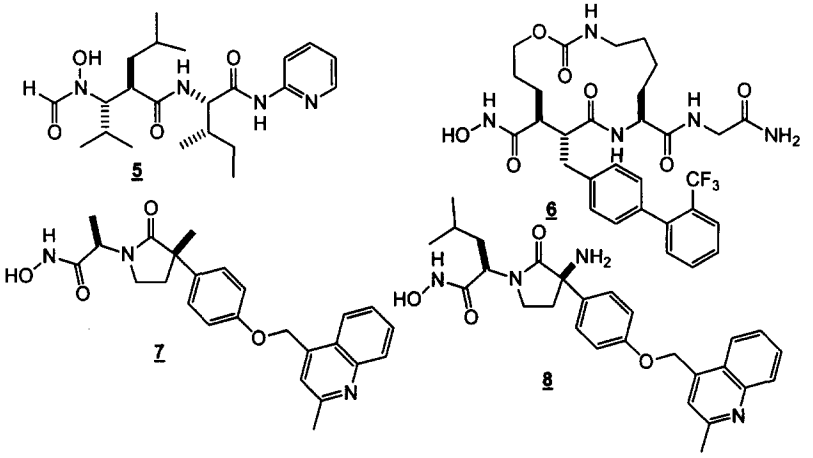
Target / Pathway	Pre-clinical Data	Example Clinical Agent	Selected Clinical Data	Reported Development Status
Inhibition of lymphocyte adhesion & recruitment				
α4/MAdCAM-VCAM	(8, 9)	Anti-α4 hmAb (Natalizumab/Antegren)	(10, 11)	III (UC & CD)
α4β7/MAdCAM	(12, 13)	Anti-α4β7 hmAb (LDP-02/MLN-02)	(14)	II (UC & CD)
ICAM/LFA-1	(15)	ICAM antisense (Alicaforsen/ISIS 2302)	(16, 17)	III (UC & CD)
Immunomodulation				
Anti-TNFα	(18)	Humanised IgG4 mAb (CDP-571/Humicade)	(19, 20)	III (UC & CD)
		Pegylated Fab fragment (CDP-870)	(21)	III (CD)
		Chimeric IgG1 mAb (Infliximab)	(22-25)	Launched for CD; UC in Ph III
		Fully-human mAb IgG1 (Adalimumab / D2E7)	(26)	Launched for RA (Ph II/II for CD)
		TNFα anti-sense (ISIS 104838)		II (CD)
		Soluble p55 TNF-receptor (Onercept)	(27)	II (CD)
		Soluble TNF-receptor-Fc fusion (Etanercept)	(28)	Launched for RA
Anti-IL-2 receptor	(29)	humanised mAb (Daclizumab)	(30)	Launched for transplant rejection (Ph II for UC)
Anti-IL-6 receptor	(31)	humanised IgG1 mAb (MRA)	(32)	II (CD)
IL-10	(33)	rhuIL-10 (Tenovil/Ilodecakin)	(34, 35)	Discontinued for CD
IL-11	(36)	rhuIL-11 (Oprelevkin)	(37)	Launched for thrombocytopenia (Ph III for CD)
IL-12	(38)	fully human mAb (J695)		II (CD)
Anti-CD40	(39)	mAb (TNX-100/5-D12)		II (CD)
Anti-OX40L (CD134)	(40)	mAb and OX40-IgG fusion protein		
GM-CSF		hrGM-CSF(Sargramostin)	(41)	II (CD)
Anti-IFNγ		humanised mAb (fontolizumab)		II (CD)
Anti-MIF	(42)			
p38/JNK	(43)	decapeptide (RDP58)		II (CD and UC)

Table 1. Clinical development of biological agents with potential for the treatment of IBD.

Anti-TNFα Biologicals - The first biological approach to be approved for the treatment of steroid-refractory severe CD and fistulating disease was the chimeric anti-TNFα mAb, Infliximab (44). Even, as an *i.v.* infusion, this agent has had remarkable market penetration and has recently been additionally indicated as therapy for the maintenance of remission. However, there are a number of long term safety concerns associated with Infliximab, both in terms of the high degree of immunogenicity it elicits and that systemic TNFα suppression has been linked to an increased incidence of non-Hodgkin's lymphoma and opportunistic bacterial infections (*eg.* tuberculosis) (45). The efficacy observed with Iinfliximab has validated the

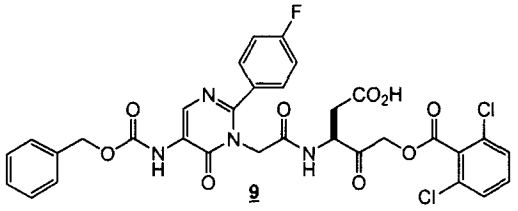
inhibition of the TNFα axis in the treatment of IBD and, as a result, a number of other anti-TNFα agents are either approved or in development (Table 1), offering potential improvements in side effect, drug delivery and long term clinical efficacy. However, caution needs to be exercised as recent clinical data with etanercept, a human soluble TNF receptor-Fc fusion protein have been disappointing, raising the possibility that not all anti-TNFα approaches will have predictable levels of efficacy (28).

TNFα-converting enzyme (TACE) – From the clinical precedence set by inhibitors of the TNFα axis, the development of TACE inhibitors have emerged. TACE is a membrane-bound zinc endopeptidase and, unlike the action of anti-TNFα mAbs, small molecule TACE inhibitors exert their effects by limiting the shedding of soluble TNFα from its active membrane-bound precursor. The concept that TACE inhibition is potentially a small molecule alternative to the anti-TNFα biological agents has been recognized for a variety of inflammatory conditions, including IBD (46). A potential disadvantage, however, is that only the soluble form of TNFα is controlled by TACE inhibition and the membrane-bound form retains its activity. Nevertheless, *ex vivo* experiments have demonstrated that a TACE/MMP inhibitor, but not the MMP inhibitor Trocade (Ro-32-3555), effectively blocks TACE activity in mucosal biopsies taken from UC patients (47). Although the TACE inhibitor was not exemplified in this publication, there are several TACE inhibitors that have been described. The *N*-hydroxy formamide TACE inhibitor, GW-3333 (**5**), has been shown to be as effective as a neutralizing anti-TNFα antibody in a rat adjuvant arthritis model (48). GW-3333 has additional activity at other MMPs that could limit its utility as an oral agent, but, as measurable increases in MMP-1-3 activity also correlate with the destruction of mucosal tissue in IBD patients, a non-selective TACE/MMP inhibitor, engineered for topical delivery and low systemic exposure, could have significant advantages over both oral TACE and biological anti-TNFα approaches. While **5** is not very selective for TACE over other MMPs, compounds with better selectivity, such as **6** and **7**, have been reported (49, 50). IK682, (**7**), is selective for TACE and has favourable pharmacokinetics in rat and dog (51). Optimisation of these lactam structures for improved cellular potency led to the clinical candidate (DPC-333/BMS-561392, **8**) at the expense of some MMP selectivity (52). In single dose Phase I trials, **8** is well tolerated and has a short mean half-life at doses <80 mg but a longer half-life (6 h) at higher doses (53). Although long-term safety data have not yet revealed any problems with arthralgia or myalgia, these musculoskeletal side effects have been a common problem with non-selective hydroxamate-based MMP inhibitors (54).

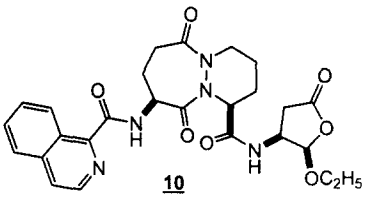


Interleukin-1β Converting Enzyme (ICE) - IL-1β and IL-18 are up-regulated in colonic tissue from CD patients and play a pivotal role in effecting Th1 and macrophage responses; significantly, neutralisation of IL-18 activity ameliorates the inflammation observed in animal models of colitis (55, 56). IL-1β and IL-18 production is determined by the protease activity of the IL-1β converting enzyme, ICE/caspase-1.

While other small molecule approaches to IL-1β inhibition have been reported (57), ICE has received the most attention. Two distinct ICE inhibition strategies designed to mimic the YVAD cleavage sequence of pro-IL-1β have been described: irreversible inhibitors, characterized by **9**, or reversible ones such as pralnacasan (**10**). Pre-clinically, chronic administration of dextran sodium sulfate (DSS) to ICE knockout mice fails to induce colitis, an effect accompanied by reduced cell activation in the draining mesenteric lymph nodes and a significant reduction in colonic IL-18, IFNγ, and IL-1β (58). Pralnacasan, an orally active ICE inhibitor, is currently in Phase II development for RA. In an acute DSS-induced mouse model of colitis, pralnacasan administration (100 mg/Kg i.p, q.d.) resulted in significant amelioration of disease activity (59).

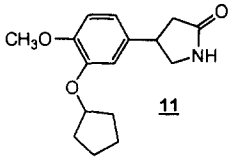


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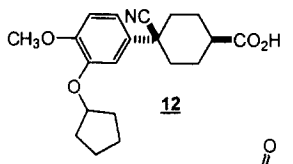


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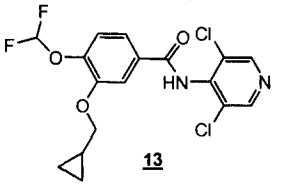
PDE4 - A decrease in intracellular cAMP following co-stimulation of CD3/CD28 is one of the key triggers for T-cell activation. PDE4 and PDE7 appear to be the predominant cAMP-dependent phosphodiesterases in inflammatory cells, and either inhibition of PDE4 activity or PDE7 expression reduces T-cell activation and the production of pro-inflammatory cytokines (60). The therapeutic potential of non-emetic PDE4 isotype-selective inhibitors for COPD and asthma has been advocated, but to date no compound has yet reached the market in spite of the fact that over a dozen inhibitors from a variety of structural series have advanced to clinical trials (59). Representative PDE4 inhibitors, including rolipram (**11**), cilomilast (**12**), roflumilast (**13**), arofylline (**14**), and lirimilast (**15**) and the full range of inhibitors along with new chemical series has recently been reviewed (61). In rodent models of colitis, efficacy for rolipram and arofylline has been reported. In particular, rolipram (5 mg/kg, b.i.d, i.p.) was able to halt the progression (but not the onset) of colitis in a DSS mouse model (62-64). The effect of rolipram in mice with existing DSS-induced colitis resulted in faster recovery after DSS discontinuation than for untreated animals. Structural differences in the reported series of PDE4 inhibitors notwithstanding, the common side effect of nearly all PDE4 inhibitors is emesis which narrows the therapeutic index. The development of non-emetic PDE4 inhibitors awaits a more complete understanding of the underlying causes of emesis or potentially through the design of novel PDE4 isotype-selective agents.



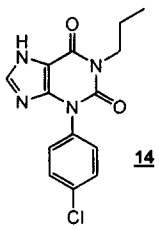
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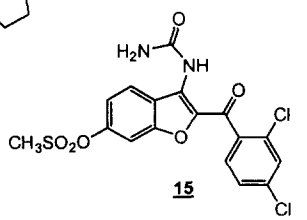
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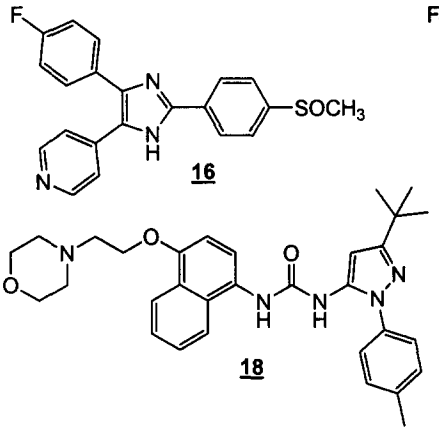


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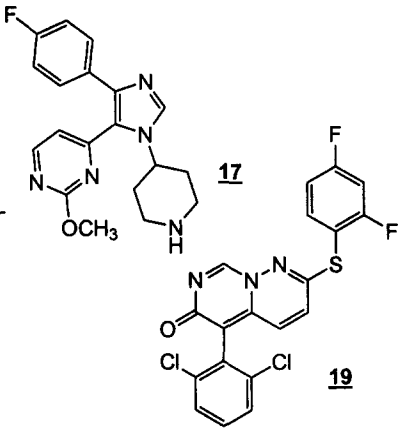
To circumvent the known side effects and short (2-3 h) half-life of rolipram in humans, a sustained release nanoparticle formulation that specifically targets the colon has been developed (65). In a rat TNBS-induced model of colitis, this formulation was as effective as dosing a rolipram solution (66). However, on discontinuation of solution dosing, treated animals relapsed, whereas the animals

given nanoparticles showed only a slight increase in the severity of their clinical scores. Significantly, the nanoparticle-treated animals showed fewer side effects associated with systemic exposure than animals dosed with a rolipram solution, as judged by the established neurotropic indicators of grooming and forepaw shaking.

p38 - Similarly, against the known efficacy of 5-ASAs and glucocorticoids, a number of other intracellular targets that modulate the NF-κB pathway have emerged. One of the most well-progressed approaches is in the design of selective p38 MAPK inhibitors. p38 signals downstream of the IL-1β receptor and LPS/TLR to regulate, in part, the transcription/translation of pro-inflammatory cytokines, including IL-1β, IL-6 and TNFα (67-69). *In vivo,* p38 MAPK inhibitors have shown activity in both acute and chronic models of inflammation (70, 71). There have been equivocal data reported on the pre-clinical efficacy of the founding p38 inhibitor, SB-203580 (**16**), in a model of TNBS-induced colitis (72). In a separate study, 12 patients with severe CD were administered CNI-1493 (25 mg/kg/day *i.v.*), a tetra-guanylhydrazone, which is reportedly a dual p38/JNK inhibitor (73). Although treatment was only 12 days, 42% of patients were in clinical remission after 8 weeks and all but one patient showed endoscopic improvement. As p38 and JNK are known to be activated in the inflamed mucosa of IBD patients only the use of more selective p38 inhibitors will be able to de-convolute the relative importance of p38 in IBD. This is also an issue for the experiments conducted with the pyridyl-imidazole SB-203580, as the compound is less than 10-fold selective for p38α over JNK (74, 75). By comparison, the recently described pyrimdyl-imidazole p38 inhibitor SB-242235 (**17**) is reportedly much more selective and does not inhibit JNK up to 10 μM (76). SB-242235 is also orally active and effective in models of LPS- and adjuvant-induced arthritis. Pharmacokinetic endpoints for SB-242235 have been described for pre-clinical species and the mean human half-life is reported to be 16.4 h (77). In a human *ex vivo* assay, greater than 75% inhibition of TNFα and IL-1β expression was achieved after 3 h at doses greater than 150 mg (78).



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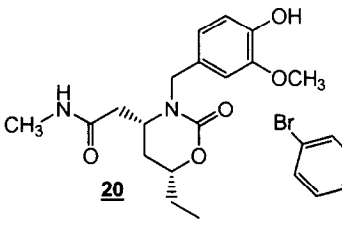
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At least two other p38 inhibitors have recently been reported to be in human clinical trials. BIRB-796 (**18**), a diaryl urea-based p38 inhibitor is in Phase II clinical trials for RA (79). Although BIRB-796 is reported to have excellent kinase selectivity, it is only so by virtue of the exquisite potency for p38α (K_d = 0.1 nM) as the IC_{50} for JNK inhibition is 0.1 μM (80). The low K_d is estimated to translate to a 23 hr off-rate and a long pharmacodynamic end-point, beyond the 6-10 hr elimination half-life (81). In a human endotoxemia model, inhibition of TNFα was observed with 50 mg doses of BIRB-796 (82). VX-745 (**19**) is another selective p38 inhibitor that has shown reductions in ACR_{20} response scores relative to placebo in a 12 week Phase II RA (83). While clinical trials have been suspended with VX-745, it would serve as a useful tool in establishing the role of p38 in IBD. Although detailed safety data is not available, a common reported issue with p38 inhibitors is the elevation of aspartate and alanine aminotransferase activity. Development of p38 inhibitors that are specifically delivered to the mucosal lining of the intestine for the treatment of IBD may avoid these unwanted effects in the liver.

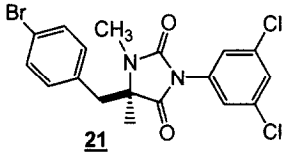
TARGETING LYMPHOCYTE RECRUITMENT TO THE INFLAMED BOWEL

Nearly 30% of patients with steroid-refractory CD do not respond to infliximab. In addition, infliximab has not yet demonstrated clinical efficacy in treating UC (84). Other immunomodulatory approaches, such as recombinant IL-10 or IL-11 (Table 1), have also demonstrated modest clinical efficacy. Other approaches in development may better address these shortfalls in potency and side effect profiles.

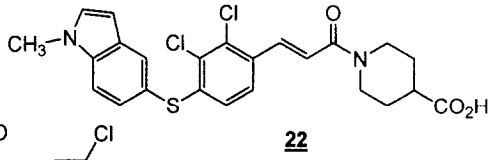
LFA/ICAM – Common between CD and UC is the recruitment of large numbers of activated CD4+ lymphocyte to the gut lamina propria and associated lymphoid tissue (85). As a general mechanism of inhibiting T-cell recruitment, the LFA/ICAM interaction has received much clinical attention through ISIS-2302/alicaforsen. ICAM expression increases substantially in the disease state. ISIS-2302/alicaforsen is an anti-sense oligonucleotide that inhibits ICAM mRNA and expression, but the results of larger clinical trials have been disappointing (see Table 1) (86). However, the finding that lovastatin targets an allosteric site on LFA-1, inhibiting its binding to ICAM, has offered new opportunities for inhibiting this axis with small molecules. The development of additional mevinolin derivatives has been recently reviewed, but, of note, the modification of the integral lactone of lovastatin to cyclic carbamates, such as **20**, improved affinity (IC_{50} = 50 nM) with equivalent activity as an anti-LFA mAb in some inflammatory models (87). Further series of compounds have developed from HTS screening, including the 5-substituted-3-phenylhydantoin derivates, such as BIRT-377, **21** (K_d = 25.8 nM) and the diaryl sulphides , represented by **22** (IC_{50}= 6 nM). There are little data reported on the potency of these compounds *in vivo*, although the encouraging effects of **22** on allergen-induced eosinophilia and LPS-induced neutrophil migration are suggestive that benefits might also be expected in animal models of colitis (88).



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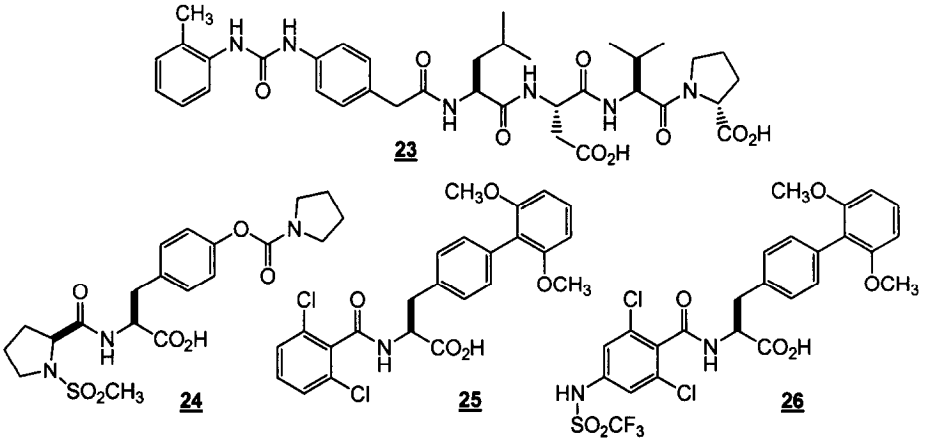


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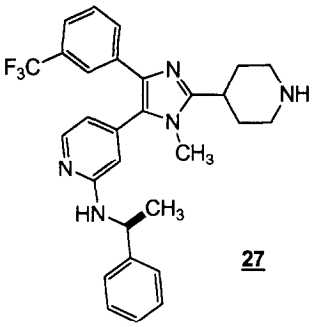
α4β7/MAdCAM - A specific system whereby lineages of lymphocytes bearing the expression of the α4β7 integrin were able to specifically home to the gut by engaging the mucosal addressin, MAdCAM has been described (89). MAdCAM is uniquely expressed on high endothelial venules supporting on the gut lamina propria and associated lymphoid tissue. The expression of MAdCAM increases tremendously at sites of inflammation, leading to an elevation in the recruitment of α4β7+ lymphocytes (90). Blocking anti-MAdCAM or anti-α4β7 antibodies are effective at blocking the rolling and adhesion of lymphocytes to the gut endothelium and reduce inflammation in animal models of colitis (13, 91). Humanised monoclonal antibodies natalizumab and LDP-02 that block the α4β7/MAdCAM interaction are in clinical development for UC and CD (Table 1). By inhibiting the recruitment of key inflammatory cells these agents have the potential to improve IBD symptoms and re-balance oral tolerance without inducing systemic immune suppression.

In terms of small molecule antagonists most of the work described in the literature has been directed at the related integrin α4β1 (or very late antigen-4, VLA-4) and its interactions with VCAM (92-95). As a byproduct of this work on selective α4β1 antagonists, compounds with good potency against α4β7 have also been generated. One of the earliest of these was BIO-1211 (**23**), with an IC_{50} <1μM against α4β7. Compound **24** is even more potent, with inhibition of α4β1 (IC_{50} = 0.5 nM) and α4β7 (IC_{50} = 4.4 nM) (96). Another is the biphenylalanine derivative **25**, TR-14035 (α4β1 IC_{50} = 7 nM; α4β7 IC_{50} = 87 nM) (97). In the mouse, intravital

microscopy has been used to show that TR-14035 (10 mg/Kg *i.v.*) blocks the binding of $\alpha4\beta7^+$ cells to MAdCAM in Peyer's patches (98). Pharmacophore modeling has been used to optimize the selectivity of these dual $\alpha4\beta1/\alpha4\beta7$ antagonists, producing highly selective compounds with only minor changes to the structure of **25** (99). Compound **26** with the additional trifluoromethansulfonamide is nearly 400-fold selective for $\alpha4\beta7$ relative to $\alpha4\beta1$ (IC_{50} = 0.5 nM, 192 nM, respectively). Although these integrin antagonists achieve desirable *in vitro* activities, as peptidomimetics they generally have poor bioavailability *in vivo* (96).



Delivery Systems – In reviewing IBD therapies, a discussion of delivery systems is essential. While the subject of colon-specific drug delivery has been reviewed elsewhere, it is worthwhile enumerating some of the current approaches and molecular properties that are desirable for drug delivery (100-102). Some of the major drug delivery strategies are: 1) pro-drug based approaches, such as that used by balsalazide and relying on bacterial azoreductase activity to release 5-ASA at the site of action; 2) pH sensitive coatings such as Eudragit®, made of aminoalkyl methacrylate copolymers that release the active agent at various pHs; 3) time release coatings that release the drug after a given period of time, usually determined by the coating thickness; 4) microflora methods that exploit the action of colon-specific enzymes (glycosidases, azoreductases) on the core coating. Each has their own limitations, including variations in the location of the disease within the gastrointestinal tract, differences in pH between the small intestine and the colon, and large differences in gastric retention times and colonic enzyme activity. With perhaps the exception of PDE4, none of the molecules for the newer approaches discussed herein have been specifically designed to work with a delivery system. Of the approaches described above, TACE, ICE and $\alpha4\beta7$ inhibitors are pre-disposed for use with delivery systems as they rely on peptidomimetic antagonists. The disadvantages of a peptidomimetic for oral drugs (high clearance and poor absorption) are potential advantages for topical or locally delivered therapy - less potential for side effect due to systemic exposure. The molecule will require a degree of epithelial absorption and the little drug that does enter systemic circulation will have to be rapidly metabolised to pharmacologically inactive, non-toxic metabolites. While many of these attributes are intrinsic to a peptidomimetic, approaches such as PDE4 and p38 are still amenable to local delivery, a strategy that could overcome some of the safety and toxicity concerns associated with these targets. Indeed, research programs that search for orally bioavailable agents readily identify compounds with the desired biological properties, but with very short systemic half-lives and/or poor absorption. If not, building in metabolic liabilities into molecules and reducing absorption by adding molecular weight and/or polar groups is something that can be achieved with regularity by medicinal chemists. In the case of p38, the large molecular weight (505), lipophilic (ClogP= 5.5), potent (p38α IC_{50} = 0.1 nM), and selective inhibitor, L-790,070 (**27**) may be a good starting point (103).



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Summary IBD is a highly morbid condition where, despite recent innovations, significant unmet medical need remains. The development and approval of Infliximab has not only been a valuable addition to the armamentarium of physicians, but has validated a pathway which may in the future prove highly exploitable with small molecules. A number of generic anti-inflammatory or immunomodulatory pathways have been identified and the use of imaginative medicinal chemistry or pharmaceutical science may allow their power to be harnessed in such a way as to ameliorate the impact of side effects. In addition, emerging data from a variety of novel biological agents may reveal a number of new targets, thus opening a new chapter in therapy for this problematic condition.

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Chapter 16. TNF-α Converting Enzyme (TACE) as a Therapeutic Target

Jerauld S. Skotnicki and Jeremy I. Levin
Wyeth Research
Pearl River, New York 10965

Introduction – TNF-α is a pleiotropic pro-inflammatory cytokine produced by a variety of cell types (1,2). It is initially expressed on the cell surface as a 233-amino acid 26 kDa membrane-bound precursor protein. Proteolytic cleavage of membrane-bound TNF-α by TNF-α converting enzyme (TACE) occurs between Ala-76 and Val-77 (3-5). The resulting mature, soluble cytokine of 17 kDa exists as a non-covalently bound trimer. The biological effects of TNF-α are mediated by two type I membrane-bound receptors, designated as p55 and p75.

Overproduction of TNF-α is associated with a number of pathological conditions including Crohn's disease, diabetes, multiple sclerosis, ulcerative colitis and congestive heart failure (6-9). Furthermore, there are compelling data to support the critical role that TNF-α plays in rheumatoid arthritis (10). In addition to inducing the release of cell adhesion molecules and other cytokines such as IL-1β and IL-6, TNF-α simulates the release of connective tissue degrading enzymes such as the matrix metalloproteinases. Elevated levels of TNF-α are observed in the synovial fluid of rheumatoid arthritis patients and these levels correlate with disease severity (11). Two clinically effective biologic agents that target TNF-α levels, etanercept (Enbrel®), a soluble TNF-α receptor-Fc dimer, and infliximab (Remicade®), an anti-TNF-α antibody, validate TNF-α as a therapeutic target. Blocking the production or action of this cytokine with orally bioavailable small molecules provides an alternative to protein therapeutics (12,13). In particular, inhibition of TACE is a major focus of drug discovery research.

TACE is an endopeptidase and member of the ADAM (a disintegrin and metalloprotease, ADAM-17) family of enzymes (3-5,14). It is a type I transmembrane protein consisting of an *N*-terminal pro-domain, a zinc-dependent metalloproteinase catalytic domain, a disintegrin domain, an EGF repeat, a transmembrane domain and a cytoplasmic tail. TACE has a furin cleavage site between the pro and catalytic domains. The catalytic domain of TACE contains the consensus HExxHxxGxxH motif, characteristic of the metzincins. The isolation, purification and cloning of TACE have been reported (15,16). Early observations that matrix metalloproteinase inhibitors block TNF-α release from cells aided in the identification of this sheddase (17-19). The crystal structure of the catalytic domain of human TACE has been solved (20). Though sharing characteristics with the matrix metalloproteinases, the distinguishing and unique feature of TACE is a deep S3' pocket merging with the S1' specificity pocket. Knowledge of structural features of the active site of TACE gained from X-ray crystallography provides a framework for inhibitor design and the generation of compounds with high affinity for the enzyme.

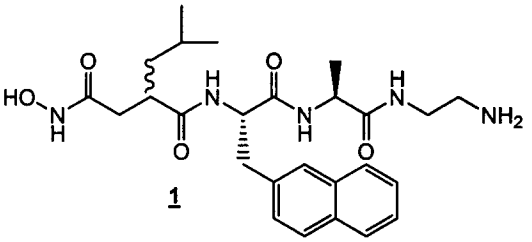
The importance and implications of proteolytic cleavage of proteins from the surface of a cell have been highlighted (21, 22). TACE "sheddase" activity extends to other membrane-bound proteins including TNF receptors p55 and p75. Shedding of a variety of ectodomains, including TGF-α, IL-6R, fractalkine, macrophage colony-stimulating factor, amyloid precursor protein, and the growth hormone receptor, is blocked in TACE knock-out cells. However, it has been shown that recombinant forms of TACE do not efficiently cleave many of these proteins (23). Evidence for TACE activity both intracellularly and on the cell surface has been reported (24, 25). The

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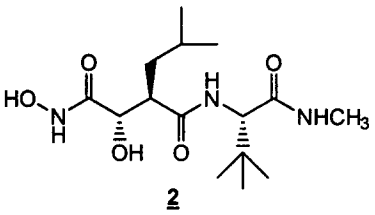
biology of TACE inhibition has been reviewed (26). It has been shown that a selective inhibitor of TACE can significantly inhibit the release of soluble TNF-α without causing a dramatic and sustained increase in active membrane pro-TNF-α (26). Efficacy superior to etanercept in a mouse CIA model has been demonstrated for a selective TACE inhibitor (26).

TACE MEDICINAL CHEMISTRY

The activity of peptidic hydroxamic acids such as TAPI, **1**, and marimastat, **2**, against TACE enzyme has been well documented, and several short reviews focusing on early TACE inhibitors have appeared (27-30). Recent work has aimed to concomitantly optimize enzyme and cell activity. Much of this effort has been structure-based design using matrix metalloproteinase (MMP) inhibitor scaffolds as leads. Homology models of TACE generated from snake venom toxins, such as atrolysin C and adamalysin II, have correctly predicted the size and unique shape of the enzyme's joined S1'-S3' subsites. This structural information has been used with great success and was subsequently confirmed by X-ray studies of enzyme-bound ligands. Advances have resulted in a diverse set of inhibitors, some with excellent potency in cells, human whole blood, and efficacy models. Broad spectrum inhibitors of MMPs and TACE, as well as some with selectivity for TACE over MMPs, have been disclosed.



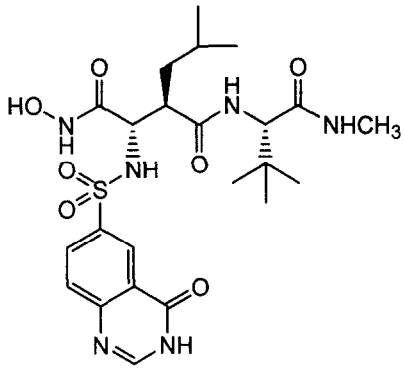
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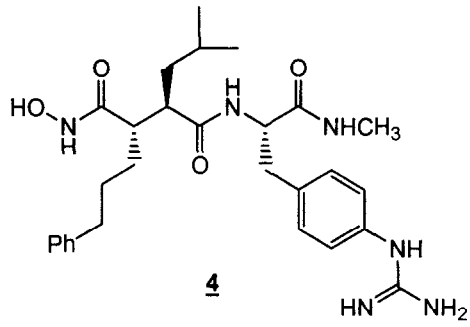
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The optimization of succinate hydroxamates as TACE inhibitors has been a major area of research. The ability of the P1 substituent of succinate hydroxamate TACE inhibitors to affect potency in human whole blood (HWB) has been examined. As for the MMPs, the *S*-configuration at the α-carbon is highly preferred. Bulky P1 groups were sought to protect the hydroxamate from metabolism and increase HWB activity, but potency correlated better with decreased lipophilicity at this position. The quinazoline sulfonamide **3** is a 0.28 μM inhibitor of TNF-α production in HWB, 25-fold more potent than marimastat in the same assay (31). The isobutyl P1' of **3** does not provide selectivity for TACE (IC_{50} = 0.6 nM) over MMP-1 (IC_{50} = 1.3 nM), but replacement with a benzyloxybutyl group to take advantage of the shallow S1' pocket of MMP-1 increases selectivity to greater than 100-fold. A 25 mg/kg po dose of **3** in marmosets gave plasma levels of 310 ng/mL after 0.5 hours. Additional analogs were prepared via solid-phase synthesis (32).

Succinate hydroxamate **4** is water-soluble by virtue of its solvent-exposed P2' phenyl-guanidine moiety and is a potent broad spectrum MMP inhibitor with an IC_{50} of 130 nM in LPS-stimulated THP-1 cells (33). Compound **4** has an approximate plasma half-life of two hours with an AUC of 13.7 μg/mL*h after a 30 mg/kg sc dose in rats. In a rat prophylactic adjuvant-induced arthritis (AIA) model, a dose of 30 mg/kg/day sc substantially reduced hindpaw swelling, joint space narrowing and bone destruction. Several early administration periods (days 0-5, 0-7, 3-7) were efficacious whereas dosing beginning after day 7 was not effective.

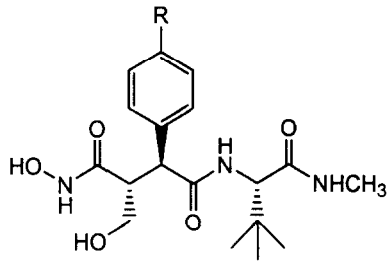


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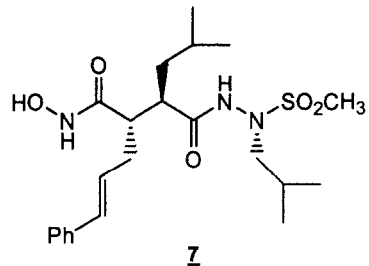


4

P1' phenyl groups also provide potent, water-soluble MMP/TACE inhibitors on the succinate scaffold. Methoxyphenyl derivative **5** (PKF242-484) is a 0.6 nM inhibitor of human TACE with similar potency against MMP-1, -2, -3, -9, and -13 (34). Though ten-fold more potent than marimastat against purified enzyme, it is 20-fold more potent (IC_{50} = 48 nM) in human PBMCs. The analogous p-methylphenyl analog **6** is more than 5-fold less active against enzyme and in cells. A route for the synthesis of multi-kilogram quantities of **5** has been reported (35). In a murine LPS challenge model, a 10 mg/kg po dose of **5** reduced neutrophil and lymphocyte accumulation, and completely inhibited TNF-α levels, in bronchoalveolar lavage three hours post-LPS dose (36). It is also active in an ovalbumin-driven model of lung allergic inflammation.

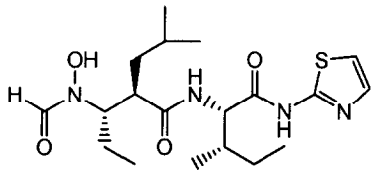


5 R = OCH_3
6 R = CH_3

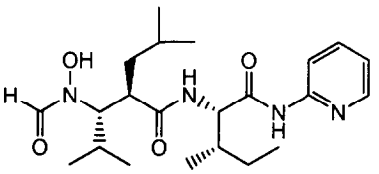


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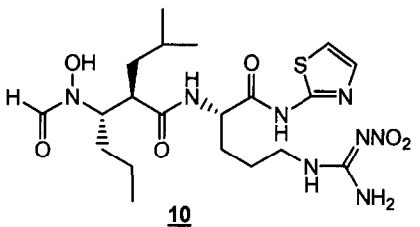
Succinate hydroxamate **7** (Ro 32-7315), in which the amino residue of **3**-**5** was replaced with a sulfonyl hydrazide that bears a P2' isobutyl substituent, is a 5 nM inhibitor of the extracellular domain of TACE. Although **7** has sub-micromolar potencies in both THP-1 cells (IC_{50} = 350 nM) and rat whole blood (IC_{50} = 110 nM), a significant loss of activity is seen in HWB (IC_{50} = 2400 nM). It is 100-fold selective over MMP-1 and moderately selective (20 to 50-fold) over MMPs-2, -3, -7, -9, and -13, and non-selective against MMP-8 and MMP-12 (37). Compound **7** is active in suppressing LPS-stimulated TNF-α production in rats with an ED_{50} of 25 mg/kg po and gives statistically significant reductions in paw swelling, lesion score and joint mobility in a prophylactic rat AIA model at doses as low as 2.5 mg/kg ip bid. The ability of Ro 32-7315 to reduce LPS-induced TNF-α release in man was assessed in an ex vivo study. Blood sampled one hour after a single 450 mg oral dose of **7** affords a mean maximal 69% inhibition of TNF-α levels. The degree of inhibition correlates well with Ro 32-7315 plasma levels. A scaleable synthetic route to **7** has been reported (38).



8



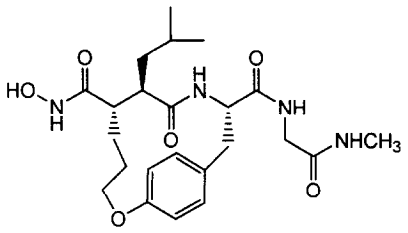
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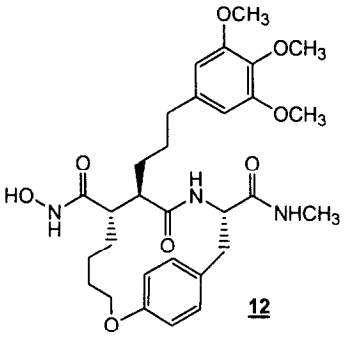
10

Analogs of the succinate TACE inhibitors in which an N-hydroxyformamide serves as the zinc chelator are represented by structures **8**-**10**. The P3' heteroaryl moiety imparts increased enzyme and cell potency. Branching of the P2' group is optimal for cell activity, as t-butyl and sec-butyl analogs are substantially more potent than the isobutyl and neopentyl derivatives. Compound **8** is a non-selective TACE inhibitor (IC_{50} = 11 nM) with an IC_{50} of 92 nM in LPS-stimulated MonoMac-6 cells (39). The P3' pyridyl analog GW3333, **9**, (TACE IC_{50} = 40 nM) is a submicromolar inhibitor (IC_{50} = 970 nM) of LPS-induced TNF-α production in human PBMCs. Compound **9** has an ED_{50} of approximately 1 mg/kg po in the rat LPS model with a duration of action of up to twelve hours and is active in the prophylactic rat AIA model at 50 mg/kg bid po and in a peptidoglycan polysaccharide rat arthritis model (40). In search of inhibitors with greater aqueous solubility than **9**, analogs with solvent exposed P2' nitro- and sulfonyl-guanidinium derivatives were prepared and evaluated in a scintillation proximity assay (41). Nitroarginine **10** has an IC_{50} of 4 nM against cell-free TACE and is a potent inhibitor of LPS-stimulated TNF-α production in MonoMac-6 cells (IC_{50} = 34 nM). While **10** displayed excellent potency after sc injection in a murine LPS model and has good water solubility, it is inactive orally. Added steric bulk in the form of branching at the P2' position does not improve oral activity. Selectivity over MMP-1 can be achieved with a P1' 5-methylthien-2-yl group.

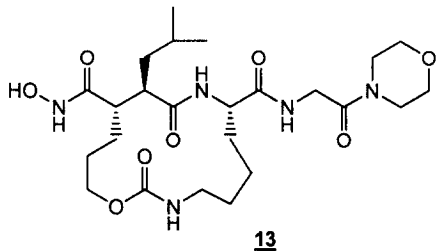
Potent succinate hydroxamate inhibitors of TACE in which the P1 and P2' groups are joined to form a macrocyclic ring are known (42). More recently, 14-membered cyclic ethers derived from P2' 4-hydroxytyrosine residues with superior activity were prepared. Glycine N-methylamide P3' analog SL422, **11**, is a 0.22 μM inhibitor of TNF-α production in HWB, 5-fold more potent than the analogous N-methylamide, and orally active in the mouse LPS model (ED_{50} = 15 mg/kg). Longer P1' groups reduce potency in this model (43). However, **11** is a broad spectrum MMP inhibitor with a 12 nM IC_{50} against porcine TACE. Excellent selectivity over MMP-1 is attained by **12** with a 15-membered ring, while retaining potency in THP-1 cells, bearing a trimethoxyphenylpropane P1' group (44). Dramatic improvement in potency is seen with macrocyclic carbamate SP057, **13**, which is extremely potent in HWB (IC_{50} = 67 nM) and the mouse LPS model (ED_{50} = 2.6 mg/kg po). Replacement of the terminal glycine residue of **13** leads to greatly reduced oral activity, although HWB activity remains potent. Compounds **11** and **13** have short half-lives and moderate bioavailability in dogs. Variation of the P1' moiety of **13** leads to biphenyl derivative **14**, which retains the enzyme and HWB potency of **13** and is more than 100-fold selective over a broad panel of MMPs (45). Docking of this compound into a homology model of TACE based on atrolysin indicates that the *ortho*-trifluoromethyl group serves to place the two phenyl rings in an orthogonal orientation resulting in a steric clash with a conserved tyrosine side chain in the MMPs.



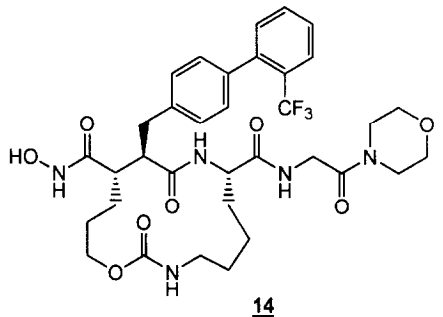
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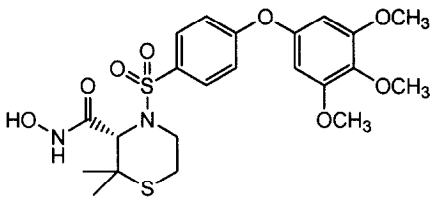


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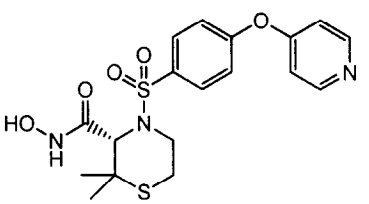


14

Sulfonamide hydroxamates comprise another major class of TACE inhibitors. Thiomorpholine **15** makes use of the P1' trimethoxyphenyl (TMP) group of macrocycle **12** to provide a 1.5 nM inhibitor of cell-free TACE having submicromolar potency in THP-1 cells. By comparison, the clinical MMP inhibitor prinomastat, **16**, is 15-fold less potent in cells (44).



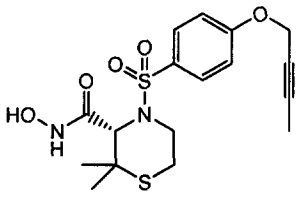
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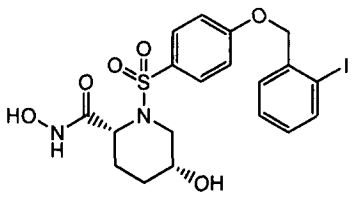
16

On the same scaffold, a P1' butynyloxy group, designed to fit in the TACE S1'-S3' channel, affords **17** which inhibits activity in THP-1 cells (IC_{50} ~100 nM) and provides 98% inhibition of TNF-α production in the murine LPS model after a 50 mg/kg po dose (46). 5-Hydroxy-pipecolic acid derivative **18** is a low nanomolar inhibitor of TACE enzyme with an IC_{50} of 1 μM in HWB. Halogen or alkyl substitution at the *ortho*-position of the terminal phenyl ring greatly enhances activity in HWB. The 4-hydroxy-pipecolates are of equivalent potency against cell-free enzyme, but less potent in HWB. An X-ray crystal structure of **18** bound to TACE shows that several residues in the S1' pocket must move in order to accommodate the ligand's bulky *ortho*-iodo group. These 3,3-unsubstituted pipecolates with P1' benzyl groups are greater than 100-fold selective for TACE over MMP-1 (47). In contrast, pipecolate hydroxamate CP-661,631, **19**, with geminal substitution at the 3-position, is a potent broad spectrum MMP/TACE inhibitor. Importantly, in human primary neuron cultures **19** inhibits the

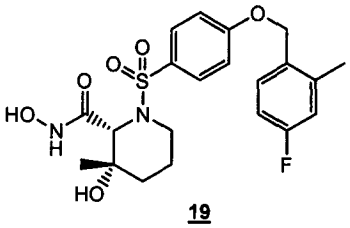
regulated secretion of amyloid precursor protein (APP), but does not result in higher levels of Aβ (48). Deposition of Aβ has been associated with Alzheimer's disease.



17

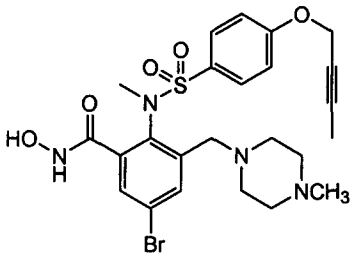


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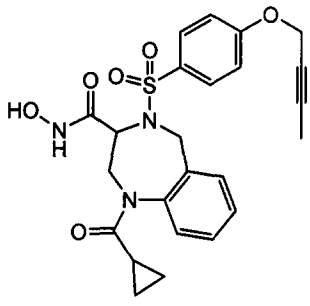


19

The butynyl P1' group of **17** has been shown to enhance activity in TACE enzyme and cellular assays when applied to a variety of other MMP inhibitor scaffolds. Optimization of the TACE activity of the anthranilate MMP inhibitors using a homology model of TACE generated from adamalysin II and the TACE X-ray structure resulted in hydroxamate **20**, an MMP-1 selective inhibitor of TACE (IC_{50} = 25 nM) with cellular activity in the range of 300 nM. Anthranilate **20** gives greater than 50% inhibition of TNF-α for over six hours in the mouse LPS model (49, 50). Lengthening the P1' butynyl group, or replacing it with a benzyl or picolyl moiety results in increased selectivity over MMP-1, but diminished cellular potency (51). Quinoline hydroxamates, and other bicyclic heteroaryl analogs of the anthranilates, are also potent inhibitors of TACE enzyme and have shown efficacy in a mouse collagen-induced arthritis (CIA) model (52). The P1' butynyloxy group of benzodiazepine **21** increases TACE activity by 7-fold over the analogous P1' methoxy analog in cell-free enzyme and affords low micromolar level potency in THP-1 cells (53).



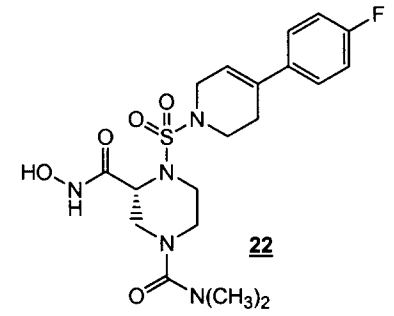
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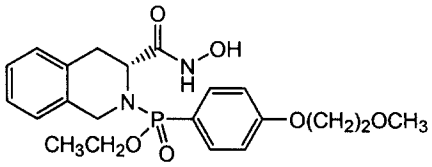
21

Variations on the sulfonamide hydroxamates include sulfamide **22** and phosphonamide **23**. The piperazine hydroxamate **22** is potent in MonoMac-6 cells (IC_{50} = 0.2 μM), the rat LPS model (ED_{50} = 11 mg/kg po) and the mouse CIA model. Solubility and pharmacokinetic properties in this series are improved for NH-sulfonamide analogs of **22** derived from acyclic amino acids such as leucine and serine (54). Phosphonamide **23** is a 3 nM inhibitor of cell-free TACE with excellent selectivity over MMP-1. In addition, it is a potent inhibitor (IC_{50} = 70 nM) of heparin-binding epidermal growth factor-like growth factor (HB-EGF) shedding (55).

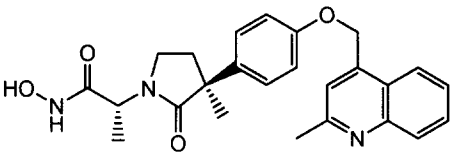
Another novel scaffold is represented by IK-682, **24**, a lactam hydroxamate, designed from modeling a sulfonamide hydroxamate into MMP-3 (56, 57). An extra methylene spacer, relative to the sulfonamides, is required between the amide carbonyl and the P1' phenyl group to allow the carbonyl to attain the proper orientation for forming a hydrogen bond with the protein. The lactam α-methyl group serves to hold the phenyl group pseudoaxial. Key to obtaining selectivity and cellular potency is the P1' 2-methylquinoline group. The quinoline can be replaced with a 3,5-disubstituted phenyl ring with little loss in enzyme potency or selectivity, since this group is still too wide to be accommodated by the MMPs, but activity in HWB is greatly diminished. The P1' quinolinylmethoxy and benzyloxy moieties have been applied to additional hydroxamic acid inhibitor scaffolds, including thiomorpholines, piperidines, and benzothiadiazepines (57, 58). In this series HWB potency appears to correlate with enzyme activity and the degree of protein binding. IK-682 **24** has a K_i of 0.6 nM against porcine TACE and is greater than 100-fold selective over a wide spectrum of MMPs. It has low clearance and long half-life (>7h) at 4 mg/kg iv, and good bioavailability (>30%) in rats and dogs at 8 mg/kg po. Optimization of IK-682 **24** yielded DPC 333 (BMS-561392), a clinical candidate with potent activity in a variety of animal models including mouse LPS (ED_{50} = 6 mg/kg po), mouse collagen antibody-induced arthritis, and rat CIA (59).



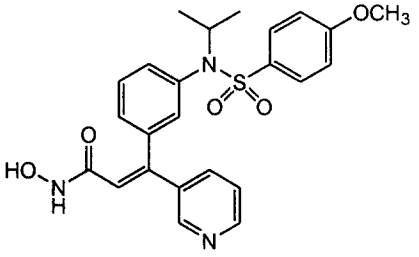
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23



24



25

Finally, a structurally novel series of propenohydroxamate TACE inhibitors with nanomolar potency against cell-free enzyme and excellent selectivity over many MMPs, exemplified by W-3646, **25**, has recently been disclosed. This series of inhibitors arose from optimization of 3,3-biphenyl propenohydroxamic acid, an HTS hit. The P2' pyridyl ring and *E*-olefin configuration are essential for optimizing the moderate THP-1 activity (IC_{50} = 2.4 μM) attained by this series. Lengthier alkyl groups on the sulfonamide nitrogen are detrimental to enzyme activity. Despite the relatively low potency of **25** in cells it is active in the mouse LPS model at 30 mg/kg po (60).

Summary - The most important unresolved issues impacting the design of future TACE inhibitors are the frequent discrepancies between activity measured in cell-free TACE assays and cellular assays of LPS-stimulated TNF-α, and the desired TACE/MMP selectivity profile to provide adequate efficacy and safety. Many factors, most notably cell permeability and protein binding have been considered in obtaining highly active

compounds in cells. The possibility that differences in the membrane bound enzyme and cell-free enzyme alter the configuration of the S1' and S3' enzyme subsites has also been addressed (61). The assessment of preferred TACE/MMP selectivity profiles awaits further clinical testing of TACE inhibitors against various pathologies. The development of new enzyme assays, including a continuous fluorometric TACE assay and a high-throughput screen based on a phage-displayed TACE catalytic domain should also aid in the rapid identification of new inhibitors (62, 63). A recent study of the binding process for hydroxamate and carboxylate TACE ligands may also be valuable for inhibitor design (64).

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SECTION IV. CANCER AND INFECTIOUS DISEASES

Editor: Jacob J. Plattner, Chiron Corp
Emeryville, CA

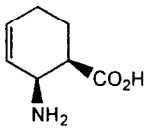
Chapter 17. Progress in Antifungal Drug Discovery

Mark B. Anderson, Terry Roemer, and Robyn Fabrey
Elitra Pharmaceuticals
3510 Dunhill Street, San Diego CA 92121

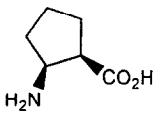
Introduction – The incidence of opportunistic fungal infections such as *Aspergillus* species, *Candida* spp. (*C. albicans* and others) and *Cryptococcus neoformans* has been increasing worldwide despite active research programs devoted to the discovery and development of novel antifungal agents (1,2). Effectively treating opportunistic fungal infections represents a significant threat to the general human condition, especially those with compromised immune systems brought on by chemotherapy treatment for cancer, organ transplants, surgery, and inflictions with HIV/AIDS or other immune compromising events (3,4). A second growing concern is the incidence of drug resistance of fungal pathogens to the currently known classes of antifungal agents used in the clinic (5,6). These two factors accentuate the urgency for the ongoing rapid identification of exploitable antifungal targets, to develop safer and more effective therapeutic agents, and to discover new chemical entities (NCE) to fight current and drug resistant fungal infections (7). This paper examines a select number of emerging antifungal compounds and captures recent advances in antifungal agents of a known mechanism of action.

EMERGING ANTIFUNGAL COMPOUNDS AND TECHNOLOGIES

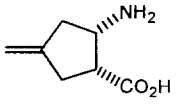
β-Amino acids – Cyclic β-amino acids have been reported with a dual mode of action where one mechanism is the inhibition of protein synthesis after concentrative uptake and second is interference with self-regulatory mechanisms of amino acid metabolism. 2-Aminocyclohex-3-enecarboxylic acid, (**1**) designed as a pyridoxal phosphate suicide inhibitor, and cispentacin (**2**), a natural β-amino acid, were both shown to be active against *C. albicans*.



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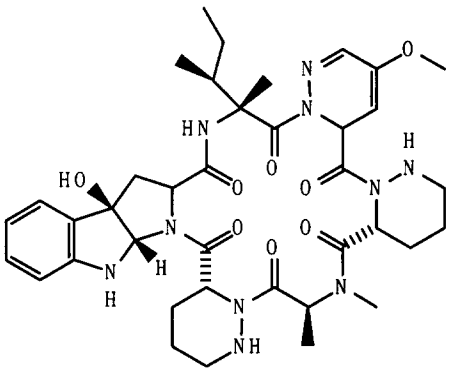
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PLD-118 (10-8888, **3**), obtained during a derivatization program, is a novel orally available β-amino acid which shows an *in vitro* IC_{50} of 0.13 mg/mL activity against *C. albicans* (8). PLD-118 is currently being investigated in Phase II clinical trials. Positive *in vivo* efficacy of orally administered PLD-118 was reported in mice using lethal systemic infection models for *C. albicans* or non-lethal kidney infection models for *C. glabrata* or *C. krusei*. PLD-118 was also effective against infections caused by fluconazole-resistant strains (9).

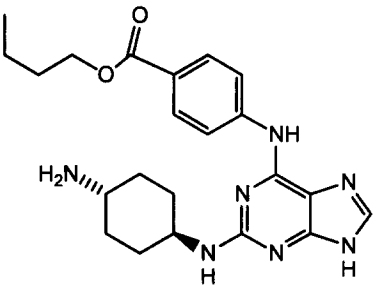
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Peptides and Polymers – A cyclic peptide SF-2822 (**4**) showed *in vitro* activity against *C. albicans* (10). Other reported cyclic peptides and cilofungin were shown to have *in vitro* antifungal activity (11). New peptides obtained from the MUCD1 domain of the saliva mucin glycoprotein MUC7 were obtained from the C-terminus of the MUC7D1 protein and range in size from 8 to 20 amino acids in length, having a net positive charge (12). An antifungal family of DNA-binding cationic polyheterocyclic molecules showing fungicidal activity against *Candida* spp. was reported. The initial lead, GL-047296, has fungicidal activity against *Candida* spp. A metabolically improved derivative, GL-663142, was reported to be active against *Candida* spp., some *Aspergillus* spp. and *C. neoformans* subtypes. It was fungicidal against *Fusarium*, dermatophytes, and certain endemic dimorphic species, with most MIC and MFC values < 10 μg/mL. The agent was active against *C. albicans* strains resistant to fluconazole and fluocytosine. The derivative GL-406349, displayed efficacy in murine systemic candidosis models (5 mg/kg) with a half-life of 166 minutes (13). *C. albicans* presents β-1,2- oligomannosides on the cell surface, and in a recent report, synthetic analogs of these oligomannosides prevent intestinal colonization (14).

Purines – Recently, a series of purine derivatives (**5**) with antifungal properties has been disclosed indicating the compounds act by inhibiting the *C. albicans* protein kinase CIV1. A representative compound inhibited *in vitro* purified CIV1 enzyme with an IC_{50} value of 5.6 μM and exhibited an MIC value against *C. albicans* of 12.5 μg/mL (15).



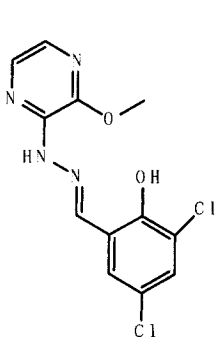
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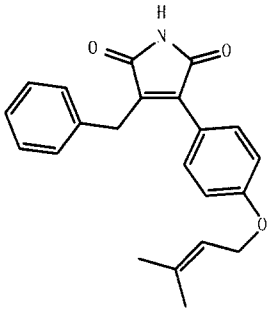
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Miscellaneous – A number of compounds exhibiting a variety of mechanisms have been described. Hydrazone (**6**), and related hydrazides and thiosemicarbazones showed *in vitro* MIC activity against *C. albicans* (≤ 1 μg/mL); natural product isolates showing antifungal activity from a *Serpula himantoides* strain gave succinimides and maleimides, for example himanimide A (**7**) (16,17). Sordarins and sordaricins selectively inhibit fungal protein synthesis by interfering with the translational complex containing co-factor Elongation Factor 2 (EF-2). Two new azasordarin derivatives GW-471552 (**8**), and GW-471558 (**9**), were shown to be active against *C. albicans* with an MIC < 1μg/mL and recently studied *in vivo* against *Pneumocystis carinii* in immunosuppressed-rat models (18,19). A new sodaricin derivative (**10**), was reported with potent *in vitro* MIC against *C. albicans* (< 0.06 μg/mL) and *C. glabrata* (0.125 μg/mL) (20,21). Benzofuran (RO-09-4609, **11**) was initially reported as an N-myristoyltransferase inhibitor with the finding that these compounds are

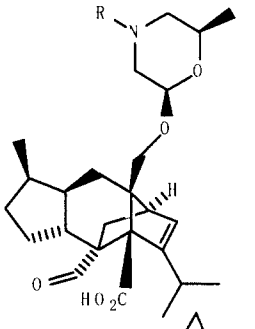
active in a murine systemic candidiasis model (22). Manool (**12**) and other hydronaphthalene ring structures have *in vitro* activity against Candida and *Aspergillus fumigatus* (23).



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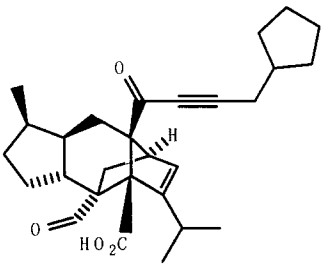


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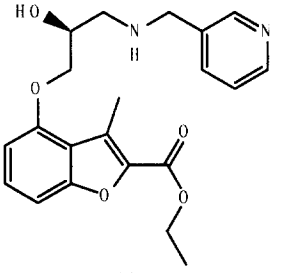


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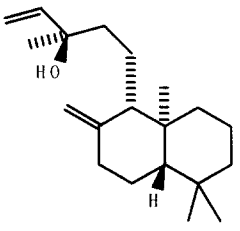
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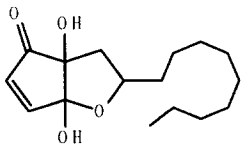


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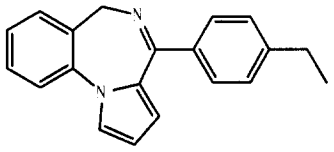


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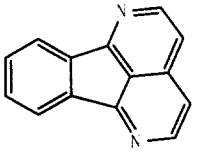
F-15784 (**13**) was cultured from *Rigidoporus lineatus* and has reported activity against *C. albicans in vitro,* and 4-substituted 5,6-dihydro-4H-pyrrolo[1,2-a][1,4]benzodiazepine (**14**), was reported to have *in vitro* MIC activity against *Candida parapsilosis* (3.2 μM) and *A. fumigatus* (1 μM) (24,25). The azafluoranthene alkaloid eupolauridine (**15**) was initially reported to be a selective inhibitor of topoisomerase I; however, recent reports indicate it to be a DNS topoisomerase II inhibitor (26).



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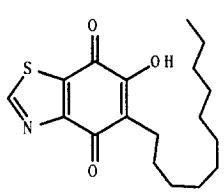
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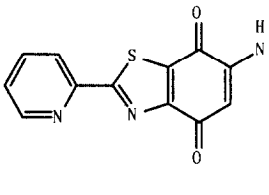
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Quinolones such as UHDBT (**16**) block mitochondrial electron transport in *Saccaromyces cerevisiae* and analog (**17**) was reported with potent *in vitro* MIC activity against *C. albicans* (3.2 μg/mL), *C. tropicalis* (0.8 μg/mL), *C. kursi* (0.4 μg/mL) and *A. niger* (6.3 μg/mL), while other analogs showed a marked decrease in potency (27). Reports of antifungal natural products include several diterpenes and withametelin from *Datura metel* (*Solanaceae*) leaves (28,29). Spiro pyrrolidines have been reported with antibacterial and antifungal activity (30). Finally, searching for

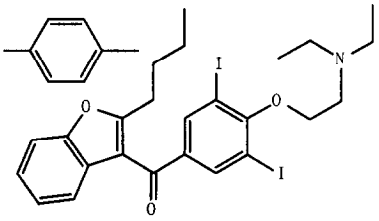
new antifungal agents by the evaluation of known drugs not previously known for antifungal activity uncovered amiodarone (**18**), which is a known antiarrhythmic agent (31,32).



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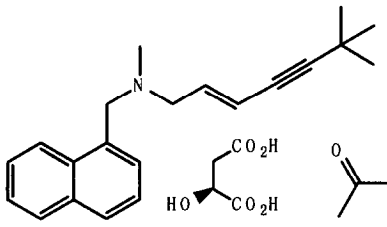


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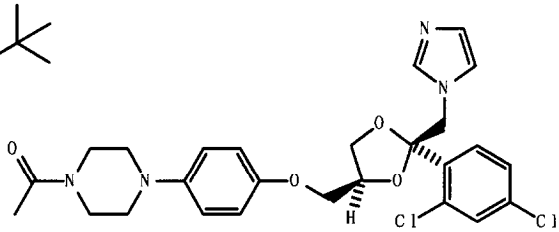
Genomics as a Tool in Discovery – Functional genomics by gene replacement and conditional expression (GRACE™) can identify essential gene products and can be used to identify antifungal compounds in a cell-based assay system (33,34). Moreover, proteomics and genomics offer emerging tools to assist in the rapid identification of essential fungal pathways and protein targets that can in turn be exploited by modern drug discovery programs (35,36).

ADVANCES IN AGENTS OF KNOWN MECHANISM OF ACTION

Allylamines – The allylamine mechanism of action reversibly inhibits squalene epoxidase, a key enzyme in ergosterol biosynthesis, resulting in accumulation of intracellular squalene, which blocks new sterol synthesis and diminishes membrane ergosterol content. The best-known compound is terbinafine (Lamisil, **19**) that is available as both an oral formulation and a topical preparation for the treatment of dermatophyte infections (37,38). Terbinafine has good antifungal activity against *C. albicans* and the maleate salts are used for the systemic and topical treatment of fungal infections, especially fungal sinusitis infection and onychomycosis (39,40).



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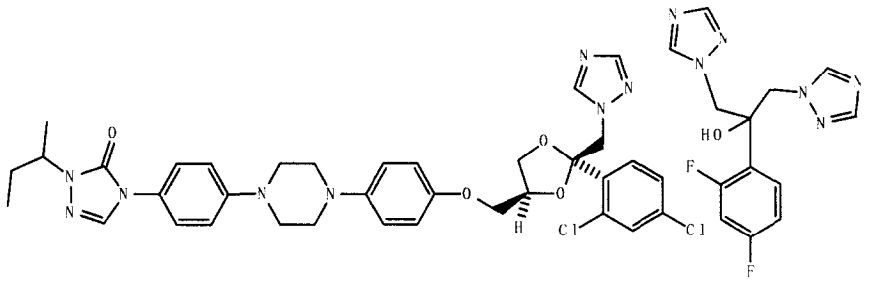


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Azoles – The azoles (imidazoles and triazoles) are fungistatic agents that inhibit fungal cytochrome P-450 3A-dependent C14- α-demethylase (*ERG11*), an essential enzyme responsible for the conversion of lanosterol to ergosterol which then leads to the depletion of the ergosterol in the fungal cell membrane (41). The introduction of azoles as an NCE significantly advanced the treatment of fungal infections and recent reviews depicting clinical isolates, structure activity relationships, and azole resistance in fungi are available (42,43). Ketoconazole (**20**), a phenethyl imidazole with a wide spectrum of antifungal activity, was the first orally available azole currently approved for treating serious fungal infections. Transdermal patches and use in combination with herbal essential oils were reported in 2002 to be active against *Candida* spp. (44,45). Itraconazole (**21**), was initially introduced in the 1980's as an alternative to ketoconazole showing reduced toxicity with better pharmacokinetics and a broader spectrum of antifungal activity. Recently, resistance

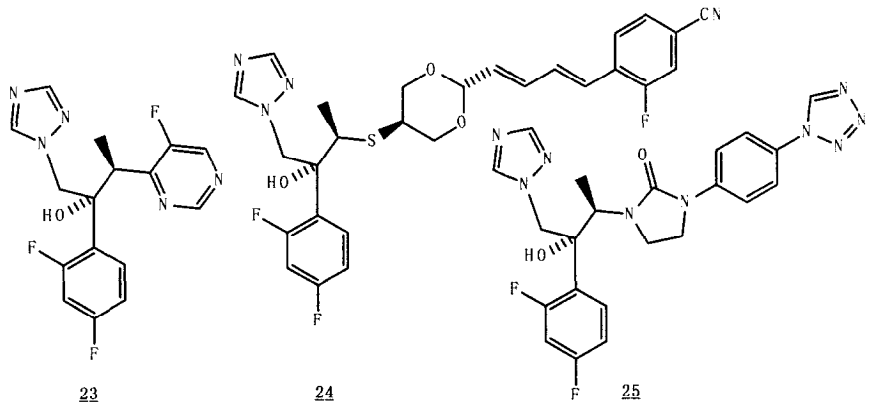
to itraconazole has been reported (46,47). Fluconazole (Diflucan, **22**) is a water-soluble bis-triazole derivative that was first approved in 1990 and exhibited good pharmacokinetics and therapeutic activity in several fungal infections (48). Fluconazole was recently reported in intensive care facilities to prevent Candida infection; however, emerging resistance has been observed (49,50). Strains of *C. neoformans* expressing heteroresistance to fluconazole were tested to characterize the heteroresistant phenotypes using 107 clinical isolates where fluconazole showed MICs ranging from 0.25 to 32 μg/mL (51).

Voriconazole (Vfend, **23**), approved May 2002 by the FDA, is a broad-spectrum triazole available as film-coated tablets for oral administration, and as a lyophilized powder for intravenous infusion for the treatment of invasive Aspergillosis (52,53). Voriconazole was also approved in Europe for treatment of serious infections caused by *Aspergillus*, *Fusarium*, *Scedosporium*, and drug resistant *Candida* spp. (*C. albicans* an MIC_{90} of 0.06 μg/mL) (54,55). Animal studies and clinical data shows Vfend to be safe and well tolerated, but a non-sight-threatening visual disturbance and drug-drug interactions require monitoring (56).



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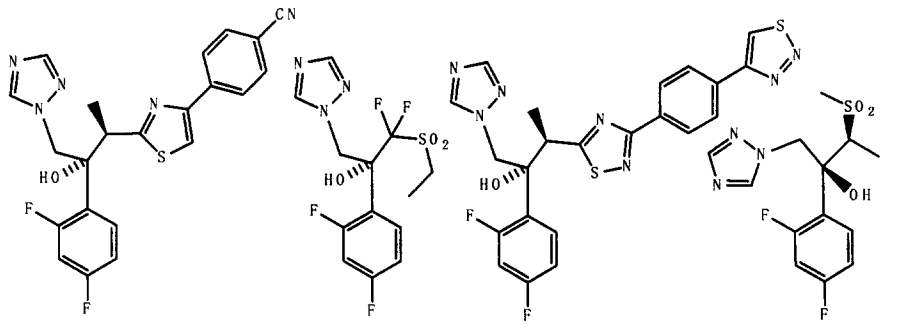
CS-758 (R-120758, **24**) shows potent and broad-spectrum activity against *Candida* spp., *Aspergillus* spp. and *C. neoformans* and *in vitro* and *in vivo* activity profile comparisons were made versus fluconazole, itraconazole and amphotericin B (57). Against *Candida* spp., CS-758 was reported as superior to fluconazole and at least comparable to itraconazole and amphotericin B, with MIC values ranging from < 0.008 μg/mL up to 1 μg/mL. CS-758 displayed excellent activity against *Aspergillus* spp. and *C. neoformans*. *In vivo* studies were performed in mice with systemic infections caused by *C. albicans, C. neoformans, A. fumigatus* and *A. flavus*, and the compound exhibited strong efficacy with ED_{50} values of 0.41-5.0 mg/kg/day after *per os* once daily for 10 days (58).



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TAK-456 (**25**) showed strong *in vitro* activity against clinical isolates of *Candida* spp., *Aspergillus* spp., and *C. neoformans*; however, activity was less for *C. glabrata*. TAK-456 inhibited sterol synthesis of *C. albicans* and *A. fumigatus* by 50% at 3 to 11 ng/mL (59). Ravuconazole (BMS-207147/ER-30346, **26**), is an orally available broad spectrum agent that recently completed a double-blind, randomized, placebo-controlled Phase I/II clinical trial. At a dose of 200 mg once daily, it is a safe and effective treatment for toenail onychomycosis (60). The pharmacokinetics, safety and efficacy at doses of 200 mg once daily, 100 mg once weekly and 400 mg once weekly versus placebo for 12 weeks were performed with a 36-week follow-up showing mycological eradication of the causative pathogens *Trichophyton rubrum* and *T. mentagrophytes*.

In vitro experiments with SS750 (**27**), demonstrated that this compound had comparable activity to itraconazole and better potency versus fluconazole against *Candida* spp. and *C. neoformans*. Introduction of an ethanesulfonyl and a gem-difluoro moiety into SS750 enhanced its *in vitro* and *in vivo* activities by increasing its lipid solubility. SS750 *per os* to immune suppressed mice having systemic and pulmonary candidiasis caused by *C. albicans* showed dose dependent increases in survival. SS750 binds with strong affinity to *C. kursi* cytochrome P-450 (61). SS750 was suggested to be useful for the treatment of deep mycoses caused by *Candida* spp. and *C. neoformans* in immune compromised patients and is currently in development (62). Ro-098246 (**28**), was reported to have potent and broad-spectrum activity and was also potent against *Aspergillus* spp. and *Mucor* spp. (63). The enantiomer of SCH 42427 (**29**) was tested in animals to evaluate its chiral inversion after oral administration. The racemate, genaconazole, was also examined. (64). Posaconazole (SCH-56592), currently in Phase III clinical trials, with a structure is similar to itraconazole, was compared to other well-known agents showing good reported antifungal activity (65).

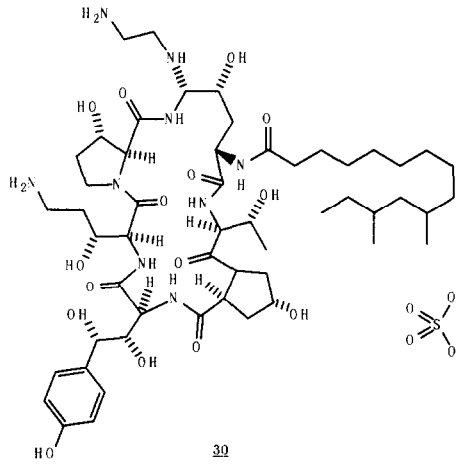


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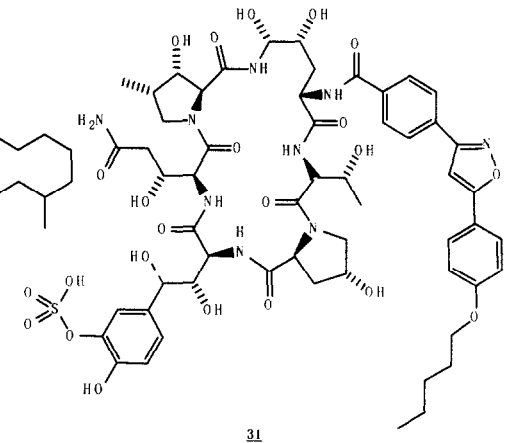
Echinocandins – The echinocandins are a new class of semi-synthetic cyclic lipopeptide fungicidal agents which act by preventing cell wall synthesis by non-competitive inhibition of essential cell wall β-(1,3)-D-glucan synthase, an essential glucan synthase enzyme in fungal cell wall synthesis. Inhibition of glucan synthase leads to increased cell wall permeability and cell lysis (66,67).

Caspofungin (**30**) was the first echinocandin approved in the US and in Europe for treatment of candidiasis and invasive aspergillosis in adult patients who are refractory to amphotericin B and/or itraconazole (68,69). Due to low intestinal absorption, an oral formulation has not been developed. Micafungin (FK-463, **31**) is a water-soluble lipopeptide semi-synthetic derivative in Phase III clinical trials against *Candida* spp. (70,71). Micafungin is structurally similar to the echinocandins and

pneumocandins, showing good MIC activity in the presence of 10, 20, and 50% human serum and plasma against *Candida* spp. European approval is anticipated in 2004 for patients suffering from cancer and AIDS. Micafungin's drug interactions were reported and human plasma protein binding indicated micafungin to be >99% plasma protein bound (72). Anidulafungin (VER002/LY-303366, **32**) a semi-synthetic agent structurally related to cilofungin was tested *in vitro* versus fluconazole and itraconazole against 460 clinical yeast isolates. The report indicates anidulafungin was superior to itraconazole and fluconazole against *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* (73).

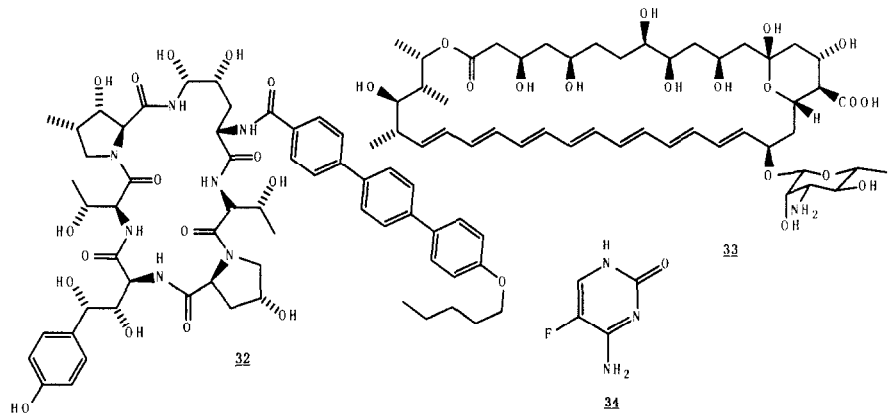


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Polyenes – Polyene antifungal agents form complexes with ergosterol and disrupt the fungal plasma membrane which results in increased membrane permeability, leakage of the cytoplasmic contents and death of the fungal cell (74). The agents have the broadest spectrum of antifungal activity including Candida, Aspergillus, and are effective in severe systemic fungal infections. They are fungicidal (75). Amphotericin B (**33**), is well-known in several formulations and is used to treat invasive fungal infections related to cancer, organ transplantation and other conditions. Because of the strong supporting data indicating reduced toxicity, an improved formulation of amphotericin B was approved by the FDA (76,77).



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The pharmacokinetics and tissue distribution reported for SPA-S-753 (a derivative of partricin) was compared to amphotericin B in a single dose trial in mice at 1.25 mg/kg by intravenous route of administration in 5% glucose. The report showed a

serum elimination half-life of 15.1 hours compared to amphotericin B of 19.8 hours and SPA-S-753 has been reported effective and nontoxic *in vivo* on murine candidiasis, aspergillosis, and cryptococcosis (78). An alternate salt form, SPK-843 was selected to undergo Phase I clinical trials in late 2001 (79,80).

Pyrimidines – Flucytosine (5-Fluorocytosine, **34**) is currently the only FDA approved fluorinated pyrimidine agent. 5-Fluorocytosine is a water-soluble agent having at least two mechanisms of action. 5-Fluorocytosine is taken up by the fungal cell via a permease system that recognizes several purines like the natural analog cytosine. Once inside of the cell, fluctosine is rapidly deaminated by the enzyme cytosine deaminase to the antimetabolite 5-FC that incorporates into the fungal RNA instead of uridine, resulting in inhibition of protein synthesis. In mammalian cells, cytosine deaminase is either absent or has a minimal activity. The second mechanism of action is the conversion of flucytosine into 5-fluorodeoxyuridine monophosphate by the enzyme uridine monophosphate pyrophosphorylase (81). This results in the subsequent inhibition of thymidylate sythetase and interferes with DNA synthesis. Unfortunately, it has a narrow spectrum of activity against *Candida* spp. and *C. neoformans*. However, recent reports tested 5-FC against 8,803 clinical isolates of *Candida* spp. (18 species) indicating that lower doses can be used to reduce host toxicity while maintaining efficacy (82).

Summary – Undoubtedly, innovative research efforts will continue in the 21st century as we seek to minimize the incidence of opportunistic fungal infections worldwide, exploit available and emerging technologies such as genomics and proteomics, and develop new chemical entities (NCE) with improved efficacy and safety profiles. Finally, strategies for combining several antifungal drugs as an alternative approach for antifungal therapy is an evolving option (83).

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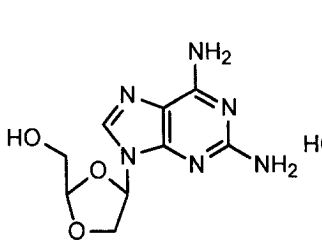
Chapter 18. Recent Advances in the Chemotherapy of HIV

Steven D. Young
Merck Research Laboratories
WP14-3, West Point, PA 19486

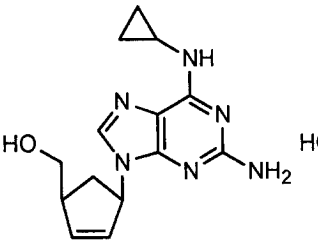
Introduction – The global pandemic of human immunodeficiency virus (HIV) infection/AIDS is showing no sign of abatement. In 2002 the number of people living with HIV infection grew by 5 million to 42 million individuals worldwide. Epidemiological projections point to that number increasing to 100 million by 2010, with most of the infected individuals living in developing countries (1). In the developed world, the number of new infections continues to grow, albeit at a much slower rate. For those patients with access to anti-retroviral medications, the emergence of viral strains resistant to the current therapeutic agents represents a significant threat (2). The challenge facing anti-retroviral drug research in 2002 and beyond is the discovery and development of new agents that address the issue of resistance, either through improvements in existing drug classes or by the discovery of agents targeting new mechanisms of action. This chapter will therefore be divided into two sections, first, new compounds for existing viral targets and second, compounds for novel viral targets.

COMPOUNDS INHIBITING HIV THROUGH EXISTING MECHANISMS

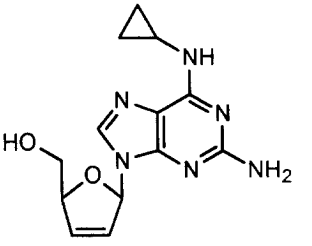
Reverse Transcriptase Inhibitors - Reverse transcriptase (RT) functions to synthesize a double stranded DNA copy of the viral genomic (+) stranded RNA. Inhibitors of RT come in two varieties: first, nucleoside mimics (NRTI's) which are phosphorylated *in vivo* to 2'-deoxynucleoside-5'-triphosphates along with prodrugs of 5'-nucleotides themselves and second, nonnucleoside inhibitors (NNRTI's) which function at an allosteric site on the enzyme (3). The mechanism of RT inhibition by NRTI's is both chain termination and competition for the deoxynucleoside triphosphate-binding site. NRTI's were the first anti-HIV drugs approved by regulatory agencies, and as such, viruses harboring mutations conferring resistance to these agents have become widespread (4,5). Promising new NRTI's in development contain both unusual ribose mimics and modified bases. In particular, the diaminopurine substituted dioxolane DAPD (amdoxivir) (**1**) is converted *in vivo* to the guanosine analog DXG through a metabolic deamination process similar to that of the 6-N-alkylpurine **2** (abacavir, 1592U89) (6,7). DAPD is potent against viral variants resistant to currently marketed NRTI's. Efforts to improve its CNS penetration produced the N-cyclopropyl prodrug, *cyclo*-D4G (**3**) (8). Compound **3** embodies the lipophilic and metabolically labile 6-cyclopropylamino functionality of **2** and avoids some of the chemical instability problems found in the parent, D4G.



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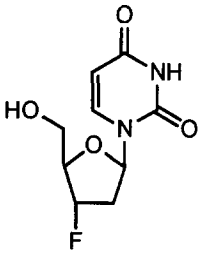
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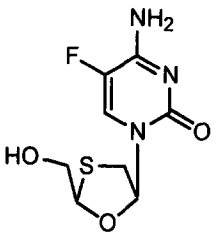
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Among the pyrimidine containing NRTI's, MIV-310 (3'-deoxy-3'-fluorothymidine) (4) and emtricitabine (FTC) (5), an analog of lamivudine (3TC), continue in phase II / III clinical development (9,10). Although more potent than 3TC in cell culture, in clinical studies, emtricitabine shows loss of susceptibility to viruses containing the 184(M-V) mutation in RT, a feature in common with lamivudine (11).

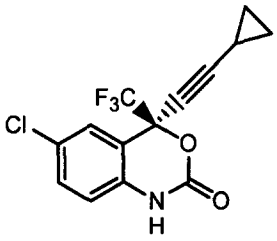


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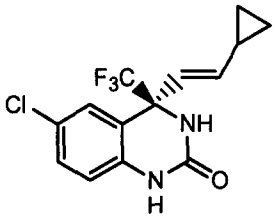


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The successful use of NNRTI's in the treatment of AIDS is well-documented (12). Efavirenz (6), a so-called second generation compound, established a new standard of care when it was employed with a combination of NRTI's clinically (13,14). Nevertheless, the development of resistance to the NNRTI's is a significant medical problem (14). Mutations in RT at residues 100 (L-I), 103 (K-N), 181 (Y-C), 190 (G-S) and 230 (M-L), singly or in combination, result in viruses that successfully evade all three of the currently approved agents (15-18). Efforts to uncover third generation compounds with clinically useful efficacy against these resistant strains continue apace. Quinazolinone analogs of 6 have been studied in detail (19). The most advanced of these, DPC-083, (7) is 3-7 fold more potent against clinical isolates harboring resistance mutations to efavirenz (20). DPC-083 was shown to be safe and effective in clinical trials, lowering virals loads in patients who previously failed therapy with nevirapine (61%) or efavirenz (39%) (21,22).

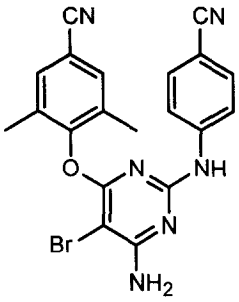


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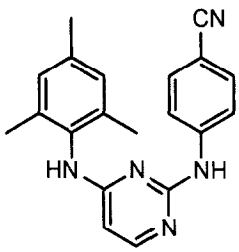


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Another third generation compound in clinical trials, dapivirine (TMC-125, R165335) (8) has been shown effective in treating infected individuals harboring phenotypic NNRTI resistant virus (23). Recent clinical studies uncovered significant drug-drug interactions with 8, an auto-inducer of CYP3A4, and other HIV drugs that are inducers of both CYP3A4 and glucuronidation (24). A more potent analog, TMC-120 (R147681) (9), also in clinical trials, requires the selection of at least a double mutation in RT 181(Y-C) + 188(Y-L) for the development of significant resistance (25). Capravine (AG1549, S-1153) 10, is effective against viruses containing the mutation in RT at residue 103(K-N). Temporarily withdrawn from clinical trials due to vasculitis in dogs, phase III studies have resumed in a treatment experienced patient population (26).

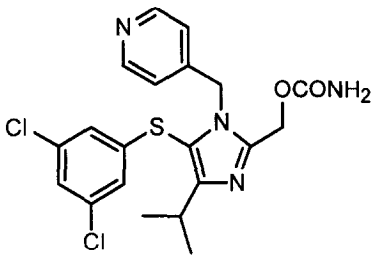


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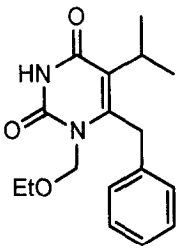


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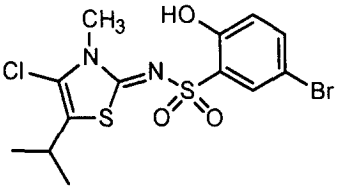
Development of emivirine (MKC-422) (11) was halted when it was shown to be less potent than other antiretrovirals clinically (27). Preclinical data for 11 indicate viruses bearing the common NNRTI selected double mutation in RT, 103(K-N) + 181(Y-C) are completely resistant to the drug. Research in the NNRTI field is still quite active with inhibitors from new chemical classes appearing each year. A noteworthy example is the thiazol-2-ylidene sulfonamide YM-215389 (12), a compound with potent inhibitory activity against both wild type and 103(K-N) + 181(Y-C) double mutant enzyme. YM-215389 shows potent antiviral activity, EC_{50} = 37 nM, against wild type virus (IIIB) in MT4 cells (28).



10



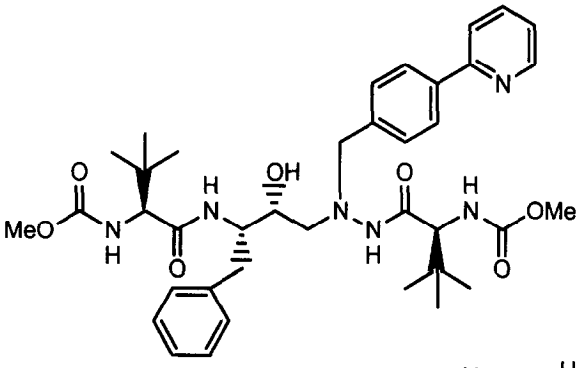
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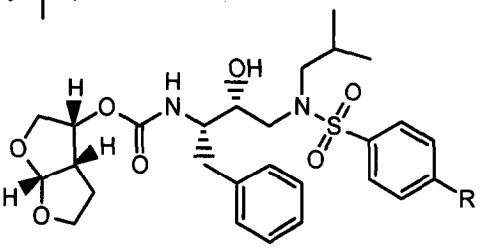
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Protease Inhibitors - As a result of early clinical successes, inhibitors of the HIV aspartyl protease (PI's) have gained wide acceptance among infectious disease physicians. As a class, the approved drugs are peptidomimetic transition-state analogs of gag-pol polyprotein cleavage sites. Critical issues surrounding the long term use of these agents are the development of resistant virus populations, modest human pharmacokinetics, compound specific side effects, and class effects on lipid metabolism, the so called "lipodystrophy syndrome" (29-31). New agents in development target one or more of these problems. A late stage development compound addressing many of these issues is atazanavir (BMS-232632) (13), an azapeptide in PIII trials being developed for once a day dosing (32,33). Atazanavir shows a distinct resistance profile compared with other PI's. Genotypic and phenotypic analyses have shown the uncommon double mutation 50(I-L) / 71(A-V) in protease is found among patient isolates having reduced susceptibility to atazanavir (34). Clinical data suggest atazanavir has little or no effect on patient LDL cholesterol and triglyceride levels (32). Also in development are the PI's TMC-126 (UIC-94003) (14) and TMC-114 (UIC-96017) (15), both sulfonamide peptidomimetic PI's with subnanomolar antiviral activity against wild type virus (35,36). TMC-126 retains low nanomolar activity (IC_{50}'s = 0.5 to 55 nM) against viral isolates from patients containing common PI resistance mutations. While *in vitro* selection pressure experiments with TMC-126 produced several common protease

resistance mutations, a novel active site mutation at position 28(A-S) was also uncovered (37). *In vitro* selection pressure experiments suggest a higher genetic barrier to resistance exists for TMC-114 than that for several approved PI's. Selected resistant viruses contained mutations at positions 41(R-T) and 70(K-E). These viruses were moderately resistant to TMC-114 but replicated poorly (38).

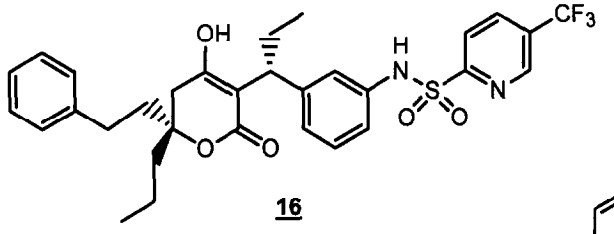


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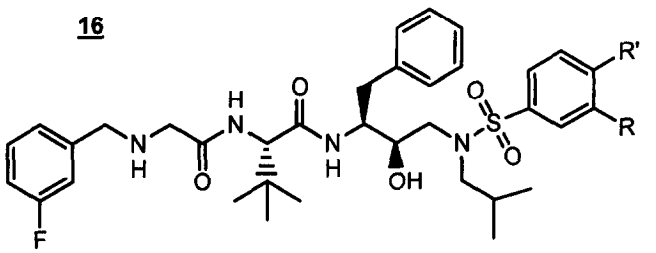


14 R = OMe; 15 R = NH_2

An agent that is unique among PI's in clinical development is tipranavir (PNU-140690) (16), a compound evolved from a non-peptide screening lead that does not employ a hydroxylic transition state mimic in its backbone (39). As such, clinical resistance to tipranavir comes from amino acid substitution patterns in protease that differ somewhat from those elicited by conventional PI's. Typical mutations in protease that engender resistance to tipranavir include the relatively uncommon changes at residues 82(V-T) and 90(L-M) (40). Tipranavir has modest innate pharmacokinetics in man, and therefore requires co-dosing or "boosting" with the P450 inhibitory PI ritonavir to maintain adequate trough levels.



16

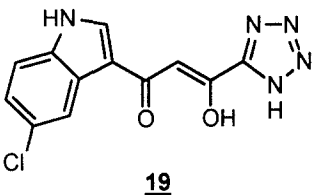


17 R = NH_2 R' = H; 18 R = H, R' = NH_2

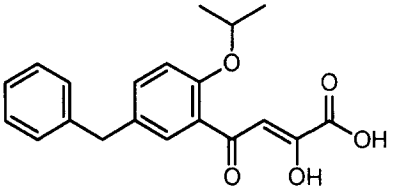
The combination of the two PI's shows potent antiviral activity in treatment experienced patients (41). Two new PI's based on more conventional backbones have entered phase I clinical trials. DPC-681 (**17**) and DPC-684 (**18**) are arylsulfonamidohydroxyethylamine transition state isosteres with low nanomolar antiviral activity (IC_{90}'s = 4-40 nM, wild type HIV) and superior resistance profiles (42). A notable feature of these compounds is their extraordinary inhibitory activity against purified enzyme. K_i's for wild type protease are 12 and 21 pM for **17** and **18** respectively, potencies in line with the clinically effective PI lopinavir (43). Also of current interest in the PI field is the phosphate prodrug of amprenavir, known as fosamprenavir (GW433908). Fosamprenavir has significantly improved oral bioavailability in man relative to the parent drug. The increased aqueous solubility of the phosphate allows for better formulation and a lower pill burden, two 700 mg tablets are equivalent to eight amprenavir capsules (44). Regulatory approval of fosamprenavir is anticipated in 2003.

COMPOUNDS INHIBITING HIV THROUGH NOVEL MECHANISMS

HIV Integrase Inhibitors - HIV integrase (IN) is the third constitutive viral enzyme required for replication, and therefore is an attractive target for chemotherapeutic intervention in the treatment of AIDS. A general lack of understanding of the molecular mechanisms of viral integration has hindered the discovery of small molecule integrase inhibitors with antiviral activity (45). Recently advances in integrase enzymology have pointed out the need to inhibit the strand transfer function of integrase to obtain significant antiviral activity (46). Accordingly, the most interesting integrase inhibitors from a drug discovery standpoint are those molecules that function as integrase strand transfer inhibitors (INSTI's). Important lead structures for medicinal chemistry efforts on INSTI drug development are a series of 1,3-dicarbonyl compounds typified by (1-(5-chloroindol-3-yl)-3-hydroxy-(2H-tetrazol-5-yl)propenone (5CITEP) (**19**) and the bioisosterically related 2,4-diketobutanoic acid **20**. The 1,3-diketone **19** has an IC_{50} = 2.1 μM for strand transfer inhibition while the 2,4-diketobutanoic acid **20** is somewhat more potent (IC_{50} = 0.01 μM) and shows good antiviral activity in cell culture (IC_{95} = 0.1 μM, iiib virus, MT4 cells) (47,48).



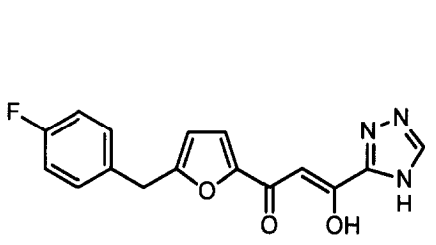
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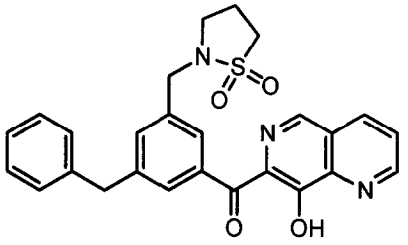
20

Improvements in potency and pharmacokinetics have been made to both prototype structures, which have resulted in clinical development candidates from both series. S-1360 (**21**) is a 1,3-diketopropane that combines features of both diaryldiketone **19** and diketoacid **20**. S-1360 has a strand transfer inhibition IC_{50} = 20 nM and an antiviral EC_{90} = 720 nM (49). Human pharmacokinetics have been reported and suggest that to maintain adequate trough levels of drug, dosing may require 500 – 2,000 mg of S-1360 two or three times a day (50). The dicarbonyl portion of these molecules is believed to interact with the catalytically important Mg^{++} contained in the active site of integrase (51). Replacement of the pyruvic acid portion of **20** with an 8-hydroxy-1,6-naphthyridine led to a series of benzophenone like molecules that mimic the metal cation interaction of the diketobutanoic acid pharmacophore. The most potent example from this series of naphthyridine ketones is compound **22**, which has an antiviral IC_{95} = 390 nM (52). Further elaboration of

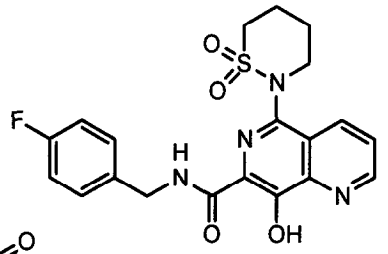
the 8-hydroxy-1,6-naphthyridine based INSTI's led to the N-benzyl carboxamide L-870,810 (**23**), a very potent antiviral agent with an IC_{95} = 0.019 μM against wild type virus (53,54). This compound entered phase I clinical trials in 2002 (55). Proof of concept studies with the related naphthyridine amide L-870,812 (**24**) demonstrated a significant antiviral effect in a group of rhesus macaques infected with the simian-human chimeric virus SHIV 89.6p. Macaques receiving a 10 mg kg^{-1} dose of L-870,812 twice a day had sustained decreases in viral RNA of 1-3 log copies ml^{-1} over an 87 day period (56). Structurally related 3-substituted-8-hydroxyquinolines have been reported as integrase strand transfer inhibitors. Although no antiviral activity is reported, compound **25** has an integrase inhibition IC_{50} = 0.331 μM (57).



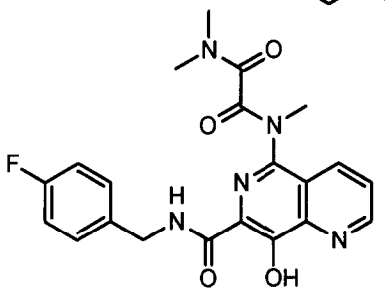
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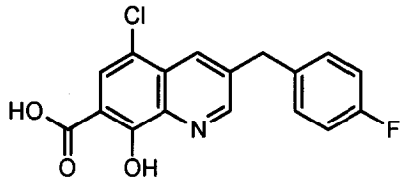
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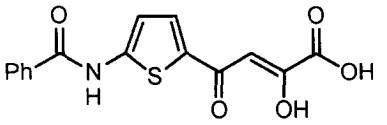
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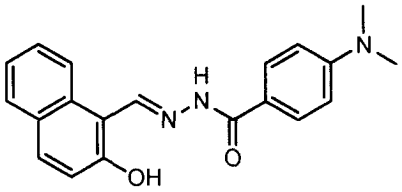
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RNase-H Inhibitors - Within the p66 subunit of the p66/p51 heterodimer of HIV reverse transcriptase lies a second catalytic region capable of breaking / making phosphodiester bonds. Known as the ribonuclease H domain (RNase-H), this region is independent of the DNA polymerase site and serves to remove the RNA template from the growing DNA:RNA heteroduplex (58,59). As an independent catalytic site with its own unique enzymic function, RNase-H is a target for antiviral drug design. Structurally there are many similarities between the RNase-H domain of RT and HIV integrase which suggest they use a common alkali metal cation/carboxylate mechanism (60). Indeed, such suggestions led to discovery of RNase-H inhibitory activity with a series of 2,4-diketobutanoic acid integrase inhibitors (61). The N-benzoylaminothiophene diketobutanoic acid **26** has an IC_{50} = 4.7 μM for RNA cleavage using an isolated RNase-H domain with Mn^{++} as the catalytic metal. Certain phenylhydrazones inhibit both the polymerase and RNase H activities of RT. Recently the 4-dimethylaminophenylhydrazone **27** was reported to show selectivity for inhibition of RNase-H over the polymerase domain of RT with an IC_{50} = 4.0 μM

for RNA cleavage (62). 1,4-Naphthoquinone and integrase inhibitory DNA aptamers also have been shown to selectively inhibit the RNase-H activity of RT (63,64).



26



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Inhibitors of Viral Entry - Entry of HIV virions into host cells involves attachment of viral coat glycoproteins to host cell receptors followed by fusion between virus and host cell membranes. In the initial phase of this process the viral envelope glycoprotein gp120 binds with high affinity to a host cell CD4 receptor. This single protein-protein interaction is insufficient to trigger fusion by itself. A second interaction, one between the gp120/CD4 complex and a host cell "co-receptor" is required. The co-receptors involved the viral entry process belong to a series of transmembrane G-protein coupled receptors for certain chemokines. The most commonly used chemokine receptors in the viral entry process are CCR5 and CXCR4. The interaction of gp120 with CD4 and a chemokine receptor is believed to be the trigger that sets in motion a membrane fusion process involving a second viral glycoprotein, gp41. Gp41, which is situated at the base of gp120, reorganizes in such a manner as to bring the viral and cellular membranes into contact. Fusion of the two membranes then permits entry of the viral nucleoprotein core (65). Inhibitors of the CD4/gp120 interaction include the antibody like tetravalent immunoglobulin molecule containing CD4 binding domains PRO542, and the 11kDa cyanobacterial protein cyanovirin-N (66,67). Cyanovirin contains two high affinity carbohydrate binding sites that interact with mannosyl disaccharide residues on gp120. The nanomolar affinity of these two binding sites is in line with the potent, broad-spectrum anti-HIV activity of cyanovirin in cell culture. While large molecules such as PRO542 and cyanovirin are effective in disrupting the gp120/CD4 interaction, a more surprising observation, that a small molecule can efficiently block this same interaction was recently reported. BMS-806 (structure not disclosed) is a small molecule inhibitor of the gp120/CD4 interaction that has submicromolar antiviral activity in cell culture (68). However, envelope heterogeneity among viruses is problematic for BMS-806, several hundred fold differences in antiviral activity were seen with a panel of diverse viral isolates. Selection of resistant variants to BMS-806 revealed amino acid substitutions near the CD4 binding domains of gp120.

Antagonists of chemokine receptors are antiviral agents that prevent the interaction of the gp120/CD4 complex by blocking the host cells' co-receptor. The CCR5 antagonist SCH-C (SCH 351125) is a potent antiviral agent that inhibits viral replication of NSI viruses with IC_{50}'s in the 0.4 to 9 nM range *in vitro*. Importantly, SCH-C has no effect on SI viruses that employ the CXCR4 co-receptor for viral entry (69). Clinical studies with SCH-C demonstrated a viral load reduction of 0.5-1.0 log in a patient group that excluded those having SI phenotype virus at baseline (70). Other small molecule CCR5 antagonists in preclinical development, notably TAK-220 and AK-602, show similar low nanomolar antiviral activity and SI phenotype selectivity *in vitro* (70,71).

Inhibitors of the gp41 mediated fusion process are the most clinically advanced viral entry inhibitors. Enfuvirtide (T-20, DP-178, pentafuside) is a 36 amino acid residue peptide that binds to one of the two heptad repeat regions on gp41. The high affinity binding between T-20 and gp41 results in subnanomolar antiviral activity

(IC_{90} = 0.3 nM) (73). In a phase II trial T-20, administered by subcutaneous injection (100 mg, b.i.d.) has been shown to effectively reduce viral load in patients through 48 weeks (74). Resistance to T-20 does occur in treated patients and a follow up fusion inhibitor, T-1249, a 39-mer which is 4-5 times more potent, has shown good efficacy in patients with phenotypic T-20 resistance (75).

Conclusions: The spread of HIV infection continues without abatement. Analysis of newly acquired infections in North America shows the spread of phenotypic drug resistant HIV has increased to 12.4 percent of new infections. Genotypic analysis suggests the situation is worse, 22.7% of new infections show one or more mutations in reverse transcriptase and/or protease genes (76). The need for new drugs to combat these resistant strains of HIV will continue to grow. Fortunately, a number of new drugs designed to meet this need are showing efficacy in clinical trials.

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Chapter 19. Antibacterial Treatment of Community-Acquired Respiratory Tract Infections

Allan S. Wagman and Mary Lee MacKichan
Chiron Corporation, Small Molecule Drug Discovery,
4560 Horton Street, M/S 4.5, Emeryville, CA 94608-2916

Introduction - Community-acquired respiratory tract infections (CARTIs) represent one of the most globally prevalent classes of infection. Acute RTIs account for approximately 75% of all antibiotic prescriptions and 20% of all medical consultations (1). Community-acquired upper respiratory tract infections (CAURTI) (pharyngitis/tonsillitis, laryngitis, otitis media and sinusitis) and viral RTIs (rhinorrhea/the common cold, influenza A/B, adenovirus, parainfluenza and syncytial virus) are typically not life-threatening unless complicated by a coinfection or an immunocompromised host (e.g. meningitis, HIV, etc.). Generally, CAURTIs respond well to front-line antibiotics such as penicillins, erythromycin, azithromycin, amoxicillin/clavulanate or cefpodoxime. Viral RTIs are usually self-limiting and only require symptomatic support (2,3). Of greater concern are lower respiratory tract infections (LRTI) which include community-acquired pneumonia (CAP) and acute exacerbations of chronic bronchitis (AECB). These LRTIs account for nearly half of all reported community-acquired infections, can be challenging to treat, place a considerable burden on the health care system, and exhibit significantly higher rates of morbidity and mortality (4). This review will present select current topics in the epidemiology and treatment of CARTIs (ca. 2000-2003).

EPIDEMIOLOGY OF CARTIs

Morbidity and Mortality - In 2000, the National Centers for Disease Control and Prevention (CDC) reported that influenza and pneumonia as a combined category were the 7th leading cause of death (5). Influenza viral pneumonia is one of the risk factors leading to bacterial invasion and severe bacterial pneumonia (especially *Staphylococcus aureus*) (6). Mortality for ambulatory outpatient treated CAP is low (<1%), although about 20% of diagnosed CAP cases eventually require hospitalization (7). Mortality rates increase dramatically to 10-25% for serious cases of CAP which typically have a bacterial etiology, while acute CAP requiring intensive care unit (ICU) admission is associated with a mortality rate of almost 40% (1,7). Other CARTIs, while not as deadly as CAP, are a leading cause of serious infection. Incidence rates for CAP in 1996 were 18 per 1000 persons, while acute bronchitis and other acute URTIs (e.g. sinusitis) which required medical attention were 46 and 113 per 1000 persons respectively (8). Associated with colds, it is estimated that about 20 million cases of otitis media and sinusitis are caused by acute bacterial complications of viral rhinosinusitis (9). CAURTIs share a common microbiology with CAP and bronchitis with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* representing some of the most frequently identified pathogenic bacteria (4,10)

Treatment Guidelines, Diagnoses and Pathogens - There is no shortage of CARTI (especially CAP) treatment guidelines, guideline summaries or expert opinions on specific syndromes, comorbidities or local case studies (6,11-13). In general, all of the guidelines for CAP recommend monotherapy with a macrolide for outpatients or inpatients having no risk factors for drug-resistant *S. pneumoniae* (DRSP) or gram-negative pathogens. For those patients with either cardiopulmonary disease or risk factors for DRSP, a combination therapy of a β-lactam and macrolide (e.g. erythromycin, clarithromycin or azithromycin) or monotherapy with an

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antipneumococcal quinolone (e.g. levofloxacin, gatifloxacin or moxifloxacin) is recommended. First-line β-lactams for outpatient use include cefpodoxime, cefuroxime, high-dose amoxicillin, amoxicillin/clavulanate and ceftriaxone *iv* followed by oral cefpodoxime. For inpatients, acceptable β-lactams are cefotaxime, ceftriaxone, amoxicillin/sulbactam and high-dose amoxicillin. Certain agents are listed in guidelines as alternatives for pneumococcal infection, such as imipenem, meropenem, cefepime and piperacillin/tazobactam because of their activity against resistant bacteria and *Pseudomonas aeruginosa*. Recommendations suggest that these agents should be reserved for serious CAP patients with risk factors for resistant gram-negative pathogens. Vancomycin has excellent utility for DRSP infections, but, like linezolide, should be reserved for difficult cases of resistant gram-positive infections such as *S. aureus*.

Treatment guidelines arise from the need for empiric therapies based on the lack of fast, accurate and cost effective diagnostic tests, as well as a need to improve patient outcomes and lower mortality (6). The most identified causative agent in nearly all studies of CAP was *S. pneumoniae* (14). From a variety of studies, other common microorganisms associated with CAP are *H. influenzae*, influenza viruses, other viruses, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. with the first two representing by far the most identified pathogens (4,14-16). Other important causes of pneumonia are *M. catarrhalis* in AECB and COPD patients, *Klebsiella pneumoniae* with chronic alcoholism, Gram-negative bacilli and *S. aureus* in nursing home and inpatients (17). The etiology of acute sinusitis and chronic bronchitis is similar to CAP, except for a greater incidence of *M. catarrhalis* and Gram-negative bacilli with chronic bronchitis. Pharyngitis is nearly always attributed to either *Streptococcus pyogenes* or viral pathogens (4,17).

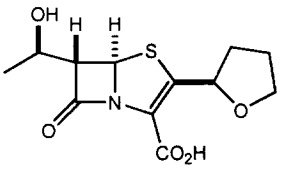
ANTIBIOTICS USED IN THE TREATMENT OF CARTIs

Developments in antibacterial research across a wide assortment of drug classes were recently reviewed (18), as well as research directed toward overcoming resistant pathogens (19,20). The current progress of many of the most promising new agents in clinical development was also evaluated (21).

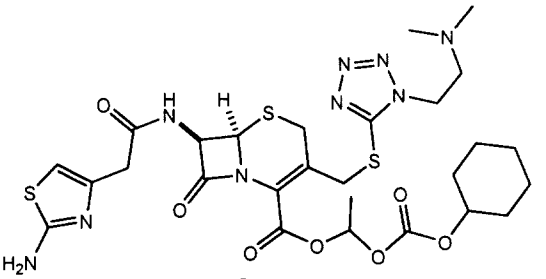
Penicillins and Cephalosporins - Agents in these two classes of β-lactam are generally considered the drugs of choice for the treatment of CARTIs due to a variety of attractive features including their broad spectrum of activity, fast bactericidal action, safety profile and oral dosing. While penicillin is typically used for treating pharyngitis, many CARTIs, including CAP, are treated with oral amoxicillin/clavulanate or an oral cephalosporin such as, cefpodoxime, cefdinir, cefproxil, or cefuroxime. Since many of these agents (alone or in combination with a β-lactamase inhibitor, e.g. ampicillin/sulbactam) are available in both oral and *iv* forms, they are ideal for transition therapy for patients hospitalized with CAP or AECB (22). Unfortunately, β-lactam agents alone do not adequately cover atypical pathogens (23,24), and with the increase in β-lactam resistance, oral cephalosporins are no longer adequate for the treatment of CAP caused by intermediate resistant *S. pneumoniae*.

The increasing threat of multidrug-resistant Gram-positive and extended-spectrum β-lactamase producing Gram-negative pathogens fuels research into new cephalosporins which tends to yield potent, but narrow spectrum agents (25). Many current cephems developed as anti-MRSA agents will not adequately cover serious Gram-negative infections and are usually only suitable for parenteral administration (26). One of the few new oral penems, faropenem **1** which is sold in Japan, has potentially less liability for CNS side effects (27), excellent MIC_{90}s (0.008, 0.25 and

1) against U.S. strains of penicillin-susceptible, -intermediate, and -resistant *S. pneumoniae* strains and good activity against β-lactamase positive *H. influenzae* and *M. catarrhalis* (28). However, feropenem showed reduced activity *in vitro* against *S. pneumoniae* in the presence of human serum (29). A prodrug form, faropenem daloxate, is being investigated as a clinical candidate for CARTIs (30).

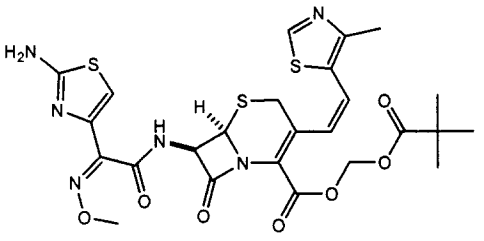


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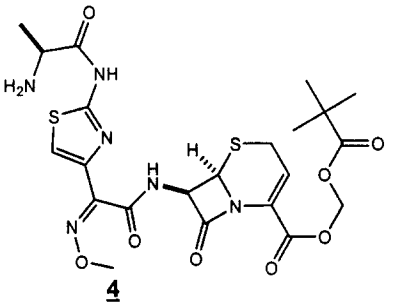


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Driven by the need for oral activity, some cephalosporins have been developed as prodrugs to reduce polarity and increase membrane permeability. Cefotiam **2**, a mainstay product in Japan, employs a cyclohexylcarbonate group to prodrug the cephem carboxyl (31). More recent third-generation cephalosporins, such as cefditoren pivoxil **3** and ceftizoxime alapivoxil **4**, use a lipophilic pivaloyloxymethyl ester. The prodrug, while stable in the gastrointestinal lumen, is rapidly hydrolyzed by intestinal esterases once absorbed to release the active drug. The activity and clinical utility of cefditoren pivoxil has been reviewed (32). Cefditoren has activity against most common CARTI bacteria, but is not active against atypical pathogens, including *C. pneumoniae*, *M. pneumoniae*, and *Legionella* spp. Cefditoren penetrates rapidly into bronchopulmonary and tonsillar tissue leading to clinical cure rates in AECB (dosed at 200 or 400mg bid for 10 days) of 88 to 89% within 48 hours of treatment completion (33). The antibacterial activity of cefditoren was compared to six other cephalosporins against 250 pneumococci, including strains resistant to macrolides and quinolones, giving the lowest MIC_{90}s for penicillin-resistant pneumococci and excellent MBCs. MICs were also exceptional for β-lactamase producing *H. influenzae* (≤0.016 mg/L), and overall showed low resistance frequencies (34).



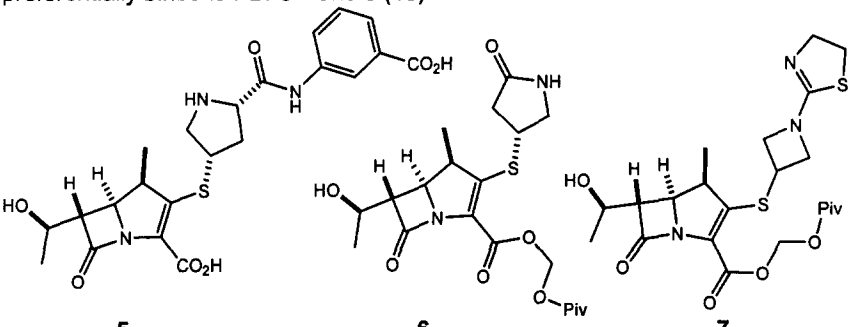
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4

Derived from a parenteral use cephalosporin, ceftizoxime alapivoxil (CFX-AP, AS-924) has an alanine appended to the weakly basic amino group of the cephem thiazole. This bifunctional prodrug strategy balances the solubility and lipophilicity of the molecule for improved physical properties and oral bioavailability. The physicochemical properties and oral absorption of ceftizoxime alapivoxil have been reported along with its antibacterial activity (35). Ceftizoxime alapivoxil has a similar spectrum of activity to other common cephems, but has particularly good MICs against Enterobacteriaceae and *H. influenzae*. Oral dosing gave potent protection in mouse models of Gram-positive and -negative infections (36).

Carbapenems and Trinems - Since the discovery of thienamycin in 1976, only three carbapenems have become widely used for serious infections in the hospital and ICU settings, namely imipenem, meropenem and panipenem. In contrast to cephalosporins, carbapenems have shown a broad spectrum of activity especially against resistant hospital-acquired infections caused by MRSA, VRE, PRSP *Pseudomonas* spp. They can overcome a wide variety of β-lactamases and demonstrate excellent *in vivo* activity. However, the lack of oral bioavailability in the broad spectrum carbapenems and trinems, their short half-life, potential for convulsive activity and high cost of manufacturing have directed research in this class toward the severe hospital infection market (37-39). Carbapenems have also been associated with nephrotoxicity. For example, imipenem is coadministered with cilastatin, an inhibitor of renal dehydropeptidase enzymes (DHP-1), and panipenem is give with betamipron which may block active transport of drugs into the renal cortex (38,40). Launched in 2002, one of the newest parenteral carbapenems, ertapenem **5** (formerly MK-826), has shown excellent efficacy against CAP (1g qd, *iv* or *im*) even in patients with comorbidities (41). Ertapenem seems to have evaded many of the toxicities and liabilities observed historically in carbapenems and has thus far been well tolerated in clinical trials (42). Ertapenem is stable to DHP-1 and preferentially binds to PBPs 2 and 3 (43).



5 **6** **7**

Motivated by the spread of β-lactam resistance, research is yielding a new generation of oral carbapenems to address these challenges (44). CS-834 **6**, an oral carbapenem antibiotic, is in phase II trials in Japan and Europe for RTI (45). CS-834 is the active prodrug of R-95867 which is stable to extended spectrum β-lactamases and active against most RTI pathogens except *Enterococcus* spp. and *Pseudomonas* spp. (39). In mouse models of PRSP infections, CS-834 has shown good efficacy with tid dosing (46). Tebipenem **7** (L-084, ebipenem pivoxil) is an oral 1-β-methylcarbapenem prodrug of LJC-11036 in phase II trials for RTI in Japan (47). Tebipenem displayed a promising safety profile in healthy adults with a half-life of 30 min (48). While tebipenem showed no useful activity against *Enterococcus* spp. and *Pseudomonas* spp. (39), it was effective *in vitro* and *in vivo* against most common CARTI bacteria with good distribution to the lungs (49). From an active series of THF carbapenems, CL-191121 was orally available as the prodrug OCA-983, stable to DHP-1 and efficacious in mice, but was dropped from development (50).

Macrolides and Ketolides - Primarily prescribed for treatment of CARTIs, erythromycin and second-generation semisynthetic derivatives, such as clarithromycin and azithromycin, together account for >$3B in sales worldwide (2001) and have been comprehensively reviewed (51,52).

Macrolides exert their antibiotic activity by inhibiting protein translation by binding to the 23S rRNA of the 50S ribosomal subunit. Structural studies suggest this binding disrupts peptide elongation, leading to chain termination (53). Macrolides are generally bacteriostatic agents with broad-spectrum activity. They are effective against the principal pathogens involved in CAP, including *S. aureas*. as well as

atypical bacteria, an advantage over β-lactams that macrolides share with fluoroquinolones (54). Second generation macrolides (e.g. clarithromycin, azithromycin) have superior activity against Gram-negative species, notably *H. influenzae*, compared to erythromycin, and improved pharmacokinetic properties. Azithromycin, in particular tends to accumulate in tissues, attaining lung levels several hundred-fold greater than plasma concentrations (55). These high tissue concentrations, particularly in phagocytic cells, allow clearance of infection with shorter treatment time (3-5D for pneumonia). In addition to their antibacterial effects, some macrolides are also reported to reduce lung inflammation in animal models and in patients (56,57). Macrolides are considered among the safest antibiotics available, with GI effects the most commonly reported problem. Drug-drug interactions due to inhibition of CYP 3A4 can limit use of specific macrolides in combination with other commonly prescribed treatments. For example, cardiotoxicity (QT interval prolongation, torsade de pointes) can result when macrolides are co-administered with an H1 antagonists or cisapride (51).

The safety and efficacy of macrolides have lead to widespread use and a parallel increase in prevalence of resistance. In the US and EU, macrolide resistance in *S. pneumoniae* has reached levels ≥30% (58). Two mechanisms account for the majority of resistance to macrolides in *S. pneumoniae*: target modification and drug efflux (59). The most common target modification is methylation of the 23S rRNA at a key contact point, adenine 2058, by *erm*-encoded methylases. *Erm*-mediated macrolide resistance in streptococci can also confer resistance to lincosamide and streptogramins, a phenotype termed MLS_B. *Erm*(B) in streptococci is encoded on transposable elements and has become widely disseminated (60). The balance of macrolide resistance is mediated by an efflux pump encoded by the *mefA* gene (M type resistance). In the US, 60-80% of macrolide-resistant *S. pneumoniae* are *mefA* positive (61), while in Europe, efflux pump-mediated macrolide resistance is less prevalent (~5%) (62). Macrolide resistance mediated by mutation in ribosomal components is also observed, either in the rRNA (ML type) or L4 ribosomal protein (MS type resistance). However, these mutations account for little of the macrolide resistance observed in CAP patients.

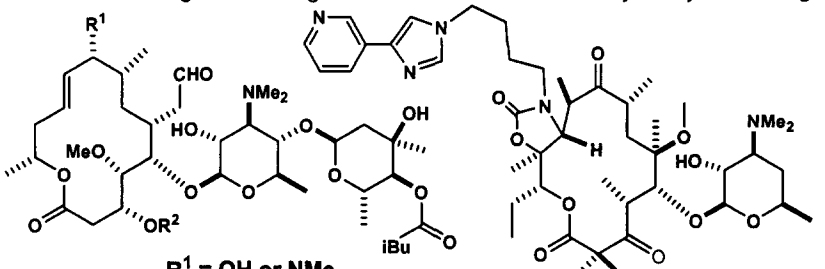
Efforts to develop new drugs in the macrolide class have been focused on identifying molecules that overcome existing resistance mediated by *mef* and *erm*. The ketolide modification at C-3 overcomes inducibility of *Erm*-mediated resistance and also confers activity against *Mef*-dependent resistance in streptococci (62, 63). Additional advantages of ketolides over earlier macrolides include increased potency against respiratory pathogens and a longer post-antibiotic effect (62). Conformational control afforded by a cyclic C-11,12 carbamate group also contributes to the potency of ketolides against both sensitive and resistant organisms. Elaboration of several 14-member ketolide series has been pursued, including 11-N-substituted, 6-O-substituted, 2-fluoro, tricyclic, and C-13-modified ketolides (52, 62). An aryl group at the 6-O or 11-N position contributes to the ability of ketolides to overcome *Erm*-mediated resistance by providing a second site of interaction with the ribosome. Addition of fluorine at C-2 can improve pharmacokinetic properties, while C-13 modifications allow elaboration of novel skeletons without loss of potency (62).

Telithromycin (previously HMR-3647), a 6-O-methyl-11,12-cyclic carbamate, is the first marketed ketolide (64,65). Telithromycin has significant *in vitro* activity against nearly all macrolide-sensitive and -resistant *S. pneumoniae*, e.g. with 99.4% of *S. pneumoniae* isolates susceptible to telithromycin in recent study of 157 isolates from CAP patients (66). Telithromycin is also effective against *H. influenzae* and atypical infections *in vitro* and *in vivo* (80). Telithromycin has shown excellent response rates in clinical trials of CAP (reviewed in 63). Clinical response rates in 3

randomized, double-blind trials ranged from 79-95% with doses of 500-1000 mg qd for 7-10 days. Telithromycin has also been shown to be effective in treating pneumococcal bacteremia in CAP patients (67). Telithromycin gained EU approval in July 2001 for community acquired infections, including those caused by resistant organisms. In 2001 an FDA advisory committee declined to approve telithromycin beyond an indication for CAP, due to concerns of potential cardiac and liver toxicity (68). Subsequently, an additional 24,000-patient Phase III trial demonstrated similar efficacy and safety for telithromycin and amoxicillin, with serious adverse event (SAE) rates of 1.3% and 1%, respectively (69). The most commonly reported SAE associated with telithromycin in this large Ph III trial was a transient blurring of vision (0.61%), while no drug-related cardiac events were observed (69). On the basis of these and previous results, the FDA advisory committee deemed telithromycin "approvable" in January 2003 for CAP, AECB, and sinusitis (70).

Cethromycin (ABT-773) is a 6-*O*-substituted ketolide that has also advanced to clinical trials. Like telithromycin, cethromycin has potent activity against both sensitive and macrolide-resistant *S. pneumoniae*, other Gram-positive organisms, and Gram-negatives including *H. influenzae* (63). In a Canadian study of CAP pathogen isolates from 1999-2002, MIC_{90} of cethromycin for all *S. pnuemoniae* was 0.008, versus 0.25 and 0.5 for clarithryomcin and azithromycin, respectively (71). In rat models of pneumonia, cethromycin demonstrated efficacy against *S. pneumoniae* and *H. influenzae* in the range of 1-20 mg/kg/day (63). No data from cethromycin clinical trial have been published to date. Another novel 6-*O*-substituted ketolide, A-217213, which has equal or superior efficacy against CAP pathogens *in vitro* and in rodent infection models compared to telithromycin has been reported (72,73).

Recent novel 2-fluoroketolides include HMR3562 **8**, HMR3787, where the 4-(3-pyridinyl)-1*H*-imidazol has been replaced with a 3*H*-imidazo[4,5-*b*]pyridine moiety, and RU64399 which is des-fluoro HMR3787. RU64399 and HMR3787 have pre-clinical activity against *H. influenzae* similar to azithromycin (74), while HMR3562 is active against enterococci, including vancomycin-resistant organisms (75). A series of clarithromycin analogues with telithromycin- or ABT-773-type side chains at C11 gave good activity against both *erm* and *mef*-mediated resistant *S. pneumoniae*. The best analogues had saturated linkers 4 or 5 carbons in length (76). Through application of a ring contracting metathesis, 16-membered josamycin analogues



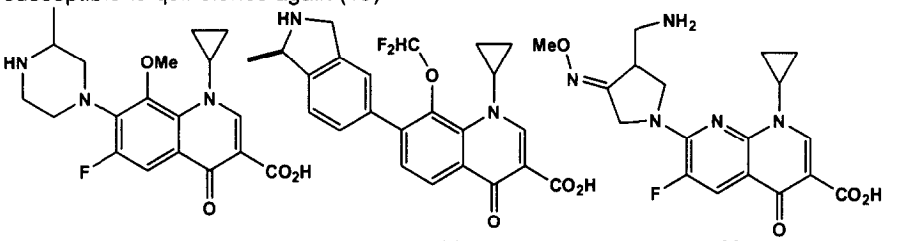
9 R^1 = OH or NMe_2
R^2 = H or Ac

8

were converted into novel 14-membered macrolides **9** which retain much of the parent's activity against *mef*-mediated resistant *S. pneumoniae* (77,78). A tricyclic ketolide, TE802, has also shown activity against drug-resistant *S. aureus* and *S. pneumoniae* (79). In addition to the ketolides, new azalides related to azithromycin but having activity against some *S. aureus* and *S. pneumoniae* strains have been reported (80).

Quinolones - Quinolone antibiotics are typically employed to treat CALRTIs, such as CAP and AECB, but are sometimes used to treat acute bacterial sinusitis. The

quinolones of choice in the outpatient setting are those with an enhanced *S. pneumoniae* activity like that of levofloxacin. The efficacy, success rates and safety of quinolones e.g., ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin **10** and trovafloxacin, in clinical studies for CAP and AECB have been recently summarized (10,81). Comprehensive reviews of quinolone clinical utility, pharmacokinetics, adverse effects and microbiology have been published (82-84). A recent review covering the most commonly prescribed fluoroquinolones examines the mechanisms which lead to adverse side effects, such as cardiotoxicity, phototoxicity and CNS effects (85). Because of their target of action, resistance toward fluoroquinolones are primarily due to mutations in *gyr A* and *parC*, although efflux may also play an important role in quinolone resistance. For resistant *S. aureus* and *S. pneumoniae*, it has been shown that inhibiting the NorA quinolone pump renders both strains susceptible to quinolones again (19).

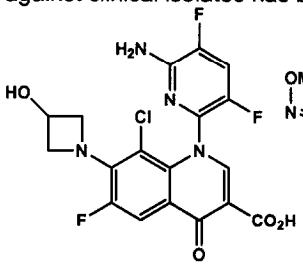


10 **11** **12**

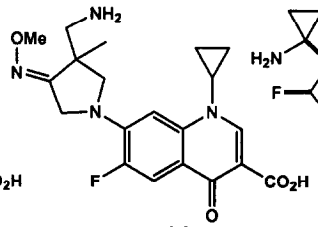
With safety as a major concern e.g., the clinical issues of grepafloxacin, clinafloxacin and trovafloxacin (86), new fluoroquinolones, such as moxifloxicin, are being scrutinized for adverse effects (87). Thus, improved safety, as well as resistance profiles, are driving current research. The latest developments in DNA gyrase inhibitors addressing these issues has been published with a focus on the C-8 methoxy fluoroquinolones moxifloxacin and gatifloxacin and new advanced research compounds (88). Quinolones with C-8 methoxy groups have shown a lower propensity to induce resistance in *S. pneumoniae* (89). A full profile of moxifloxicin's microbiology, lower resistance frequency rates for *S. pneumoniae* and improved safety profile is available (87). The clinical effectiveness and results of postmarketing surveillance have also been published (90). A full evaluation of gatifloxacin's antibacterial activity, clinical PK and efficacy has also been reported (91). A new des-fluoro(6)-quinolone ganefloxacin (BMS-284756, **11**) in late stage development was reported to be safe and well tolerated in phase II studies of CAP and AECB (92,93). Multiple *iv* administration did not prolong the QTc interval (94), and less then 2% of patients were reported having CNS toxicity (95). No phototoxicity has been seen (96). As part of the SENTRY program, ganefloxacin was the most potent quinolone overall against *Staphylococcus* spp. (MIC50 $\leq$ 0.03 mg/L) and Streptococcus spp. (MIC50 0.06 mg/L), but less effective against the enteric Gram-neg. bacilli when compared to ciprofloxacin, gatifloxacin and levofloxacin (97). Ganefloxacin is highly effective against antimicrobial resistant pneumonococcal isolates and *S. aureus* strains, but less effective against the ciprofloxacin-nonsusceptible *S. aureus* (98,99). It is also potent against *C. pneumoniae* and *Legionella* spp. (100,101).

A new fluoro-1,8-naphthyridinone with a unique oxime pyrrolidine moiety, gemifloxacin **12**, has been in phase III studies for CARTIs. A complete profile of this quinolone has been published (102). While the *in vitro* activity of gemifloxacin is excellent against the majority of CARTI pathogens (103), there was some indication of phototoxicity and CNS effects in clinical trials (102). Currently in phase II clinical trials, ABT-492 **13** has been highly effective in pre-clinical studies showing excellent oral activity against *S. pneumoniae* and *H. influenzae* in a rat lung model and enhanced activity against Gram-positive species (104,105). ABT-492 showed excellent antibacterial activity against 300 strains of aerobic and anaerobic

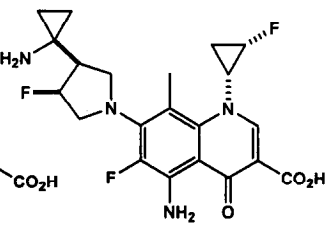
pathogens from sinusitis isolates, including quinolone resistant species, and 410 *Legionella* spp. (106,107). Pazufloxacin, an orally active quinolone, was launched in Japan in 2002 (108,109). Other late stage, orally active research compounds include WCK-919 which is efficacious against fluoroquinolone resistant *S. pneumoniae* in a mouse lung model (110), olamufloxacin (111), DK-507k which showed very good activity against *S. pneumoniae* (112), as did DW-286 **14** both *in vitro* and *in vivo* (113). DW-286 was also potent against quinolone-resistant *S. aureus* (114). DQ-113 **15** was active against PRSP and vancomycin-insensitive *S. aureus* in mouse models of pulmonary infection (115,116), and it's *in vitro* activity against clinical isolates has been reported (117).



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Chapter 20. Beyond Kinases: Purine-Binding Enzymes as Cancer Targets

Zhenhai Gao, David M. Duhl and Stephen D. Harrison
Chiron Corporation, Emeryville, CA 94608

Introduction - The ideal target for small molecule drug discovery is one that is essential for the disease state, yet non-essential for normal tissues. From a practical perspective, the ideal class of target has a proven track record of drugability, i.e. a history of the identification of agonists or antagonists with acceptable pharmaceutical properties. One recently emerged class of drugable targets is the protein kinases (1), exemplified by bcr-abl, the target of the highly successful new CML drug Gleevec (2). The common drugable feature of the kinase class is the ability to bind ATP. The ATP purine moiety is bound in a hydrophobic environment of the active site and stabilized by key hydrogen bonds (3). Such a site favors binding of flat, aromatic heterocycles, a class of compound that has proven amenable to optimization and drug development, thus making kinases eminently drugable. However, kinases are not the only class of enzyme that binds purines through key hydrogen bonds and hydrophobic stacking interactions; other such purine-binding enzymes include ATPases, GTPases, sulfotransferases and others. Thus, these enzymes offer additional possibilities for drug discovery. To illustrate the potential of this emerging class of drug targets we will focus our discussion on oncology and specifically on ATPases (Hsp90 and the kinesin KSP), GTPases and Sulfotransferases.

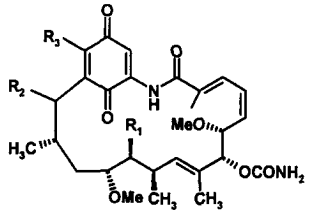
ATPases

Heat-shock Protein 90 (Hsp90) - Hsp90s are ATP-dependent molecular chaperones that are preferentially over-expressed (2-10 fold) in some cancer cells (4). Crystallographic studies have revealed the existence of a non-conventional low affinity ATP binding cleft at their N-terminal domain that is well-conserved among the four family members: Hsp90 α and β, Grp94 and Trap-1 (5). The occupancy of the ATP binding site by the ansamycin antibiotics geldanamycin (GM), **1**, and herbimycin A (HA), **2**, as well as the structurally unrelated fungal metabolite radicicol, **3**, inhibits the intrinsic weak ATPase of Hsp90, and results in simultaneous destabilization and eventual ubiquitin-dependent degradation of its client proteins (6-9). Uniquely, many of the Hsp90 client proteins are well-known oncoproteins that are frequently mutated or over-expressed in cancer cells, such as the mutated p53, Bcr-Abl, Raf-1, Akt, ErbB2, and steroid receptors (9). The association with Hsp90 allows these otherwise unstable oncoproteins to function properly in multiple oncogenic signaling pathways, which are essential in maintaining the uncontrolled proliferation and the malignant phenotypes of tumor cells. Depending on cellular contexts, Hsp90 inhibitors effectively cause growth arrest, differentiation, or apoptosis of tumor cells both in *vitro* and in *vivo* (10-12). It is also conceivable that Hsp90s may be the key components of the very machinery that allows certain cancer cells to evoke alternative or overlapping signaling, and to efficiently develop resistance to a specific drug treatment (13). Hence, inhibitors of Hsp90, by concurrently disrupting a wide range of oncogenic pathways, may prove to be a very effective approach to combat a variety of hard-to-treat tumors.

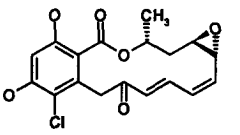
17AAG, **4**, a derivative of GM, is the first-in-class Hsp90 inhibitor that has entered human clinical trials in patients with solid tumors. It has recently completed phase I trials and is progressing to phase II (5). In the extensive preclinical studies, 17AAG has been shown to be very effective in blocking tumor growth in various human tumor xenograft models (5, 10). Although the efficacy of 17AAG was not the primary point of evaluation in the Phase I trial, very encouraging disease stabilization was observed in some patients with advanced solid tumors (14). In addition, 17AAG

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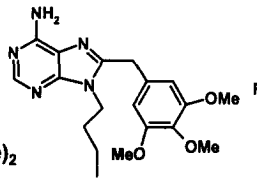
also caused the expected reduction in the expression level of several "surrogate markers" such as Raf-1, Lck, and cdk4 in treated patients (14) Nonetheless, 17AAG is generally being viewed only as a valuable proof-of-concept anti-tumor agent, and may not eventually become a marketed drug due to several salient drawbacks: 1) poor solubility and difficulty in formulation; 2) lack of an easy synthetic route and dependence on fermentation; 3) hepatotoxicity associated with benzoquinone moiety in its structure; 4) lack of selectivity among the four Hsp90 family members, and 5) ineffectiveness on a subset of cancer cells (15). Likewise, the clinical development of radicicol derivatives has also been impeded by the fact that these compounds cause cataracts, an off-target effect in animals (16). Several GM analogs with pharmaceutical properties (i.e. potency and solubility) superior to those of 17AAG have recently been developed, e.g. the water-soluble 17-(dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, **5**) (17). The clinical advantages and benefits of these improved GM derivatives remain to be determined.



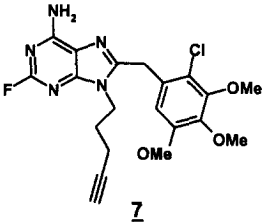
1: R_1=OH, R_2=H, R_3=OCH_3
2: R_1=OCH_3, R_2=OCH_3, R_3=H
4: R_1=OH, R_2=H, R_3=$NHCH_2CH{=}CH_2$
5: R_1=OH, R_2=H, R_3=$NHCH_2CH_2N(Me)_2$



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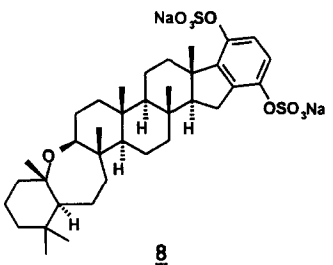
The published high-resolution crystal structures of Hsp90, in complex with adenosine nucleotides, GM or radicicol have raised the exciting possibility of developing selective Hsp90 inhibitors using structure-based rationale drug design (6, 8). ATP binds to Hsp90 in an unusual kinked conformation that distinguishes Hsp90 from other chaperone proteins or kinases. By computer-modeling the structure requirements, the first purine-based small molecule Hsp90 inhibitor, PU3, **6**, was designed and synthesized (15). The relative binding affinity of PU3 for Hsp90 is 15 to 20μM. Subsequent optimization and screening of a small biased library of compounds with purine scaffolds, has led to the identification a compound, PU24FCl, **7**, that displays Hsp90 binding affinity, potency and a physiological profile comparable to those of 17AAG (16). In addition, a homogenous fluorescence-based conformational assay suitable for high-throughput screening (HTS) has recently been designed, which may help accelerate the pace of identification of potent and selective small molecule Hsp90 inhibitors with favorable drug-like properties (18).

While current drug development efforts have been focusing on the N-terminal ATP binding site, recent studies have suggested the existence of a cryptic ATP binding pocket located at the C-terminus of Hsp90 (19). More importantly, occupancy of this site by the gyrase inhibitor novobiocin, has also been shown to impair the chaperone activity of Hsp90, and cause cellular depletion of multiple client proteins (19). Therefore, targeting the C-terminal ATP binding site may represent a novel strategy to fully harness the anti-cancer potential of Hsp90 inhibitors.

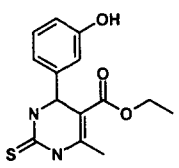
Kinesin Spindle Protein (KSP) - Pharmaceutical agents that disrupt the function of the mitotic spindle and arrest cells in mitosis have proven to be remarkably effective

and powerful chemotherapies for a broad spectrum of advanced tumors. Currently, all clinical anti-mitotic drugs act upon β-tubulin and affect the dynamics of microtubule (MT) polymerization and depolymerization, leading to cell cycle arrest and programmed cell death (20). One such agent, paclitaxel (Taxol®) has been widely recognized as one of the most clinically and commercially successful anti-tumor therapeutics developed in the past decades. Nonetheless, peripheral neuropathy, a notable side effect that is predictable from the critical role of MT in axonal transport in neuronal cells, has severely limited long-term use of these tubulin-binding compounds (20).

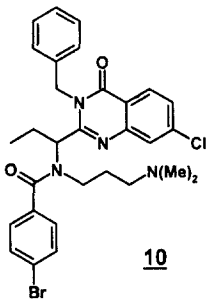
The recent advance in understanding the complex process of mitosis has led to the identification of several drugable targets that are not only essential but also more specific to the mitotic spindle apparatus. Of particular interest is KSP (also called Eg5). KSP is a member of kinesin superfamily of MT motors, which convert the energy released from ATP hydrolysis into mechanical force for transport along microtubules in the cell (21). A functional KSP is required for the establishment and maintenance of mitotic spindle bipolarity, and the proper segregation of replicated DNA into two daughter cells (22, 23). Moreover, KSP is predominantly localized to the mitotic spindle and appears to assume no function outside mitosis. Thus, specific inhibitors of KSP could have superior therapeutic value compared to tubulin-binding drugs in that they could effectively cause mitotic arrest and apoptosis in proliferating cancer cells, but may not incur the liability of producing significant neurotoxicity.



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Both ATP hydrolysis and MT binding occur at the N-terminal motor domain of KSP (24). The nucleotide binding state regulates the KSP affinity for MT, and binding of MT in turn promotes ADP release and enhances ATP turnover rate. The nonhydrolysable nucleotide analogs (AMPPNP and AMPPCP) and adocia-sulfate-2, **8**, a compound isolated from a marine sponge, are well known and characterized KSP inhibitors that are competitive with ATP and MT binding, respectively (25, 26). They are not, however, specific to KSP or cell-permeable, precluding the possible clinical use of these compounds. To date, development of potent and selective ATP- or MT-competitive KSP inhibitors has not been successful, presumably due to the fact that N-terminal motor domain shares significant structural and sequence similarity with other kinesins. An early attempt at structure-based rationale drug design using K560 (another member of kinesin superfamily) has led to the identification of several organic compounds that appear to be competitive for MT binding but not for ATP binding, and showed somewhat low selectivity (2-5 fold) for members of kinesin superfamily (27). As more and more crystal structures of kinesin proteins are becoming available, it would be interesting to determine if any of the observed subtle structural differences can be translated into the design of specific KSP inhibitors that target either ATP or MT binding pockets.

An alternative and appealing strategy to develop a KSP-specific small molecule inhibitor is to target an allosteric binding site that would interfere with the MT-

dependent ATPase activity. This approach appears to be feasible as demonstrated by recent identification of the first KSP-selective allosteric inhibitor, monasterol (IC_{50}=14-20 μM), **9**, by a phenotype-based screening of a 16,320-compound library (28). Subsequent high-throughput screening has resulted in the discovery of more potent and selective allosteric inhibitors, such as quinazolinone-based compounds CK-0106023, **10**, and CK-0238273 (SB-715992) with binding affinity values (K_i) below 20 nM (29). Detailed kinetic studies with monasterol and CK-010623 have shown that both compounds drastically reduce the MT-stimulated and basal rates of ADP release, but do not compete with either ATP or MT binding to KSP, indicating a novel allosteric inhibition mechanism (30, 31). Although all these inhibitors appear to interact with the N-terminal motor domain, the exact binding location and structural determinants remain elusive. Thus, the x-ray crystal structure of KSP in complex with an allosteric inhibitor will be extremely valuable for future design and optimization of KSP-selective small molecule inhibitors.

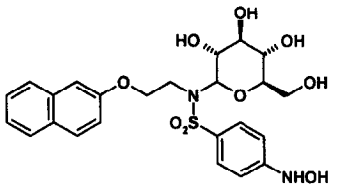
CK-0238273 has recently entered into phase I clinical trial. In preclinical studies, CK-0238273 (K_i=0.6 nM) is very potent (IC_{50}, 1.2-9.8 nM) in causing mitotic arrest in various cultured human cancer cells (e.g. Colo205 and HT-29) with the appearance of a monopolar spindle, a hallmark of KSP malfunction during mitosis (32). In a broad panel of murine and human tumor xenograft models, CK-0238278 shows striking anti-tumor activities, with complete tumor regression observed with mice implanted with Colo205, Colo201, and Madison 109 lung carcinoma, and significant growth inhibition with HT29 and LL/2 Lewis lung carcinoma (32-34).

An important observation is that in a mouse model, paclitaxel clearly produces peripheral neuropathy, whereas this side effect is absent from CK-0238278 treated mice (32). This result is consistent with the notion that KSP function is restricted to the mitotic spindle in rapidly dividing cells, and bolsters confidence that selective KSP inhibitors could be superior anti-mitotic chemotherapeutic agents with a novel mechanism of action for advanced tumors.

GTPases

GTPase proteins regulate intracellular signaling pathways by cycling between an active (GTP bound) conformation and an inactive (GDP bound) conformation (35-37). When GTP is bound, the GTPase interacts with a diverse population of effector proteins to propagate the desired signal. The signaling cascade thus initiated by the activated GTPase will continue until the GTP is hydrolyzed into GDP and the protein assumes the inactive (GDP bound) conformation. The GTPases are themselves regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activation proteins (GAPs). The GEFs act to accelerate the exchange of GTP for GDP, and therefore facilitate the transition of the GTPase to the active state. The GAPs accelerate the hydrolysis of GTP to GDP, and therefore facilitate the transition of the GTPase to the inactive state. Thus far, interest in GTPases as anti-cancer drug targets has focused on two families of proteins: the RAS/RHO family of small monomeric GTPases (20-30 kilo Dalton), and the heterotrimeric family of guanine binding proteins (commonly known as G proteins)(38, 39).

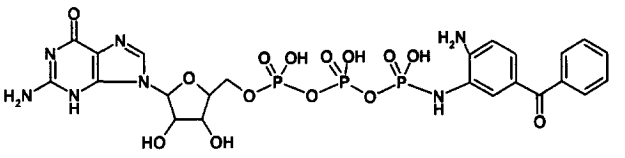
The high intercellular concentrations of GTP and the high binding affinity of the RAS/RHO family for GTP and GDP have been seen by many to be an overwhelming hurdle to targeting GTPases. Nevertheless, two promising approaches to cancer drug discovery have been identified: small molecules that affect the exchange of GTP for GDP by binding outside of the guanine binding pocket, and modifications of GTP which can facilitate GTP hydrolysis by mutated forms of RAS (substrate assisted catalysis).



11

GTPases are known to have a highly flexible and mobile active site, and this flexibility has been seen as the reason for their relatively poor catalytic efficiency (40). GEFs and GAPs are thought to stabilize conformations of the protein that facilitate the exchange of GTP for GDP and the hydrolysis of GTP, respectively (41, 42). In theory, it should be possible to stabilize the inactive state. One possibility might be to identify GDP analogs that lock the enzyme in its inactive conformations. An alternative would be to identify small molecules that bind to the GTPase in the inactive (GDP bound) state and inhibit the exchange of the GDP for GTP, thus potentially inhibiting the oncogenic nature of GTPases. Taveras and co-workers, in an attempt to develop compounds that bind RAS, have identified such an inhibitor (43). The authors use NMR, mass spectrometry, and molecular modeling to demonstrate that SCH-54292, **11**, is bound to RAS-GDP in a major cleft of the switch-2 region. The highly flexible switch-2 region is known to change its conformation depending on whether GTP or GDP is bound to RAS. This result suggests that, with the appropriately designed screen, it should be possible to identify compounds that bind the switch-2 region, and stabilize the inactive conformation.

Substrate assisted catalysis (SAC) has been reviewed in detail elsewhere (44). In short, SAC envisions that both the enzyme and the substrate (GTP in the case of GTPases) play a role in the rate of catalysis. The crystal structures of RAS in both the GDP bound state and a transition state form of the enzyme (GDP-aluminum fluoride bound), have identified two amino acids in the binding pocket of all GTPases that are crucial for catalysis of GTP (Gln_{cat} & Arg_{cat}) (41, 42, 45). In both the small monomeric and heterotrimeric GTPases, Gln_{cat} is thought to function by polarizing the nucleophile and by orienting it for attack on the γ phosphate (46). Mutations of Gln_{cat} decrease the intrinsic rate of hydrolysis by up to two orders of magnitude. The second essential catalytic amino acid is Arg_{cat}, which is thought to stabilize the transition state of hydrolysis. In small monomeric GTPases, Arg_{cat} is provided by insertion of an amino acid finger from a GAP (45), while in heterotrimeric GTPases Arg_{cat} is intrinsic to the protein itself. Similar to Gln_{cat} mutations, mutations of Arg_{cat} decrease the intrinsic rate of hydrolysis by up to two orders of magnitude. 3,4-Diaminobenzophenone-phosphoroamidate-GTP (DABP-GTP, (47)), **12**, has been shown to restore the activity of Gln_{cat} mutants to near wild-type rates, but does not restore the activity when Arg_{cat} is inactivated with cholera toxin. DABP-GTP has been shown to restore GTP hydrolysis to oncogenic GTPase deficient RAS mutated at either Gln61 or Gln12 (the most common mutation in tumors).

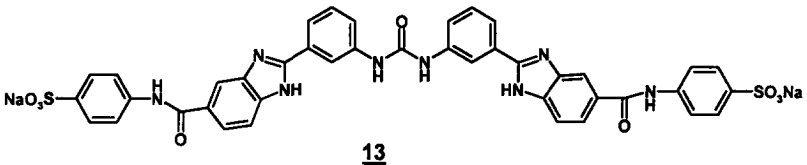


12

The Gα subunits of heterotrimeric GTPases contain the GTP catalytic domain. By grouping subunits with sequence identity of 50% or greater, Gα subunits are subdivided into four classifications (α_s, $\alpha_{i/o/t}$, α_q, $\alpha_{12/13}$) (48). Of the 16 mammalian Gα-subunits identified to date, $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{16}$, $G\alpha_z$, $G\alpha_{12}$ and $G\alpha_{13}$ have been identified as having oncogenic potential *in vitro*, with $G\alpha_{12}$ and $G\alpha_{13}$ being the most oncogenic (49). Also mutations of $G\alpha_s$ have been found in tumors

(pituitary tumors, thyroid adenomas and thyroid carcinomas). In addition to their oncogenic potential, Gα subunits can also be seen as targets for anti-cancer drug discovery by virtue of their role in downstream signaling from numerous oncogenic G-protein coupled receptors (50). Specifically, $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to directly regulate potentially oncogenic RHO GTPases through the interaction of RGS containing GEFs. Since unmutated RHO has been shown to be over-expressed in breast, colon, and lung tumors, inhibition of $G\alpha_{12}$, and $G\alpha_{13}$ may lead to the down regulation of this potent oncogene (51-54).

In support of the concept that binding outside of the guanine pocket may stabilize GTPase inactive conformations (outlined above), results similar to those with RAS and SCH-54292 have been found with heterotrimeric GTPases. Suramin, a symmetric polysulphonated napthylamine-benzamide-derivative used to treat African sleeping sickness, has been shown to inhibit the release of GDP from purified Gα subunits (55). As discussed for SCH-54292, Suramin is thought to bind the GTPase outside of the guanine pocket. Synthesis of Suramin-like compounds has lead to the identification of two compounds (NF503, **13**, and NF449) with increased specificity for $G\alpha_s$ over the other Gα subunits (56).

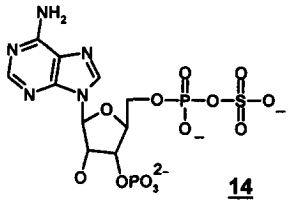


13

Through the convergence of nascent medicinal chemical efforts and an increased understanding of the biology, structure, and enzymology of GTPases, anti-cancer drug discovery within this area seems poised to release its untapped potential.

Sulfotransferases

Sulfotransferase (ST) enzymes catalyze the transfer of sulfate from the donor molecule, purine-based PAPS (3'phosphoadenosine 5'phosphosulfate, **14**) to a variety of intracellular substrates, ranging from small xenobiotics to macromolecules. These enzymes fall into two classes. Cytosolic ST enzymes, also referred to as SULTs, detoxify xenobiotics and modify small endogenous molecules, such as hormones and neurotransmitters. The ST enzymes from the second class, numbering over 30 individual types, are found within the Golgi membrane and catalyze the sulfation of carbohydrates (including glycoproteins, proteoglycans, and glycolipids) and proteins (57).



14

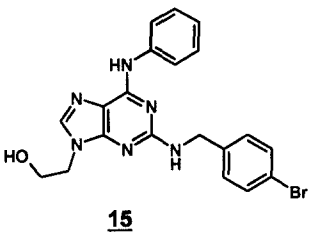
ST enzymes are being considered as therapeutic targets for a number of disease indications, including inflammation and cancer. The former application has previously been reviewed and we will focus on the potential for these enzymes as anticancer targets (58). It has been argued that members of both the cytosolic (SULTS) and Golgi ST classes have potential for the treatment of cancer. Because of the role of SULTs in xenobiotic detoxification, inhibitors of these enzymes may have use in potentiating the effect of cytotoxic chemotherapeutics. Estrogen sulfotransferase (EST) has also been suggested as a target because unusually high levels of its product, estrogen sulfate, have been found in breast cancer cells (59).

Amongst the Golgi enzymes, much interest has been focused on the carbohydrate sulfating enzymes. Carbohydrate sulfation modulates a number of processes that are important in cancer, such as cell-cell interaction and cell-matrix association and it is established that growth factor activity, such as that of fibroblast growth factor, is dependent on the presence of heparan sulfate. Chondroitin sulfation is associated with cancer progression, poor prognosis, tumor adhesion and metastasis (60-62). Thus inhibition of chondroitin ST enzymes may have therapeutic benefit. The protein tyrosine targeting ST enzymes may also have a role in tumorigenesis, as protein tyrosine sulfation, modifies extracellular protein/protein associations and hence potentially cell activation and environmental interactions (63).

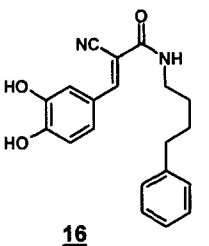
The mechanisms of action of ST enzymes suggest that this class may be susceptible to inhibition by drug-like small molecules. The sulfate group from the purine PAPS is transferred to a hydroxyl or amine, in a manner analogous to the phosphate transfer from the purine ATP to amino-acid hydroxyls during kinase-catalyzed phosphorylations. The precedent of identifying selective purine site inhibitors for kinases raises hope that similar selectivity will be achieved for ST enzymes. Indeed apart from the conserved PAPS phosphate binding sequences (see below), there is considerable sequence diversity between ST enzymes, reflecting a broad array of substrates. For example, even amongst the subset of carbohydrate-targeting Golgi enzymes there are a variety of sulfate acceptor sites, including either the 4' or 6' hydroxyl of N-acetylglucosamine (as in chondroitin 4-O-ST and chondroitin 6-ST respectively) and the 3' hydroxyl of glucoronic acid (as in HNK-ST) (64).

The design and optimization of chemical inhibitors of ST enzymes will be greatly aided by the availability of crystal structures of four such proteins (EST, hydroxysteroid ST, catecholamine ST and heparan sulfate N-deacetylase/N-ST) (65). As mentioned, the adenine-binding sites of these enzymes are related to those of kinases. Despite this similarity, the ST class possesses two characteristic nucleotide-binding motifs not seen in kinases. The PSB loop contains a P-loop such as found in ATPases (66) that interacts with the 5'phosphate of PAPS and the 3'PB domain associates with the 3'-phosphate that is found in PAPS, but not ATP (67).

Several inhibitors of different ST enzymes have been identified using a variety of approaches. The screening of *in vitro* enzyme assays has yielded several low potency inhibitors (68, 69). By monitoring inhibition of the Nacetylglucosamine-6-ST, NodH, from *Rhizobium meliloti*, several inhibitors were identified from a purine-based library (e.g. **15**: IC_{50}, 20μM) in addition to a single tyrphostin (**16**: IC_{50}, approximately 50μM). The purines showed some selectivity against other ST enzymes, including N-acetylglucosamine-6-O-ST from human high endothelium venules and keratin sulfate ST. In an alternative approach, a tyrosylprotein ST (hTPST-2) assay was screened against a library of small aromatic O-methyl oximes at high concentration (200μM) (63). Weak enzyme binders were then dimerized via a short linker to yield bidentate compounds in an attempt to promote synergistic binding. Two compounds (**17a**: IC_{50}, 30μM and **17b**: IC_{50}, 40μM) were identified, although strikingly these compounds turned out to be much more potent antagonists of EST (IC_{50}s, 250nM and 3μM respectively) and may form the basis for an optimization approach to generate EST inhibitors. A more directed

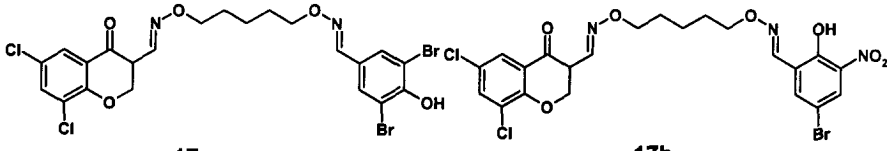


15

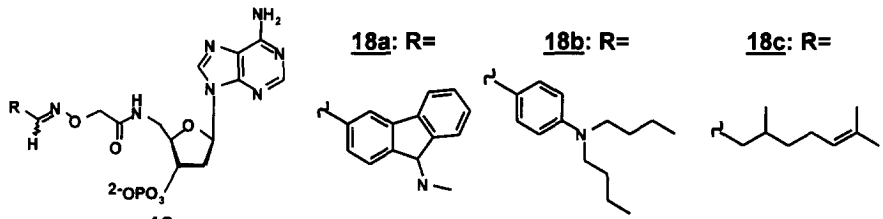


16

approach to bidentate inhibition has been suggested by analysis of ST crystal structures. Bertozzi and coworkers have attempted to generate potent EST ligands from a library of compounds based on a PAPS mimetic fused to potential analogs of the estrogen substrate, **18** (70). This approach yielded several weak binders (**18a**, **18b** and **18c**: 80-90% EST inhibition at 200μM). In a subsequent study, a PAPS/estrogen hybrid with closer similarity to the presumed transition state of EST catalysis was generated and displayed very high affinity binding (**19**, IC_{50}, 3-4nM). Although given that the two substrates have Km values comparable to this binding affinity, it is unlikely that synergistic association was achieved.

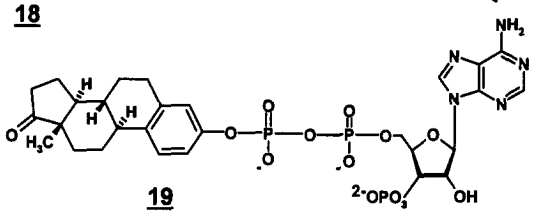


17a **17b**



18a: R= **18b**: R= **18c**: R=

18



19

This promising inhibitor screening data, coupled with the possibility of structure-based drug design and increasingly attractive rationales for targeting ST enzymes, suggests that this class of enzymes may soon become as popular as kinases as targets for cancer chemotherapeutics.

Conclusions - The recent success in bringing Gleevec to market and the advancement of other kinase inhibitors into late stage clinical trials has proven that kinases are drugable targets for cancer therapeutics. A deeper understanding of the ATP-enzyme binding interaction suggests that the utility of kinases as targets can be expanded to include many purine-binding enzymes. By increasing the diversity of drugable targets, there is the potential for broadening our repertoire of small molecules that can bind the purine pocket, as well as the opportunity to develop compounds that allosterically interfere with the binding interaction. While we have illustrated the potential of purine-binding enzyme targets with reference to a limited number of enzyme classes, many others exist, including topoisomerases, ion pumps, P-glycoprotein and other drug transporters. This wide array of potential drugable targets promises to be a fertile source of new drug leads over the coming years.

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Chapter 21. New Therapies for Malaria

Patrick M. Woster
Wayne State University
Detroit, MI 48202

Introduction - There is no doubt that significant advancements in antiinfective therapy have improved the quality of life in highly developed nations. However, in underdeveloped countries, there exist major infectious diseases that account for a large portion of global morbidity. Some of these diseases have the potential to become a threat to those living in North America. Malaria is the focus of this chapter, but many other life-threatening parasitic diseases exist. Tuberculosis claims an estimated 2 million lives each year, and drug-resistant strains originally found in New York and Russia are now being identified in other locations. Malaria, African trypanosomiasis and leishmaniasis accounted for an additional 1,210,000 deaths in 1999, and estimates suggest that these numbers are rising rapidly (1). Current therapies for parasitic infection are inadequate, especially in light of the emergence of drug-resistant parasitic strains. Many of the drugs currently used are toxic or non-efficacious, and there are no effective treatments for some parasitic diseases. Drug discovery efforts against the diseases mentioned above are limited, either because infected persons in underdeveloped areas cannot afford even a single course of therapy, or because the infected population is too small to justify the required research expenditures. Efforts to fight parasitic diseases in Third World nations are often hampered by economic issues and political turmoil. This virtually assures that the world's most impoverished people will continue to bear the major burden of parasitic disease. Clearly, there is a need for new antiinfective agents that are potent, non-toxic and inexpensive to manufacture. This report describes recent progress towards identifying suitable agents to treat malaria.

MALARIA

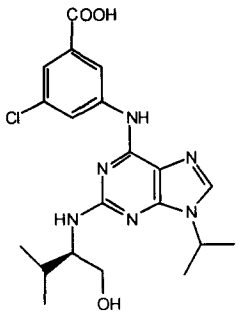
Background - Malaria is still one of the world's most deadly diseases that threatens 40% of the world's population, and infects approximately 300 million people worldwide. In Africa alone, more than one million children under the age of 5 die from malaria each year (2,3), translating to one death from malaria every 30 seconds. The World Health Organization has estimated that funding for malaria control alone, including only existing methods for vector control, will need to increase to $2.5 billion annually by 2007, and $3.1 billion annually by 2015 (4). Malaria-carrying mosquitoes have recently been found in the United States, causing infection in individuals who had not traveled to areas where the disease is endemic. In 2002, two pools of malarial mosquitoes were discovered near the Potomac River, one 4 miles and the other 6 miles from the homes of the two teenagers who were diagnosed with malaria. Authorities say it is the first case in at least two decades in which malaria has been detected in mosquitoes and humans in an American community (5). Outbreaks of malaria in the US could become more common due to global warming.

Human infection can be caused by four distinct species of the protozoon *Plasmodium*, but *P. vivax* and *P. falciparum* account for more than 95% of malaria cases. Nearly all deaths caused by malaria are due to infection by *P. falciparum* (3,6). Malaria is transmitted by the bite of the female anopheles mosquito, at which time sporozoites of *P. falciparum* are discharged into the puncture wound. Sporozoites are then carried to the liver, where they enter hepatic mesenchymal

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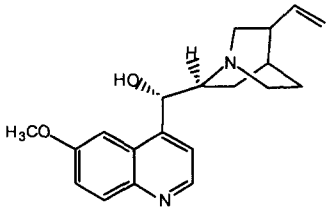
cells and begin to grow. Lysis of the hepatocyte then releases the merozoite form of *P. falciparum,* which invades host red blood cells (RBC's), feeding on hemoglobin during the erythrocytic portion of its life cycle (7). In the RBC, the parasite expresses a number of polypeptide products that are exported to the surface of the RBC, rendering it antigenic. These peptides are products of the *var, rif,* and *clag* genes located on chromosome 2 and 3 of the *P. falciparum* genome, and allow the infected RBC's to adhere to vessel walls, and to accumulate in specific organs such as the brain (2,3). In order to escape the host immune system, the parasite regularly exchanges these peptides in a process called antigenic variation. Although *P. falciparum* has a complex biochemistry and life cycle, recent research has revealed a number of potential new drug targets (6). Malaria has become more difficult to treat, due to an increase in multi-drug resistant strains (8). Indeed, the reemergence of malaria as a worldwide epidemic can be largely attributed to the rapid development of parasite resistance. Some progress has been made in identifying resistance markers in parasites that fail conventional therapy, such as the recent discovery of codon 268 mutations in the parasite cytochrome bc1 gene (9). Such approaches will lead to the identification of specific cellular changes that mediate resistance. Clearly, malaria research has and will continue to benefit from recent advances in genetics, as well as coupled efforts toward new drug discovery. Current major areas of antimalarial research include prevention and vector control, identification of new targets, development of an effective vaccine and design and synthesis of new antimalarial agents. The latter three areas of research focus will be discussed in this report.

Identification of New Targets for Antimalarial Therapy - In the past three years, remarkable advances have been made with respect to sequencing complete genomes for a variety of species. The recent publication of the human genome, the genome for *Plasmodium falciparum* and that of the vector mosquito *Anopheles gambiae* (10-13) have been collectively termed the "hat trick" of antimalarial research. Examination of differences in the regulation of gene products between host, vector and parasite can result in identification of target sites or alterations in biochemical regulation, as in the recent report of divergent regulation of the *Plasmodium* and human forms of dihydrofolate reductase (14). Using these and other approaches, a number of new targets for intervention have emerged. The aspartyl proteases plasmepsin I and II, discovered by the *P. falciparum* Genome Project, are hemoglobin degrading aspartyl proteases that represent promising targets for the design of parasite-specific inhibitors, since these proteases are critical to the survival the parasite (15-17). *Plasmodium* has been shown to induce changes in host erythrocyte membrane transport, and as such these processes may become drug targets (18). Three parasite-specific transport systems for hexoses, nucleosides and aquaglycerylporin have been functionally characterized in a *Xenopus* oocyte heterologous expression system, and the same system is now being used to study the P-type ATPases responsible for maintaining intracellular Ca^{++} homeostasis. Quinoline antimalarials have long been thought to act by binding to hemazoin, a crystalline form of heme that is not toxic to the organism. The binding of quinolines prevents proper crystallization of hemazoin, allowing the expression of free heme-mediated toxicity, and may also alter the pH inside the acidic food vacuole. A recent crystal study has identified a putative non-covalent binding site for quinoline antimalarials which may also represent a new target for antimalarial drug design (19). Finally, cyclin-dependent kinases (CDKs), which control cell division, apoptosis, transcription and differentiation in normal and tumor cells, are promising targets for inhibitors of growth in *Plasmodium* and other parasitic organisms (20). *Plasmodia* expresses CDKs which, despite a 40-60% sequence homology with human CDKs, are sufficiently divergent from their closest mammalian homologues to be considered distinct drug targets. The parasitic CDK *Pf*CK1 (casein kinase 1) was recently found to be a major target for the known CDK inhibitor purvalanol, **1**.

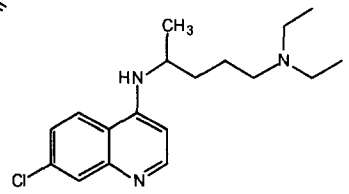


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Antimalarial Vaccines - A multilateral effort known as the Multi-Stage DNA-based Malaria Vaccine Operation (MuStDO) has been launched to develop DNA vaccines against *Plasmodium falciparum* (21). These vaccines are to be targeted against the pre-erythrocytic and erythrocytic forms of *P. falciparum*, and are intended to protect travelers and short term residents of high risk areas, as well as reduce morbidity in endemic populations. Vaccines targeted against 5 genes that encode for pre-erythrocytic form antigens (termed MuStDO 5) will neutralize sporozoite and early liver stage parasites, while vaccines targeting 10 additional genes (MuStDO 10) are designed to produce immune responses against merozoites and infected erythrocytes. Other targets for and approaches to the development of a suitable antimalarial vaccine have recently been reviewed (22). Proposed targets include antibodies to antigens expressed in the sporozoite stage (blocking invasion of hepatocytes) and the merozoite stage (blocking invasion of the red blood cell), as well as against toxins produced by *Plasmodium* that produce clinical disease and severe malaria. One such malarial toxin, a glycophosphatidylinositol-based hexasaccharide, has recently been synthesized using a solid phase method, and is being used for vaccine development (23). Genes for the potential transmission-blocking antigens Pvs25 and Pvs28 from *P. vivax* have been cloned and expressed in yeast,, and then used to generate antibodies in mice (24). These antibodies completely prevented transmission of *P. vivax* from infected sera, and have led to clinical trials, as well as extension of the work to the Pfs25 *P. falciparum* ortholog. Other antimalarial vaccines are currently in phase 1 and 2 clinical trials, including the RTS/S/AS02 antisporozoite antibody, and a series of DNA and recombinant viral vaccine products (25).



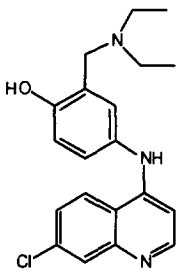
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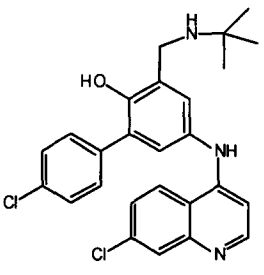
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Synthetic Analogues Related to Quinine and Chloroquine - The cinchona alkaloid quinine, 2, was the first compound to exhibit significant antimalarial activity. Subsequent studies produced the synthetic analogue chloroquine, 3, which was initially an excellent treatment for malaria. However, the evolution of chloroquine-resistant strains of *P. falciparum* have rendered this drug virtually useless in certain

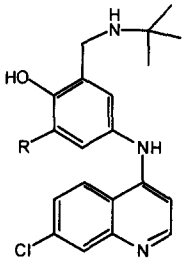
areas of the world (26). To address this resistance, a number of Mannich bases of chloroquine were synthesized, producing the active quinoline analogues amodiaquine, **4** and tebuquine, **5**. These analogues are significantly more active against *Plasmodia*, but unfortunately, chronic toxicity limits their use. A series of



4

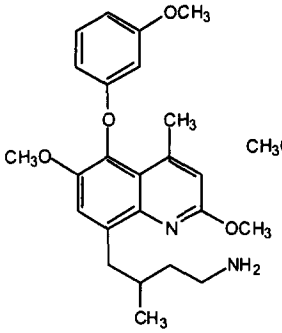


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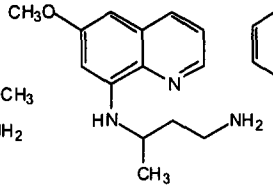


6 (R = propyl)
7 (R = isopropyl)

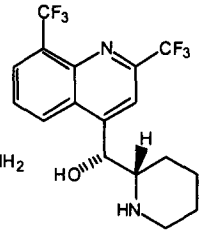
derivatives of **5** have been described in which the diethylamino moiety has been replaced by a tert-butylamino group, a modification which reduces cross-resistance (26). The 5'-substituted analogues **6** and **7**, proved to be the most effective antimalarials in vitro, with IC_{50} values of 0.98 and 1.24 nM against *P. falciparum*, and favorable cross-resistance profiles. Other quinolines of interest include tafenoquine **8**, which is a second generation agent related to primoquine, **9** and mefoquine, **10**. Tafenoquine, also known as WR 238605, appears to be an effective prophylactic agent for the prevention of *P. falciparum* malaria (27). Newly synthesized short chain quinolines such as **11** have been described that retain good antimalarial activity against the K1 chloroquine resistant strain of *p. falciparum* (28).



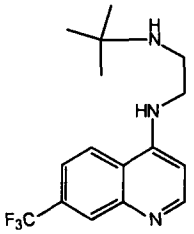
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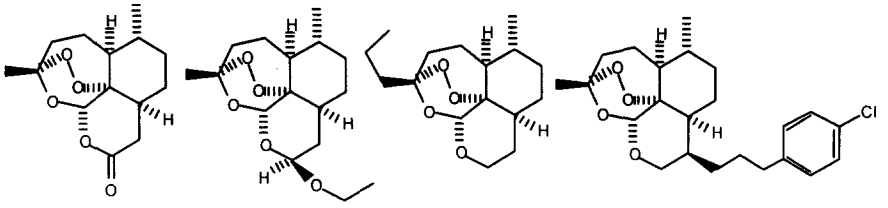


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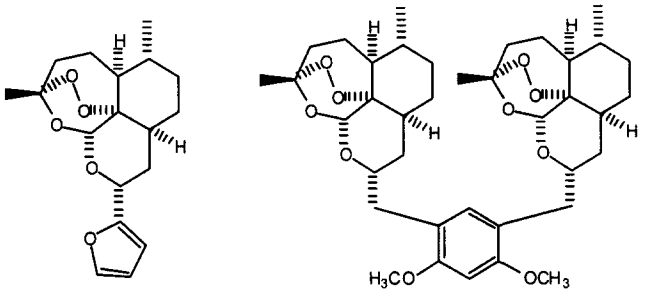


11

Artemisinin and Its Analogues - Perhaps the most promising advance in the treatment of malaria is the discovery of artemisinin, **12**, which is also known by the Chinese name qinghaosu, and the related compound arteether, **13**. These analogues are 1,2,4-trioxosesquiterpenes that produce oxidative stress in *P. falciparum*, and they are reduced by the organism in an Fe(II)-dependent process to produce cytotoxic radical intermediates (29). Artemisinin itself is a potent antimalarial, with an IC_{50} of 7.3 nM against *P. falciparum*. In the 10-deoxyartemisinin series, replacement of the 3-methyl group with an n-propyl moiety, as in **14**, produced a 7-fold increase in activity with respect to artemisinin, while substitution at the 9-position produced a 70-fold increase (compound **15**) (30). Artemisinin and its derivatives have limited oral bioavailability, and are hydrolytically unstable, problems which have been addressed by the synthesis of analogues related to **16** and **17**, in

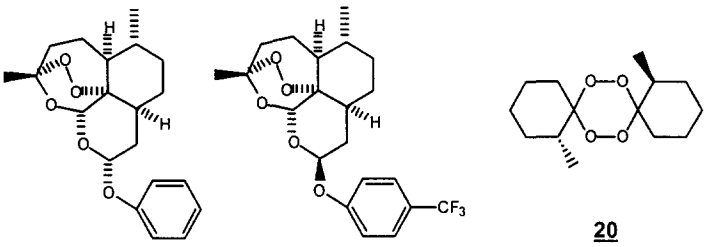


12 13 14 15



16 17

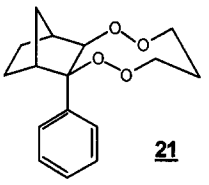
which the 10-position has been functionalized. The semisynthetic analogue **16** is hydrolytically stable, and retains an IC_{50} value of 1.4 nM (31). It is also orally available, and is more potent *in vivo* than artemisinin. Artemisinin-derived trioxane dimers such as **17** are also stable to hydrolysis; **17** has an IC_{50} of 1.3 nM against cultured *P. falciparum* (32). In a series of C10-phenoxy analogues of artemisinin, excellent activity was retained, and the analogues were hydrolytically stable (33). Interestingly, the most active analogues were **18**, a C10α derivative, and **19**, a C10β derivative, with IC_{50} values of 2.61 and 3.90 nM, respectively. Considerable research



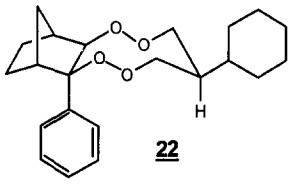
18 19 20

has been done aimed at elucidating the potential mechanism of artemisinin and similar compounds, and there are multiple theories as to the fate of the initial adduct produced by iron-mediated opening of the characteristic endoperoxide moiety (34) The promising antimalarial activity of artemisinin and its derivatives prompted the evaluation of dispiro-1,2,4,5-tetraoxanes such as **20**, WR148999. Compound **20** possesses antimalarial activity comparable to artemisinin, but also shares the characteristics of being hydrolytically unstable and poorly bioavailable by oral administration (35). Other simpler molecules also possess good activity against *Plasmodium*, such as the 1,2,5-trioxy- and 1,2,5,6-tetraoxycycloalkanes typified by

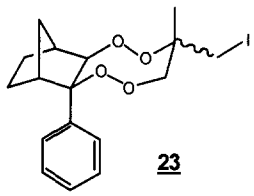
21-**23** (36). Compounds **21**-**23** showed EC_{50} values of 0.19, 0.21 and 0.005 μM against *P. falciparum* in vitro, and **23** showed a 3000-fold selectivity for the parasite over FM3A mouse mammary cells. Compounds **21** and **22** were also effective in vivo against *P. berghei* in a murine model, showing ED_{50} values of 13 and 70 mg/kg by intraperitoneal administration.



21

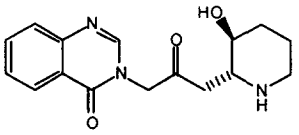


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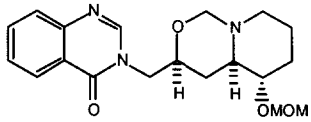


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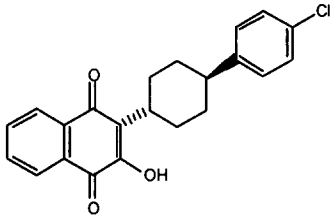
Miscellaneous Antimalarial Agents - A number of synthetic agents and natural product derivatives have been shown to possess antimalarial activity. The herbal natural product (+)-febrifugine, **24** (Chinese name: chang shan) and its derivatives



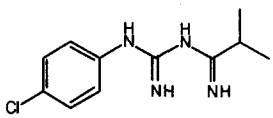
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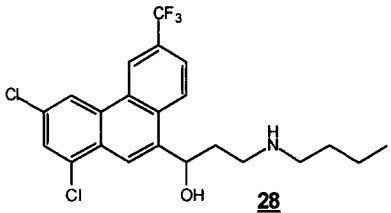
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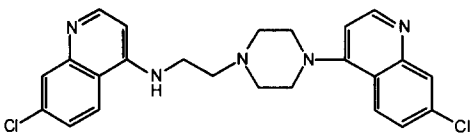
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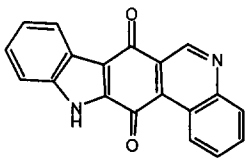
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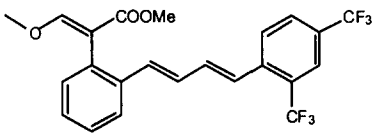
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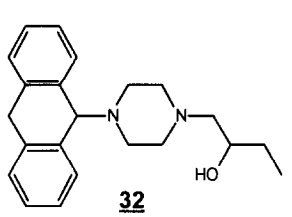
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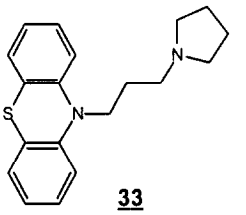
31

possess significant antimalarial activity (37). Recently described semisynthetic febrifugine analogues such as **25** have remarkable antimalarial activity, with EC_{50} values as low as 8.6 X 10^{-9} M against *P. falciparum* in vitro (38). Promising cure rates in humans have been attained by using atovaquone, **26** and proguanil, **27** in combination (39). The recently discovered phenanthrene halofantrine, **28**, has also shown significant antimalarial activity *in vitro* (27), and bisquinoline

heteroalkanediamines such as **29** (IC_{50} = 1.2 nM) are effective against *P. falciparum* *in vitro* and *in vivo* (40). Potential new pharmacophores for antimalarial lead optimization are typified by calothrixins such as **30**, which exhibit nanomolar IC_{50} values (41) and phenyl β-methoxyacrylates such as **31**, which has an *in vitro* IC_{50} of 0.06 ng/mL, and an *in vivo* effect at 100 mg/kg (42). Dibenzosuberanylpiperazine

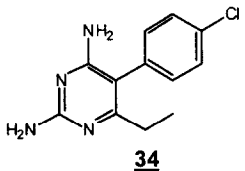


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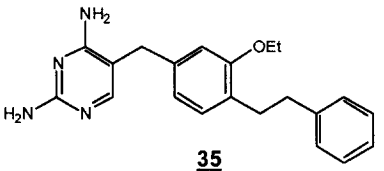


33

derivatives such as **32** possess weak to modest antimalarial activity alone, but have been shown to serve as "chemosensitizers", in that they effectively reverse chloroquine resistance in *P. chabaudi* in a murine model (43). Similar compounds such as **33** are more effective than verapamil, a well known multidrug resistance reverser, as chemosensitizers in the chloroquine resistant W2 clone of *P. falciparum* (44). 2,4-Diaminopyrimidine dihydrofolate reductase inhibitors related to pyrimethamine and trimethoprim show promising antimalarial activity against pyrimethamine-resistant strains of *P. falciparum*, typified by **34** (IC_{50} = 0.05 μM) and **35** (IC_{50} = 0.5 μM), respectively (45).



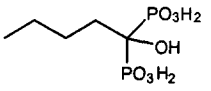
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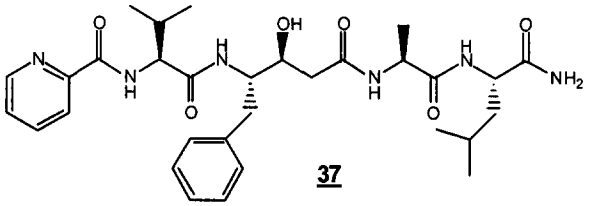
35

Screening of known bis-phosphonates such as **36** (IC_{50} = 5.1 μM) has shown that these compounds can exhibit good antimalarial activity, and also are effective against other parasitic organisms such as trypanosomes and leishmania (46). Finally, a novel series of phenylalanine-statin analogues have been described that are potent, specific inhibitors of the aspartyl protease plasmepsin II (e.g. compound **37**, Ki = 0.54 nM)(47). Unfortunately, these compounds do not traverse cell membranes effectively, and thus have relatively low IC_{50} values against *P. falciparum*. Second generation inhibitors have now been developed that are still effective plasmepsin II inhibitors, but show greater efficacy in an infected erythrocyte in vitro assay (e.g. compound **38**, Ki = 68 nM, ED_{50} = 1.6 μM) (48). Additional studies are required to optimize the structure of plasmepsin II inhibitors.

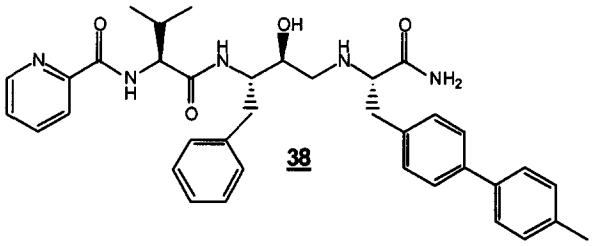
Conclusion - It is evident from the studies described above that there are many new and promising avenues for antimalarial research. Identification and exploitation of newly identified, parasite-specific targets has and will continue to enhance the ability to design effective small molecules for use as antimalarial agents. However, careful examination of the references appended to this report reveals that much of the effort to discover effective treatments for malaria is being expended in Europe, or in Third World countries in which the disease is endemic. In light of the impact of resistant strains of *Plasmodium*, additional funding will be required to fuel antimalarial research, and more of this work will need to be done in developed nations, including the United States. If these steps are taken, there is great hope that malaria can be essentially eliminated as a major cause of global morbidity.



36



37



38

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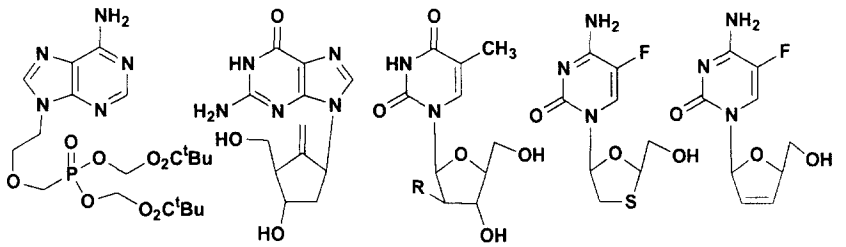
Chapter 22. Non-HIV Antiviral Agents

Nicholas A. Meanwell, Michael H. Serrano-Wu and Lawrence B. Snyder
Department of Chemistry, The Bristol-Myers Squibb Pharmaceutical Research Institute
5 Research Parkway, Wallingford, CT 06492

Introduction – The development of antiviral agents to treat non-HIV infections is largely focussed on therapies for the treatment of the chronic hepatitis infections B and C (1). Nucleoside analogues continue to be the mainstay of HBV therapeutics and clinical development of several continued during 2002. The last year has seen the first small molecule inhibitor of HCV, the NS3 protease inhibitor BILN-2061, enter phase 2 (P2) clinical trials, producing a striking reduction in viral load in treated individuals. The development of the first HCV replicon system in 1998 and its application to screening for antiviral agents is beginning to provide tangible benefit with the disclosure of mechanistically and structurally diverse HCV inhibitors. There remains considerable interest in inhibitors of herpes simplex and human cytomegalovirus viruses, particularly non-nucleoside compounds. Developments in the area of respiratory virus inhibitors have focussed more on respiratory syncytial virus with a description of the first antiviral active in animal models following oral administration. The West Nile virus outbreak in the US, originally confined to the East coast, broadened considerably during the summer of 2002, claiming 230 lives. In the wake of the events of September 11th, 2001, smallpox was a prominent concern as a potential agent of bioterrorism. Developments in each of these areas will be reviewed.

INHIBITORS OF HEPATITIS B AND C VIRUS

Inhibitors of Hepatitis B Virus (HBV) – Adefovir dipivoxil (Hepsera™) (**1**) was approved in the US for the treatment of HBV on September 20th, 2002 and in the European Union on March 11th, 2003, providing a second small molecule antiviral to add to lamivudine (3TC) and the injectable protein IFNα as the only approved agents for treating HBV infection. Adefovir is an effective inhibitor of 3TC-resistant HBV caused by the rtM204I and rtL180M + rtM204V mutations in the reverse transcriptase (2) and resistance to adefovir has not been seen after 48 weeks of monotherapy (3,4).



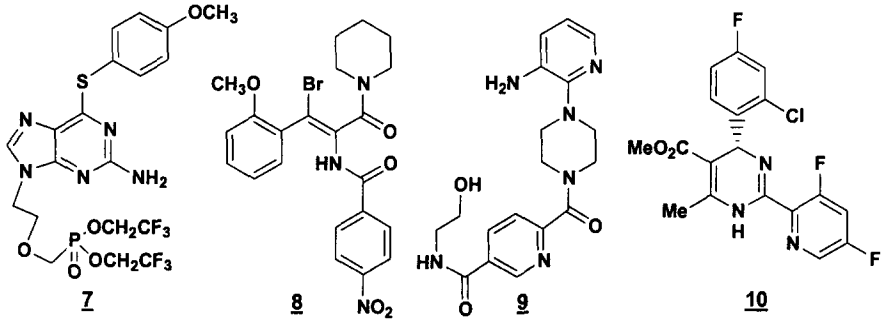
1 **2** **3**: R = H **4**: R = F **5** **6**

A clinical study with entecavir (**2**), currently undergoing P3 trials, compared a dose of 0.1-1 mg/day of **2** to 3TC (100 mg/day) for 48 days in 181 patients previously unresponsive to 3TC treatment (5). Compared to 3TC, treatment with **2** resulted in lower overall viral loads, lower ALT levels and a higher proportion of patients with undetectable HBV DNA levels. Moreover, adverse events for **2** were less than those observed with 3TC. A separate P2 trial of **2** in 177 treatment-naive patients for 22 weeks at a dose of 0.5 mg/day showed that reduction in viral DNA levels was

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independent of baseline ALT levels, with treatment resulting in a 4.7-4.8 log_{10} reduction in HBV DNA (6).

L-nucleosides represent a promising area of antiviral research (7). LdT (telbivudine, **3**) is currently in P3 clinical trials designed to evaluate 1200 patients for safety and efficacy compared with standard treatment in HBeAg+ and HBeAg- patients (8). Recent P2b data was released from a trial involving 104 adults randomized to receive 3TC plus **3** or 3TC monotherapy once daily for 1 year. Viral load reductions of greater than 6 log_{10} were seen for all patients in the study arms containing **3** and no treatment-limiting or dose-related adverse events were reported. The mechanism of action of L-FMAU (clevudine) (**4**), currently in P1/P2 trials, is not firmly understood but recent molecular dynamics simulation experiments have suggested that the triphosphate derivative of **4** may act as a competitive inhibitor rather than as a substrate of HBV polymerase (9,10). Emtricitabine (Coviracil™) (**5**) is also currently in P3 clinical trials for the treatment of HBV. An NDA seeking approval to market **5** for the treatment of HIV was filed in September 2002 (9). Results from a P1/P2 clinical trial with ACH-126443 (elvucitabine, **6**) have been released (11-13). In 36 HBV treatment-naive patients receiving single daily doses of 1-100 mg of **6**, mean declines in plasma HBV DNA of up to 2.5 log_{10} were observed after 14 days of treatment. Furthermore, plasma levels in excess of the IC_{50} values for wild type and YMDD mutants were achieved in the low dose arms. It is therefore anticipated that **6** will be efficacious against 3TC resistant infections in an ongoing P2 trial.



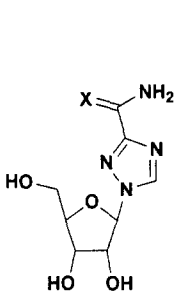
LY-582563 (MCC-478, **7**) is more potent than 3TC in HB611 cells, EC_{50} = 27 nM and 2.2 μM, respectively, and retains activity towards HBV carrying the recently identified G1896A mutation (14-16). This mutation, which arises in response to 3TC therapy, is found in the precore region and confers HBeAg negativity. The major metabolite of **7** formed in rat or human serum is the mono-ester, which is a more potent HBV inhibitor, EC_{50} = 70 nM, than 3TC. It is postulated that the arylthio moiety is responsible for the specificity towards HBV and lower cytotoxicity than PMEA, the active component of adefovir dipivoxil. Additional analogs in this structural class have been prepared with the phenylthio- and 3-methoxyphenylthio ethers showing the most promise whilst other aromatic thioethers exhibited higher cytotoxicity (14,15).

Non-nucleoside inhibitors of HBV are beginning to emerge that are anticipated to show reduced cross-resistance with nucleoside analogues. AT130 (**8**) and its close analog AT61 are active against wild type HBV and the rtL180M, rtM204I, and rtL180M+rtL204V mutants (EC_{50} = 2-5 μM) in HepG2-derived cells (17,18). It has been postulated that **8** interferes with the packaging of pregenomic viral RNA resulting in inhibition of viral reverse transcription. Pyridinedicarboxamide **9** represents the first report of a non-nucleoside inhibitor of HBV reverse transcriptase (19). The unique mechanism of action of Bay 41-4109 (**10**), a potent HBV inhibitor *in vitro*, EC_{50} in HepG2.2.15 cells = 50 nM, has recently been elucidated (20,21). Only the *(R)*-

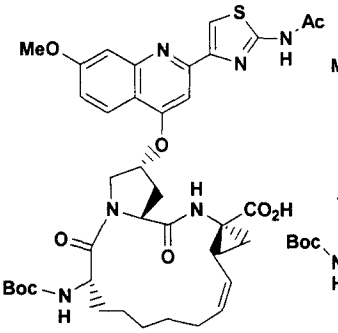
enantiomer of **10** is active in cell culture and appears to prevent proper formation of the viral nucleocapsid.

Inhibitors of Hepatitis C Virus (HCV) – Nearly 170 million individuals are infected with hepatitis C virus (HCV) worldwide and HCV infection is responsible for 8,000-10,000 deaths annually in the United States, a burden expected to increase significantly (22,23). A second pegylated interferon-α (IFN), Roche's Pegasys, was approved in 2003, both as mono therapy and in conjunction with ribavirin (**11**) (Copegus™) (24). However, safety concerns with combination therapy remain, as the accumulation of **11** in erythrocytes can lead to hemolytic anemia. This has prompted a search for safer interferon co-therapies which include the active enantiomer of **11**, levovirin, and the prodrug viramidine (**12**), which improves liver at the expense of erythrocyte exposure (25,26).

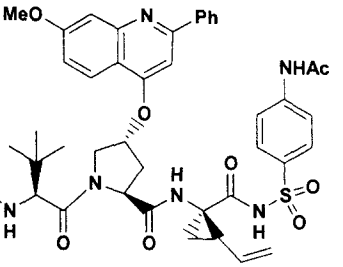
The development of safe, efficacious, and HCV-specific antiviral agents remains an important goal and the development of subgenomic HCV replicons has dramatically enhanced the potential to identify inhibitors (27,28). A significant advance towards establishing a correlation between replicon inhibition and clinical efficacy was recently accomplished with the disclosure of preliminary clinical data for BILN-2061, a selective inhibitor of the NS3 serine protease of HCV that is structurally related to **13**. This highly modified macrocylic tripeptide derivative is extremely potent *in vitro*, with K_i values of 0.3 nM and 0.66 nM towards HCV-1a and HCV-1b NS3 proteases, respectively (29). These figures are similar to the potency observed in cell culture in the cognate HCV replicons, EC_{50} = 4 nM and 3 nM, respectively (29). Antiviral efficacy was established in a study conducted with 10 patients with chronic HCV and significant liver fibrosis, where all patients treated with BILN-2061 (200 mg p.o. b.i.d.) displayed a decrease in serum HCV RNA levels of at least 2.0 $\log_{10}$ copies/mL after two days of treatment (30). Four of these patients recorded a reduction in viral load of more than 3 orders of magnitude and viral titers returned to baseline following cessation of therapy, with no drug-related safety issues identified (31).



11, X = O
12, X = NH



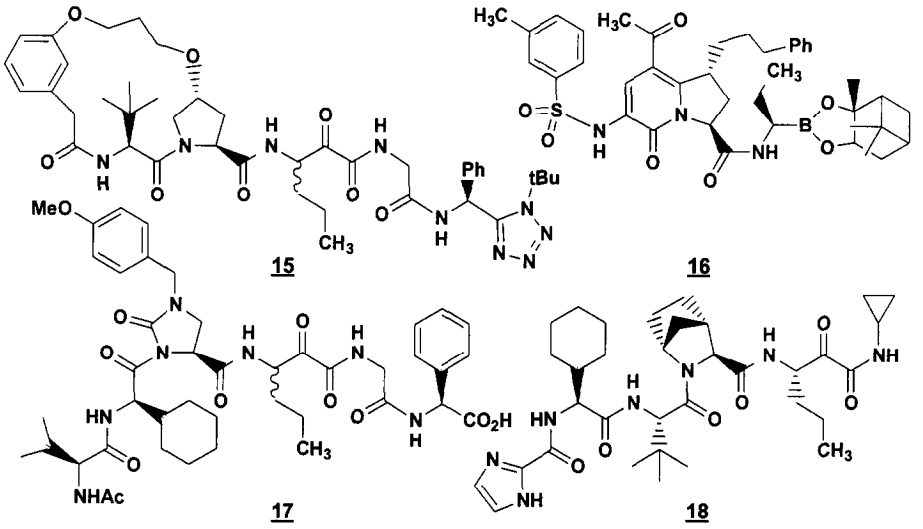
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14

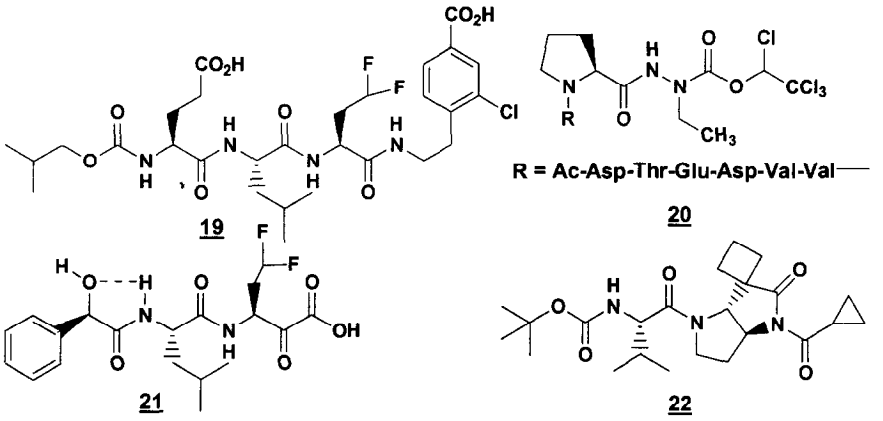
The intensity of effort devoted towards the discovery of inhibitors of HCV NS3 has continued, with the focus largely on peptide-based molecules that are required to effectively complement the active site and proximal regions of the protease (32-34). The crystal structure of a macrocyclic inhibitor related to **13** bound to HCV protease has been disclosed and an acyclic tripeptidic inhibitor has been used to generate resistant subgenomic replicons in which mutations mapped to the protease (35,36). Amongst several strategies disclosed, the C-terminal carboxylate of peptide inhibitors may be replaced with an N-acyl sulfonamide moiety, as exemplified by **14** (37). Novel approaches to constrain or mimic the peptidic backbone are represented by macrocycle **15**, the tetrahydroindolizine **16** (IC_{50} = 0.12 μM), the imidazolone **17** and

the bicyclic proline derivative **18** (K_I = 0.042 μM, IC_{50} = 0.251 μM) (38-46). These inhibitors are constructed around either an α-keto amide or a boronic acid moiety, well-precedented as serine protease inhibitor motifs that engage the catalytic serine residue in a covalent but reversible interaction.



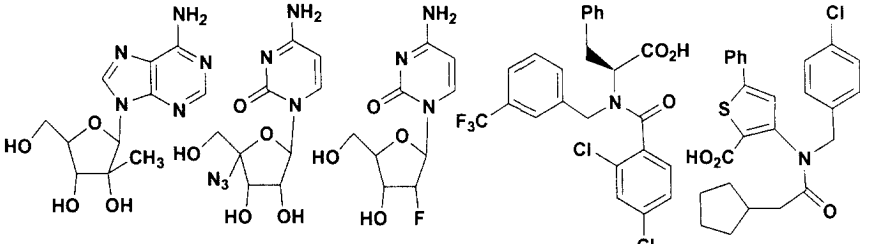
15 **16** **17** **18**

However, chemical reactivity is not a prerequisite for potent inhibition in a peptidic background since phenethylamide **19** and the azapeptide **20** inhibit HCV NS3 with K_I values of 0.6 and 0.2 μM, respectively (47,48). The more active diastereomer of the α-hydroxy amide P3 element explored in the context of **21** was found to possess the (*R*)-configuration, unanticipated and explained by the presence of an intramolecular hydrogen bond that orients the lipophilic moiety of this isomer into S3, as depicted (49). Non-peptidic inhibitors of NS3/NS4A protease are much less common but some progress has been made in this direction. The bicyclic lactam **22** is a mechanism-based inhibitor of HCV NS3 whilst additional examples of bis-benzimidazole derivatives that rely upon Zn^{2+} to consolidate the enzyme-inhibitor complex have been described (50,51).



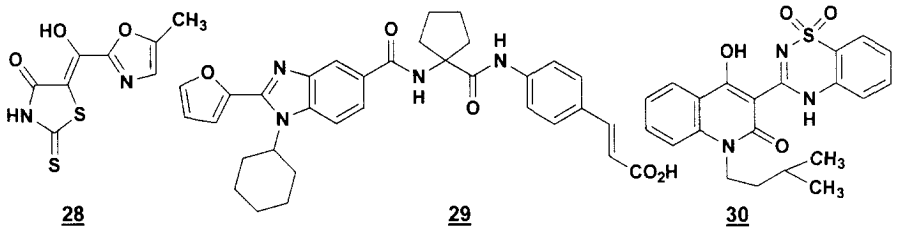
19 **20** **21** **22**

The NS5B RNA polymerase is another structurally-characterized viral protein that is an attractive target for therapeutic intervention (52-55). Inhibitors of HCV polymerase can be broadly divided into nucleoside and non-nucleoside derivatives. The nucleoside analog ribavirin (**11**) has been suggested to interfere with both the initiation and elongation steps of HCV RNA replication (52). Several HCV NS5b inhibitors incorporate modified D-ribose elements and include the 2'-Me derivative **23**, EC_{50} = 0.25 μM, the 4'-azido analog **24**, EC_{50} = 1.2 μM, and the 2'-deoxy-2'-fluoro cytidine derivative **25**, EC_{50} = 0.74 μM (56-59).



23 **24** **25** **26** **27**

Several non-nucleoside inhibitors of HCV NS5B have been reported, including a series of phenylalanine derivatives of which compound **26**, K_I = 2.2 μM, is representative (60,61). This compound has been co-crystallized with NS5B and appears to bind to the inactive, open conformation of the polymerase almost 35 Å from the active site (62). Other scaffolds with which HCV polymerase inhibitors have been discovered include an amino thiophene, represented by **27**, the enolic rhodanine **28** (IC_{50} = 1.0 μM), and structural variations of previously disclosed benzimidazole derivatives **29** (IC_{50} < 0.5 μM) claimed to be active in replicons (61,63-67). Mechanistic studies with the benzo[1,2,4]thiadiazine polymerase inhibitor **30** suggest interference with the initiation step of viral RNA synthesis, allowing for a potential synergy with existing elongation inhibitors (68).



28 **29** **30**

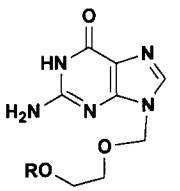
Novel approaches to HCV therapy include blocking the viral RNA internal ribosomal entry site (IRES), binding of the viral E2 envelope glycoprotein or attachement (69-72). The highly conserved IRES has been targeted by oligonucleotides and artificial ribozymes, but little progress has been made in the development of small molecule inhibitors (70,71). P2 clinical evaluation of the antisense 20-mer oligonucleotide ISIS-14803 revealed a 1-2 log_{10} reduction in plasma HCV RNA levels in approximately 30% of the patients after 4 weeks of treatment (73). Another approach, which may prove complementary to virus-specific HCV therapy, is the induction of interferon production in host cells. Small molecules that act *via* toll-like receptor activation have been identified as activators of an immune response (74). RNA interference (RNAi) is a rapidly emerging technology that has proven to be a powerful means of selectively controlling protein production in cell culture. Inhibition of HCV replication in replicons has been accomplished using this procedure whilst the

demonstration of selective targeting of the liver protein Fas *in vivo* using RNAi holds promise for the treatment of HCV (75-78).

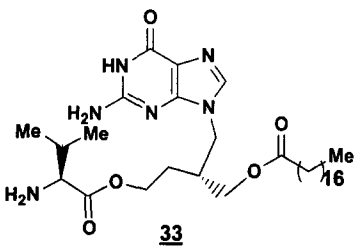
INHIBITORS OF HERPES SIMPLEX VIRUS AND HUMAN CYTOMEGALOVIRUS

The eight human herpes viruses cause a variety of pathophysiological conditions ranging in severity from mild cold sores to life threatening illnesses in immunocompromised patients. While herpes simplex virus (HSV) types 1 and 2 typically cause localized cold sores and genital herpes, other members of the herpesviridae family can be more problematic. Varicella zoster virus (VZV) is the causative agent in chicken pox whilst human cytomegalovirus (HCMV) is particularly difficult for the immunocompromised population, including AIDS patients where clinical manifestations include retinitis, colitis, oesophagitis, and pneumonia (78). Epstein-Barr virus (EBV) is responsible for mononucleosis in immunocompetent patients and lymphoma in immunocompromised individuals. Finally, HHV-6, HHV-7 and HHV-8 are the remaining known pathogenic herpes viruses of which HHV-8 is responsible for the debilitating effects of Kaposi's sarcoma. Nine antiviral agents are licensed to treat infections caused by the herpes virus family, all but one of which, fomiversen, terminate viral DNA synthesis by inhibiting the viral DNA polymerase (80,81). Resistance to current antiherpetics is modulated primarily by the thymidine kinase (TK), the UL97 phosphotransferase in HCMV and the DNA polymerase (82-86).

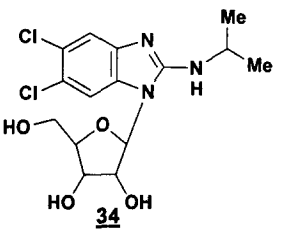
Valacyclovir hydrochloride (Valtrex™) **31** was approved in September 2002 for the treatment of cold sores in healthy adults and acyclovir (Zovirax™) **32** cream was approved for the treatment of recurrent herpes labialis or cold sores (87). Valomaciclovir stearate (MIV-606, **33**) has shown promise in P2 clinical trials for the treatment of herpes zoster with P3 trials planned (88). Maribavir (1263W94, **34**), an inhibitor of the HCMV UL97 protein kinase (IC_{50} = 3 nM), has been dropped from P2 clinical development for the treatment of HCMV infection (89-91). Data from a P1 clinical trial in HIV-1 infected men with asymptomatic HCMV shedding has been released (92,93). Maribavir demonstrated *in vivo* anti-HCMV activity in all of the dosage regimens tested (100, 200, and 400 mg tid, and 600 mg bid), with mean reductions in semen HCMV titers of 2.9 to 3.7 log_{10} PFU/mL (92).



31 R = Valine
32 R = H

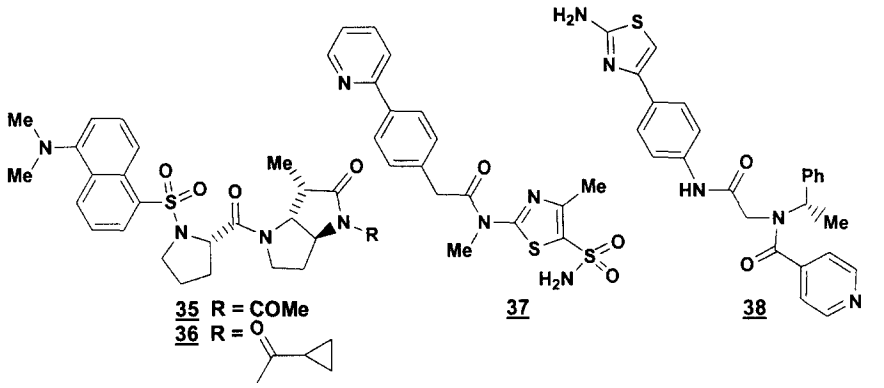


33



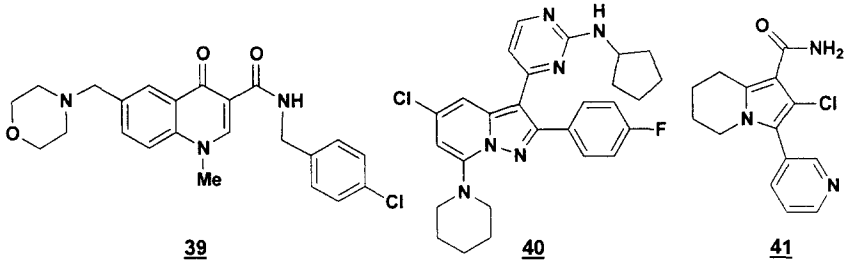
34

The HCMV serine protease has been perceived as an attractive antiviral target. Recent crystal structure data demonstrated significant conformational flexibility in the S3 binding pocket of protein complexed with two peptidomimetic inhibtors (94). A series of *trans*-lactams, represented by **35** and **36**, IC_{50} = 0.54 and 0.34 μM, respectively, are derived from the same structural platform as the HCV NS3 inhibitor **22** but inhibit the HCMV serine protease with excellent selectivity (95-97). Mechanistic studies are consistent with acylation of the active site Ser_{132} of HCMV protease in a reversible and time-dependent manner.



All but one of the currently licensed drugs available to treat HSV act by inhibiting the viral DNA polymerase, providing a suitable backdrop for the emergence of resistant virus and a rationale for identifying inhibitors of other viral proteins (98,99). Two groups have reported novel thiazole-containing inhibitors of the HSV helicase-primase. BAY 57-1293 (**37**) is a leading pre-clinical candidate that is more potent (EC_{50} = 12 nM) than any anti-herpetic currently used to treat HSV infections (100,101). In a murine lethal challenge model of HSV-1 and HSV-2, **37** was protective with an ED_{50} value of 0.5 mg/kg, which compares with the much higher doses of 22 and 16 mg/kg for HSV-1 and HSV-2, respectively, required for acyclovir to show efficacy. Additional patent applications that extend this promising chemotype have appeared (102,103). A second series of HSV helicase-primase inhibitors, of which BILS-179 BS (**38**) is representative, has been disclosed (104). BILS 179 BS inhibits viral growth with an EC_{50} of 27 nM, displays an excellent therapeutic index of >2000 and reduces cutaneous HSV-1 and genital HSV-2 disease in a murine model when treatment is initiated 3 hours post-infection. Interestingly, when treatment was initiated 65 hours after infection, **38** reduced HSV-1 pathology by 75% and HSV-2 mortality by 75% (200 mg/Kg/day) when compared to acyclovir or untreated animals (104).

A series of 4-oxo-dihydroquinolone derivatives that are potent and broad spectrum non-nucleoside inhibitors of DNA polymerases of the herpesvirus family, including HCMV, HSV-1, HHV-8 and VZV, have been the subject of a number of recent disclosures (105). These compounds exhibit no significant inhibitory activity towards human α-, γ-, or δ-polymerases. PNU-183792 (**39**) is an effective antiviral in cell culture, potently inhibiting HCMV (EC_{50} = 0.69 μM), VZV (EC_{50} = 0.37 μM) and HSV (EC_{50} = 0.58 μM), that is active towards ganciclovir- and cidofovir-resistant HCMV and acyclovir-resistant HSV (106). Excellent oral bioavailability and a protective effect in a murine CMV animal model were also reported. A series of related analogs have been reported in the recent patent literature (107,108).



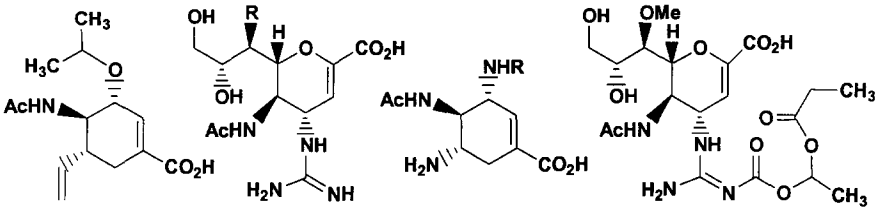
Pyrazolopyridine derivatives have been reported to possess activity against HSV-1 in Vero 76 cells with **40** having an EC_{50} of 0.12 μM (109-112). Structurally related imidazopyridine derivatives are active against HSV types 1-8 with an EC_{50} of 0.14 μM (113). CMV-423 (**41**) is a potent inhibitor of HCMV, EC_{50} = 4-7 nM, that appears to act at a step in viral replication preceding DNA polymerization (114).

INHIBITORS OF RESPIRATORY VIRUSES

Overview – Respiratory viruses continue to be a significant source of mortality and morbidity. The annual death rates due to influenza in the US are estimated to have doubled over the last 20 years, attributed to an aging of the population (115,116). This study also revealed a greater appreciation of the contribution of respiratory syncytial virus to mortality. The recently discovered human metapneumovirus (hMPV) was identified as a significant cause of wheezing in infants (117,118). The influenza inhibitor oseltamivir (Tamiflu™) was approved for the treatment of influenza in adults and children and for prevention in adults and adolescents by the European Community in June 2002, consolidating its position as the market leading neuraminidase (NA) inhibitor. However, development of the third neuraminidase inhibitor peramivir was terminated by BioCryst in June after disappointing P3 results in which the orally bioavailable compound failed to meet the key efficacy endpoint of reducing the time to onset of relief of symptoms. In March, 2003 an outbreak of severe acute respiratory syndrome (SARS) emerged in Southeast Asia for which the culprit was quickly identified as a new coronavirus distinct from any previously identified human coronavirus.

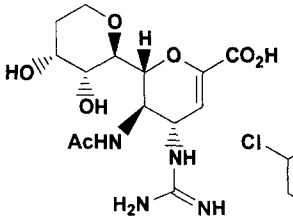
Inhibitors of Influenza Virus – Characterization of the 1918 influenza continues and chimeric viruses containing the 1918 NA or M1 ion channel showed susceptibility to oseltamivir and amantadine or rimantadine, respectively, both *in vitro* an *in vivo*. (119,120).

The role of NA inhibitors in pandemic influenza has been reviewed and the identification of structurally novel NA inhibitors has continued (121). Substitution of the primary amine moiety of oseltamivir with a vinyl group afforded the potent influenza B NA inhibitor **42**, K_i = 45 nM. (122) Additional SAR studies around zanamivir have focussed on the C-7 hydroxyl where replacement by F or methylation affords the potent NA inhibitors **43** and **44**, IC_{50} values of 0.8 and 6.1 nM, respectively, which compares favorably with an IC_{50} of 5-10 nM for the prototype (123,124). Polymeric analogues of zanamivir linked to a polyglutamate backbone via the 7 position showed enhanced influenza inhibitory activity compared to monovalent analogues (125). A dynamic combinatorial approach in which diamine **45** was equilibrated with a mixture of ketones in the presence of influenza NA amplified the concentration of structurally complementary imines, isolated as the corresponding secondary amines after reduction with nBu_4NBH_3CN (126). The isopropyl analogue **46** emerged as the most potent NA inhibitor identified, K_i = 85 nM, which compared to 1.3 nM for oseltamivir (126).

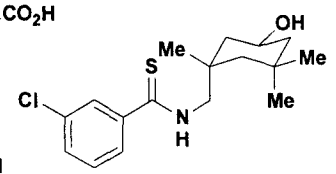


42 **43** R = F; **44** R = OMe **45** R = H; **46** R = iPr **47**

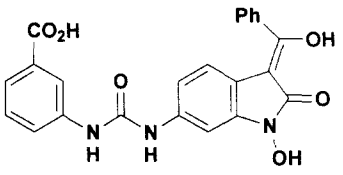
The O-methyl analogue of zanamivir is claimed to protect mice against a lethal influenza infection following oral administration of the prodrug **47** whilst the bicyclic ether **48** is the first zanamivir derivative to demonstrate oral efficacy in the mouse model (127,128). The optimization of a screening lead into potent, cyclopentane-based inhibitors of neuraminidase using a combination of structure-based design and combinatorial chemistry has been described in detail (129).



48



49

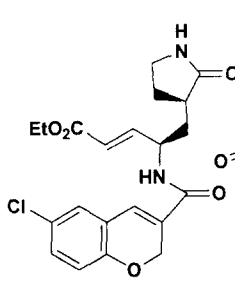


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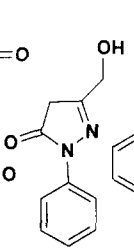
The thioamide **49** was the most potent of a series of influenza fusion inhibitors whilst the tetramic acid **50** was the most potent of a new class of influenza endonuclease inhibitors, IC_{50} = 6.6 μM (130,131).

Inhibitors of Human Rhinovirus (HRV) – The NDA for the HRV uncoating inhibitor pleconaril (Picovir™) was rejected by the FDA on May 31st with the agency requesting additional drug interactions studies.

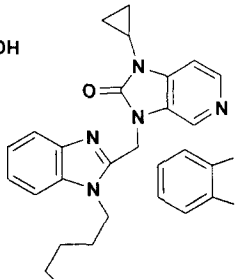
Mechanism-based inhibitors of the HRV 3C cysteine protease have been probed using a combination of structure-based design principles and parallel synthesis methods in an effort to find less peptidic inhibitors (132). The chroman **51** emerged as an inhibitor of HRV-14 replication in cell culture, EC_{50} = 160 nM; however, the serotype coverage of this compound was poor with much reduced potency against other subtypes, an observation rationalized in the context of structural data (132). The HRV 2A cysteine protease releases itself from the viral P2 polyprotein, cleaves the P2 in both a *cis* and *trans* fashion to release the 2B and 2C proteins and also proteolyzes the host cap binding complex in order to compromise host cell transcription. A series of *N*-phenylated pyrazole derivatives have been claimed as inhibitors of this essential enzyme with **52** an effective antiviral agent in cell culture, EC_{50} = 1.8 μM, CC_{50} = 388 μM (133). The HRV RNA-dependent RNA polymerase from HRV-16, a potential drug discovery target, has been cloned, expressed and purified from *E. coli* and shown to be enzymatically active (134).



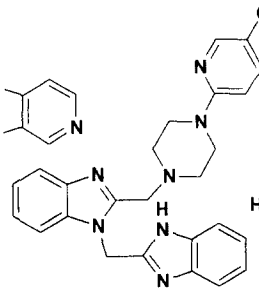
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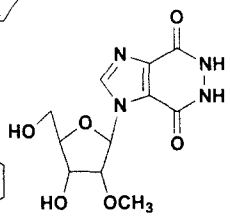
52



OH **53**



54



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Inhibitors of Respiratory Syncytial Virus (RSV) – The role of RSV in morbidity and mortality continues to be a focus of research designed to provide a more accurate perspective of this virus as a mediator of significant disease burden. (115,116,135,136). The enhanced awareness of RSV has stimulated interest in this virus as a therapeutic target with the recent emergence of new structural classes of inhibitor. However, clinical proof-of-principle remains to be established for RSV antivirals. Viropharma has suspended development of VP-14637, an RSV fusion inhibitor under examination as a topically administered agent. The benzimidazole BMS-433771 (**53**) has been described as the first RSV inhibitor to demonstrate antiviral activity in animal models following oral administration (137,138). Mechanism of action studies indicate that **53** is an inhibitor of the fusion of viral and host cell membranes and targets the RSV F (fusion) protein (137). Analogues of **53** form the basis of proprietary claims (139,140) whilst Trimeris has disclosed a series of benzimidazole-based inhibitors of RSV fusion (141). Representative of this series is **54**, which is active in cell culture, EC_{50} = 10 ng/mL.

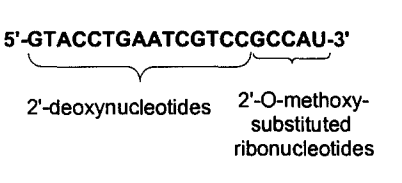
Coronaviruses – Coronaviruses are enveloped, single-strand, positive-sense RNA viruses most commonly associated with respiratory infections in man (142). Two coronaviruses are the underlying cause of approximately 30% of upper respiratory tract infections, usually mild to moderate in severity and generally recognized as the common cold, although in the immunocompromised population coronavirus infections can lead to pneumonia. However, in March, 2003 an outbreak of severe acute respiratory syndrome (SARS) emerged in Southeast Asia that very quickly spread to 27 countries, carried largely by air travelers (143-145). More than 6500 SARS infections were documented worldwide within the following 2 months that were associated with substantial morbidity, including fever, non-productive cough, malaise, chills, headache and dyspnea, and significant mortality, with over 460 deaths reported (146). Whilst SARS appears to have originated in Southern China in November, 2002, reports of major outbreaks in Hong Kong and Toronto, Canada in March 2003 brought the disease to world prominence (143-145). The attributes of modern technology, communication and international teamwork led to the rapid isolation and sequencing of the virus causing SARS, identified as a novel coronavirus with little similarity to previously known human coronaviruses (147-153). Final confirmation came with the demonstration that infection of monkeys with SARS produced a syndrome identical to that seen in man and that virus could subsequently recovered (154). These experiments were rapidly followed by the development of diagnostic assays based on a real-time quantitative PCR method and a TaqMan protocol (148, 155). Transmission of SARS appears to be via person-person contact with infected droplets rather than airborne and, possibly, a fecal-oral route. Inhibitors of coronaviruses are largely unknown although treatment with ribavirin (**11**) and oseltamivir have been examined in an empirical fashion (143, 156).

INHIBITORS OF WEST NILE VIRUS AND PAPILLOMA VIRUS

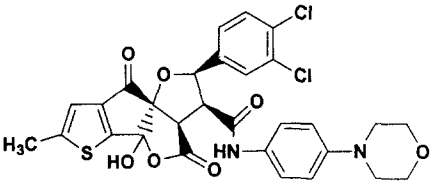
Inhibitors of West Nile Virus (WNV) – The geographical scope of the mosquito-borne West Nile virus outbreak in the US broadened considerably in the summer of 2003 extending to almost all states and causing over 2,500 cases of encephalitis and 230 deaths (157,158). The virus presents several conventional proteins as targets suitable for intervention including the NS3 protein, which expresses serine protease, helicase and nucleoside triphosphatase activities, and the NS5 RNA-dependent RNA polymerase (159). Moreover, screening using a cell culture assay provides a panoply of less well understood targets. However, few potent and selective WNV inhibitors have been described to date. Ribavirin (**11**) inhibits WNV RNA production in oligodendroglial cells, a human neural cell line, with an EC_{50} of ~ 60 μM (160). The nucleoside analogue HMC-HO4 (**55**) interferes with the helicase activity of WNV NS3 with an IC_{50} of 30 μM and is a similarly potent antiviral in cell culture (161).

Inhibitors of Vaccinia Virus (Smallpox) – Smallpox has aroused considerable concern with discussion prominently focussed on its potential as an agent of bioterrorism (162-167). Many inhibitors of vaccinia virus replication interfere with host cell pathways and include **11** (inosine monophosphate dehydrogenase), the cyclopentenyl nucleoside analogue neplanocin A (S-adenosylhomocysteine hydrolase) and pyrazofurin (OMP decarboxylase) (168, 169). The cytidine and 5-F cytidine analogues of neplanocin A are equally potent inhibitors of several orthopox viruses, including smallpox (170). The nucleoside phosphonate cidofovir (HPMPC), licensed in 1996 for the treatment of HCMV retinitis in HIV patients, inhibits vaccinia virus *in vitro* (EC_{50} = 18 μg/mL) and protects mice from a lethal vaccinia infection following a single systemic (intraperitoneal) or intranasal (aerosolized) dose (171). 5-Iodo-2'-deoxyuridine, an inhibitor of vaccinia virus DNA synthesis *in vitro*, delays the effects of a lethal vaccinia infection, following subcutaneous dosing, by 15 days (172).

Inhibitors of Papilloma Virus – The contribution of HPV-16 and HPV-18 to the etiology of cervical cancer was discussed as part of a broader review of the role of viruses in the development of cancer (173). ORI-1001 (**56**), a 20-mer phosphorothioate hybrid oligonucleotide, is being developed as a topical agent for the treatment of genital warts (174). ORI-1001, which complements the E1 mRNA start codon of HPV and has demonstrated efficacy in two animal models, is currently undergoing P1/2 clinical evaluation. The fused tetracyclic amide **57** is claimed to inhibit the E2-dependent binding of papilloma virus E1 to DNA, a critical step in viral replication, with an IC_{50} of <500 nM (175). A vaccine derived from an HPV-16 virus-like particle, completely prevented the incidence of cervical neoplasias in HPV-16-naïve young women (176). This impressive result provides strong encouragement to the development of a vaccine with a spectrum that, in addition, encompasses the broader range of HPVs (18, 31, 33 and 35) responsible for the majority of cervical cancers (177). A combined, multivalent vaccine based on self-assembling virus-like particles and directed towards HPV-16 and HPV-18, recognized as MEDI-517, is entering P2 trials (178).



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Chapter 23. Recent Advances in Antibody Drug Conjugates for Cancer Therapy

Damon L. Meyer and Peter D. Senter
Seattle Genetics, Inc. 21823 30th Dr. SE, Bothell, WA 98021

Introduction - In the past few years, monoclonal antibodies (mAbs) and mAb-based reagents have demonstrated considerable utility in the clinical treatment of cancer (1,2). Activities of unmodified mAbs such as Rituxan (rituximab) in non-Hodgkin's lymphoma (3), Panorex (edrecolomab) in colorectal carcinoma (4), and Herceptin (trastuzamab) for metastatic breast cancer (5, 6), have led to renewed interest in using mAbs as vehicles for the delivery of cytotoxic agents to tumor cells. The objective of this approach is to enhance drug efficacy through targeted delivery, while sparing non-target tissues from chemotherapeutic damage (7, 8).

While the use of mAb-drug conjugates for cancer therapy is conceptually appealing, significant limitations have been identified. These result from the physiological barriers to mAb extravasation and intratumoral penetration, mAb immunogenicity, normal tissue expression of the targeted antigen, low drug potency, inefficient drug release from the mAb, and difficulties in releasing drugs in their active states (7-9). Consequently, recent work in the field has focused on chimeric and humanized mAbs that are relatively non-immunogenic and have high affinities for tumor associated antigens, mAbs that are efficiently internalized into cells once they bind to the target antigen, engineered mAbs that are designed for efficient drug delivery, new drugs with high potencies, and linker technology to accommodate these novel agents.

In this review, we describe mAb-drug conjugates that have shown particular promise in preclinical tumor models and are either moving forward towards clinical trials, or are currently in clinical trials. One of the agents, Mylotarg, has recently been approved for treating acute myelogenous leukemia, and represents the first clinically approved mAb-drug conjugate for the treatment of cancer.

HYDRAZONE-LINKED CONJUGATES

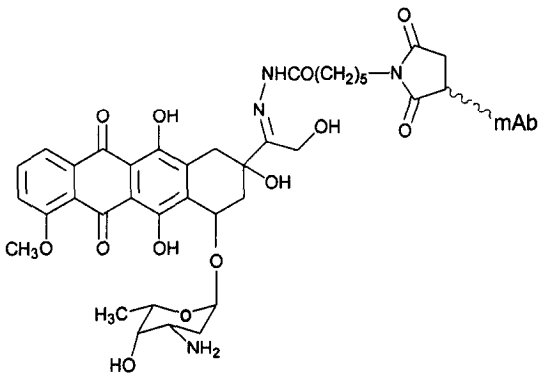
Upon binding to cell-surface antigens, many mAbs are internalized through a process known as receptor mediated endocytosis, which carries the mAb into lysosomes that are both acidic and rich in proteolytic enzymes (7). Considerable attention has been directed at developing linkers that are relatively stable at neutral pH, but undergo hydrolysis under the mildly acidic (circa pH 5) conditions within the lysosomes. The cleavable linker system that has been most extensively exploited contains the hydrazone functionality.

There are two general methods for producing mAb-drug conjugates through hydrazone bond formation. Treatment of mAbs with sodium periodate generates aldehydes and ketones through carbohydrate oxidation. Addition of hydrazido drug derivatives leads to the formation of hydrazones that are hydrolyzed under acidic conditions (10-12). One advantage of this methodology is that the mAbs are modified regiospecifically, since the carbohydrates on mAbs are largely restricted to the Fc region. However, the oxidation method leads to a variety of reactive species, and the resulting hydrazones are poorly defined. In addition, the oxidative conditions used for hydrazone formation can also lead to methionine oxidation, and this can be highly detrimental to mAb binding activity (13). An alternative approach to forming

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mAb-hydrazone linked conjugates is to attach an aldehyde, ketone, or preformed hydrazone to the surface of an mAb by acylation of lysine amino groups. This approach allows for much greater control over the relative hydrolysis rates of the hydrazone bond. Aromatic ketones generally appear to have the most promising characteristics since they are stable for several days under neutral pH conditions, but are much less stable at pH 5 (10,13,14). However, there are some examples of aliphatic hydrazones that are conditionally labile under mildly acidic conditions.

BR96-doxorubicin - A maleimido derivative of doxorubicin was conjugated to the anticarcinoma chimeric mAb cBR96 through a hydrazone that was selected for stability at pH 7 and drug release at pH 5 (15). Conjugates, having structures represented by 1, were formed by reduction of interchain disulfides, then adding the maleimido drug derivative containing the hydrazone linkage. As many as eight drugs could be attached to each mAb with complete retention of binding activity. This methodology was later extended to include branched linkers, allowing for more drug to be attached to each thiol group in the mAb (16).



1

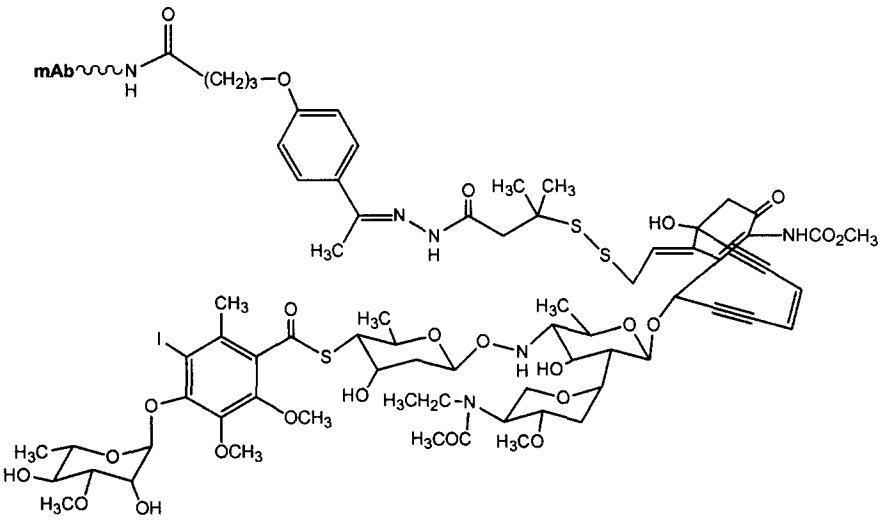
Preclinical studies demonstrated immunologically specific cures at well-tolerated doses in both mice and rats (15). However, the amount of conjugate needed to achieve these effects was very high (>100 mg conjugate/kg), reflecting the relatively low potency of the targeted drug. Pharmacokinetic studies indicated that the intratumoral drug concentration was much higher in animals treated with conjugate than in animals treated with the maximum tolerated dose (MTD) of unconjugated doxorubicin (17).

In a Phase I clinical trial, the MTD of BR96-doxorubicin was found to be 600-700 mg/m^2 with gastrointestinal dose-limiting toxicities (18). The half-life of drug release from circulating conjugate was approximately 43 hours, which is suboptimal, given that the half-life of the mAb in circulation was approximately 12 days. The conjugate was marginally active in this trial. A subsequent Phase II trial confirmed that the response rate was low, with gastrointestinal dose limiting toxicities (19). This study showed that unconjugated BR96 mAb elicited the same toxicities as the conjugate, suggesting that normal tissue cross-reactivity and mAb-mediated activities might have contributed to the toxicity. One of the noteworthy findings in this study was that active drug was detected within tumor masses, providing support for the concept of using mAbs for anticancer drug delivery.

The results from these clinical studies prompted further investigation into the use of conjugates in combination with other drugs. One such study demonstrated

that paclitaxel strongly synergized with BR96-doxorubicin (20). It was found that this combination was highly effective in lung, colon, and breast tumor xenograft models. Synergistic activities between BR96-doxorubicin and docetaxel were also reported, and a mechanism for synergy was proposed based on conjugate-mediated G2 cell-cycle arrest, leading to enhanced sensitization of the cells to taxanes (21). The promising *in vitro* and *in vivo* synergy studies provide the basis for ongoing clinical trials in which BR96-doxorubicin is combined with chemotherapeutic agents (22).

Anti-CD33-calicheamicin conjugates - Mylotarg, the first mAb-drug conjugate to be approved for human use, is a radical departure from previously described conjugates. The cytotoxic element in Mylotarg is N-acetyl-gamma calicheamicin, a minor groove binder that produces DNA double strand breaks (23) with an IC_{50} in the low ng/mL range (24). The linkage between the mAb and the drug incorporates two labile bonds as shown in structure **2**: a hydrazone and a sterically hindered disulfide. It is believed that the hydrazone is cleaved before the disulfide is reduced.



2

The mAb moiety in Mylotarg is a humanized form of P67.6, a murine mAb that binds to the CD33 antigen present on myeloid cells, notably transformed cells of acute myeloid leukemia (AML) patients (25). CD33 has been shown to internalize and is not present on pluripotent stem cells (26). Any non-transformed myeloid cells eliminated by the conjugate can therefore be replaced. Greater than 80% of AML patients express the CD33 antigen (27).

Mylotarg consists of a 1:1 mixture of hP67.6, a humanized IgG4, with hP67.6 conjugated to 4-6 moles N-acetyl-gamma calicheamicin, providing an average drug loading ratio of 2-3 drugs/mAb (28). In conjugates prepared with murine P67.6, the hydrazone linkage was generated through periodate oxidation of the mAb carbohydrate, followed by condensation of the resulting putative aldehydes with an acyl hydrazide derivative of N-acetyl-gamma calicheamicin. However, the humanized form of the mAb, hP67.6, lost binding activity on periodate treatment, possibly because of a sensitive methionine residue in the antigen binding region (13). Consequently, the conjugate was formed by reacting the drug-hydrazone-linker complex with mAb lysine residues. The hydrazone in Mylotarg is a derivative of p-hydroxyacetophenone, which underwent 6% hydrolysis in 24 hr at pH 7.4 and

was cleaved quantitatively under the same conditions at pH 4.5 (13). The role that conjugation technology can play in efficacy was further underscored by the finding that humanized lysine-linked conjugate was substantially more potent (45 fold on HL-60 cells) and more selective (70 fold on HL-60 cells) *in vitro* and somewhat more active *in vivo* than the murine carbohydrate linked version.

A Phase I clinical trial of Mylotarg in refractory or relapsed AML patients demonstrated tolerable levels of toxicity at doses up to 9 mg/m^2 (29). Responses were documented in 8 of 40 patients. Toxicities included fever and chills, reversible elevation of liver enzymes, myelosupression with severe neutropenia, and veno-occlusive disease (29, 30). The CD33 antigen was saturated in treated patients within 30 min at the 9 mg/m^2 dose. Response correlated with a combination of antigen saturation and low tumor cell efflux activity. The serum half-life of the conjugate was found to be 38 ± 21 hours. Immune response to the calicheamicin was detected in 2 of 40 patients, but no anti-hP67.7 response was observed. In the Phase II trial, patients were given up to 3 doses at 9 mg/m^2 at intervals of 14 to 28 days apart, and the overall response rate was 30%, with a relapse-free survival time of 6.8 months (31). In May, 2000, Mylotarg was clinically approved for the treatment of AML. Being the first such mAb-drug conjugate, this represents a significant breakthrough in the field.

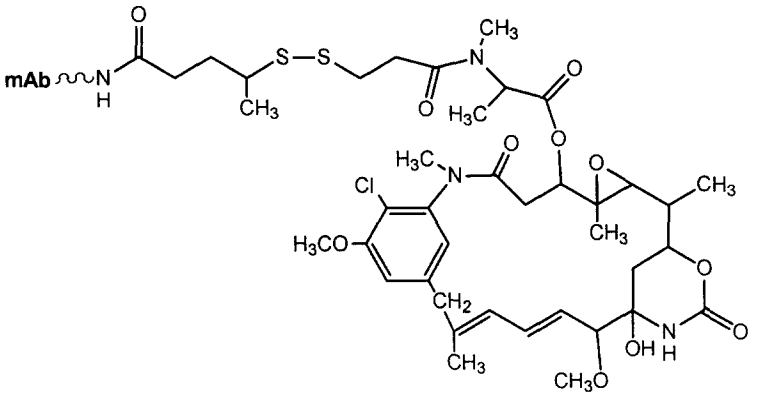
DISULFIDE-LINKED CONJUGATES

Seminal work with immunotoxin conjugates, such as mAb-ricin A chain, strongly suggested that disulfide linkers allowed for reversible drug attachment and significant selectivity, since thiol concentrations are much higher inside cells compared to in the serum (32). Even so, the immunotoxins were cleaved in the circulation, which prompted the development of hindered disulfides that were significantly more stable (33). Hindered disulfides have since played a significant role as linkers for mAb-drug conjugates.

mAb-maytansinoid conjugates - Cantuzumab mertansine (**3**) is comprised of the humanized mAb huC242 conjugated to the highly potent anti-tubulin agent DM1, a derivative of the natural product maytansine. HuC242, which was humanized using a technique known as "variable domain resurfacing" (34), recognizes a glycoform of MUC1 known as CanAg which is strongly expressed on most pancreatic, biliary, and colorectal cancers, and on 40-55% of non-small cell lung, gastric, uterine, and bladder cancers. CanAg is internalized upon binding to the conjugate (35).

DM1 is highly potent, with an IC_{50} in the picomolar range. To prepare DM1 conjugates, lysine amino groups on the mAb were first acylated with NHS esters of thiopyridyl disulfides. DM1, activated by treatment of a disulfide precursor with DTT and HPLC purified, was attached through disulfide exchange, releasing the thiopyridyl chromophore. A secondary methyl group adjacent to the disulfide provides steric stabilization of the disulfide *in vivo*. In nude mice bearing COLO 205 xenografts, cures were obtained at 16 mg mAb component/kg/day for 5 days (36).

Results from a Phase I dose escalation clinical trial have been reported (37). Doses were escalated in the trial from 22 to 295 mg/m^2, which was the approximate MTD. Analyses of serum samples from these patients indicated that the disulfide bond linking the drug and mAb cleaved in circulation and that the conjugate was cleared more rapidly than unconjugated mAb. The net result was an increase in the ratio of mAb to conjugate in the blood over a period of days after each injection. While evidence for minor responses was obtained, it is likely that the therapeutic potential of huC242-DM1 will be improved by increasing the systemic stability of the linker used to join the drug to the mAb.



3

mAb-minor groove binder conjugates - An analogue of CC1065, a potent minor groove binder alkylating agent was conjugated to murine and chimeric mAbs through a similar hindered disulfide linkage as described for DM1 (38). The resulting conjugates contained an average of 4-5 drugs per mAb and were highly potent *in vitro* with IC_{50} values in the range of 10 ng/mL. In SCID mouse models, the acute LD_{50} was 14 mg conjugate/kg, and the MTD was 5 mg conjugate/kg when administered over 5 consecutive days. In a disseminated lymphoma model, the conjugate provided a 2.7 fold increase in median survival time at its MTD, while drug alone and non-binding control conjugates provided substantially smaller increases in median survival time. Although the therapeutic window was very modest, the results provide further evidence that potent cytotoxic agents linked to mAbs through disulfide bonds have efficacy in preclinical models.

mAb-taxane conjugates - Disulfide linkages have been used to link taxoids to mAbs against the epidermal growth factor receptor (39). A form of paclitaxel was used that was 100-1000 times more potent than the parent drug on drug resistant cell lines. The drug was conjugated through the same hindered disulfide used to link DM1 to mAbs, and the resulting conjugate was active *in vitro* at sub-saturating concentrations. Tumor growth in nude mouse xenograft models was completely inhibited with a dose of 10 mg conjugate/kg given on five consecutive days, a dose that elicited no toxic side-effects. The taxoid conjugate described warrants further study, since the class of drugs is of strong clinical interest, and the particular drug chosen is highly potent. As with the mAb-DM1 conjugate, limitations may result from disulfide linker instability. The potential of this particular drug to circumvent common drug resistant pathways requires further study, and should be of considerable interest, given that this was identified as an issue with Mylotarg (29).

Anti-MUC1-calicheamicin conjugate - Conjugates of calicheamicin were prepared using the murine anti-MUC1 mAb, CTM01 (12). The drug was attached to mAb lysines *via* the same sterically hindered disulfide bond that is present in Mylotarg, but no hydrazone was incorporated. It was found that carcinoma cells do not require the acid labile linkage necessary for full activity for the anti-CD33 conjugate. CTM01-calicheamicin showed potent and specific anti-tumor efficacy in preclinical models of ovarian carcinoma. A version of this conjugate using a humanized version of the CTM01 was tested in a Phase I clinical study (40). The MTD was 16 mg/m^2, and there was some evidence for minor clinical responses. One of the issues

surrounding the use of MUC1 as a target for potent mAb-drug conjugates is normal tissue expression of the antigen.

PEPTIDE-LINKED CONJUGATES

The inherent instability of hydrazone and disulfide linkers has prompted several studies towards the identification of peptide sequences that can be used to attach drugs to mAbs. Such peptides are designed for rapid lysosomal hydrolysis and high serum stability. Initial studies indicated that cathepsin B, one of the most abundant of the lysosomal enzymes readily cleaved doxorubicin-peptide derivatives, but only if a self-immolative spacer was inserted between the peptide and the drug (41). Several dipeptides were found to be suitable for drug attachment, based on indefinite stabilities in serum and neutral pH, and rapid cleavage by lysosomal extracts or purified cathepsin B. This work was extended to include other drugs, such as paclitaxel and mitomycin C (42). This approach is particularly attractive, since the proteases that lead to drug release are mainly intracellular, and are less active in the blood, since they have acidic pH optima and are strongly inhibited by serum protease inhibitors.

BR96-peptide-doxorubicin conjugates - From a series of several dipeptide derivatives, BR96-phenylalanine-lysine-p-aminobenzyl-doxorubicin (BR96-Phe-Lys-Dox) and the corresponding valine-citrulline derivative, BR96-Val-Cit-Dox, were found to be serum stable and to undergo rapid cleavage by cathepsin B, leading to the release of doxorubicin (43). One of the enzymes responsible for drug release was cathepsin B, but kinetic data using crude lysosomal preparations indicated that other enzymes might also play a role. The stability characteristics of these conjugates were evident from *in vitro* assays, in which high levels of specificity were observed even upon prolonged treatment.

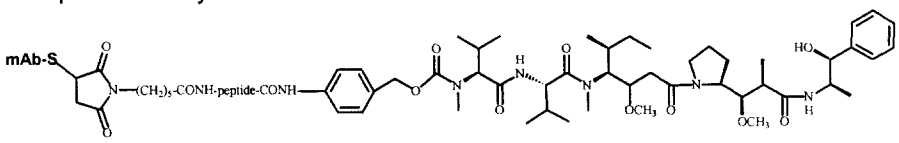
While it appears that the peptide-linked doxorubicin conjugates present significant advantages over the corresponding hydrazone conjugates described earlier, low drug potency remains an issue. For that reason, the investigators explored bivalent linkage systems that allowed for increased levels of drug substitution (44). Although the conjugates had enhanced potency, the substitution with as many as 16 drugs/mAb led to non-covalent dimerization. To circumvent this, hydrophilic ethyleneglycol hydrazides were appended in a reversible manner to the free carbonyl group of doxorubicin, and the resulting highly substituted conjugates were mostly monomeric (45). This approach, while interesting, suffers from significant complexity, since two independent events, proteolysis, followed by acid catalyzed hydrazone hydrolysis, must take place for intracellular drug activation.

mAb-auristatin conjugates - The auristatins are structurally related to dolastatin 10, a pentapeptide natural product that has been the subject of several human clinical trials for cancer therapy (46). Molecules in this family exert potent antitumor activities through the inhibition of tubulin polymerization, and may also lead to intratumoral vascular damage. The activities are generally 100-1,000 times more potent than doxorubicin. Auristatins can be prepared in large quantities through total synthesis, and unlike calicheamicin and the CC1065-like minor groove binders described earlier, the drugs tend to be exceedingly stable.

Peptide derivatives of the synthetic auristatin MMAE were prepared using methodologies similar to those described for the peptide-linked doxorubicin conjugates (47). mAb-Val-Cit-MMAE and mAb-Phe-Lys-MMAE conjugates (**4**) were prepared using the mAb interchain mAb disulfides, producing conjugates with approximately 8 drugs/mAb. Unlike the doxorubicin conjugates, mAb-peptide-MMAE conjugates effected immunologically specific cell kill at concentrations well below

that required for antigen saturation. The conjugates were highly stable in human serum, with projected half-lives of greater than 80 days. By comparison, the hydrazone-linked mAb-AEVB conjugate released free drug a $t_{½}$ of only 2-3 days in plasma. Thus, the peptide linker is much more stable than an optimized hydrazone linker.

In vivo studies in both carcinoma and hematologic tumor xenograft models demonstrated that the mAb-Val-Cit-MMAE conjugate was highly effective, immunologically specific, and well-tolerated. Studies were reported with cAC10-Val-Cit-MMAE, a conjugate that bound to the CD30 antigen on hematologic malignancies, in which cures of established tumors were obtained at doses as little as 1/60th the MTD. Therapeutic windows this high have not been reported in the drug conjugate field, and the results underscore the importance of drug potency, linker design, and mAb trafficking in developing conjugates with optimal chances for therapeutic efficacy.



4

ANTIBODY ENGINEERING

Recombinant technologies have been applied towards addressing issues surrounding the pharmacokinetics and biodistributions of mAbs, particularly to mAbs that bind to tumor-associated antigens. The goals of much of this work are to produce new mAbs, mAb fragments, and mAb constructs that have high tumor to non-tumor binding ratios, and that also have high intratumoral localization characteristics. For example, recombinant Fab' fragments were produced that had unpaired cysteine residues, which were subsequently modified with electrophilic cross-linking reagents (49). Alternatively, the modified Fab's were site-specifically coupled with polyethylene glycol and the resulting conjugates had extended serum half lives and intratumoral uptake characteristics (50). Another elegant approach towards making new mAb constructs with controlled pharmacokinetic characteristics involves the construction of Fab-peptide fusion proteins, in which the peptides are selected for their abilities to bind to human albumin albumin (51). Such relatively low molecular weight constructs would be expected to have enhanced serum half-lives compared to mAb fragments, and may be able to penetrate solid tumor masses better than whole IgGs. A further example of how recombinant technologies can be applied towards developing new mAb forms for cancer therapy involves mAbs with modified carbohydrate substitution patterns. These molecules were selected for dramatically reduced binding to Fc receptors on non-tumor cells, which could alter conjugate toxicity profiles (52). Finally, a recent study mapping the interactions of mAb hinge region residues with various Fc receptors should prove useful in designing new constructs with controlled pharmacokinetic parameters and reduced binding to non-target tissues (53). Antibody engineering will undoubtedly play a role in the new generation of drug conjugates that are being developed.

CONCLUSIONS

With the approval of Mylotarg, mAb-drug conjugates have demonstrated a new level of clinical utility. One of the major challenges that lies ahead is to develop new-generation mAb-drug conjugates that can be used for treating solid tumors and

lymphomas. Recent work in the field strongly support the use of highly cytotoxic agents attached to mAbs with linkers that are stable in circulation. Protease cleavable peptide linkers have substantially improved *in vivo* stabilities relative to hydrazones and disulfides, and may represent a major advance for mAb-drug conjugate technology. Further progress in the field of mAb-drug conjugates for cancer therapy should come by combining the advancements in drug and linker technologies with re-engineered mAbs that are designed to have optimal targeting properties.

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SECTION V. TOPICS IN BIOLOGY

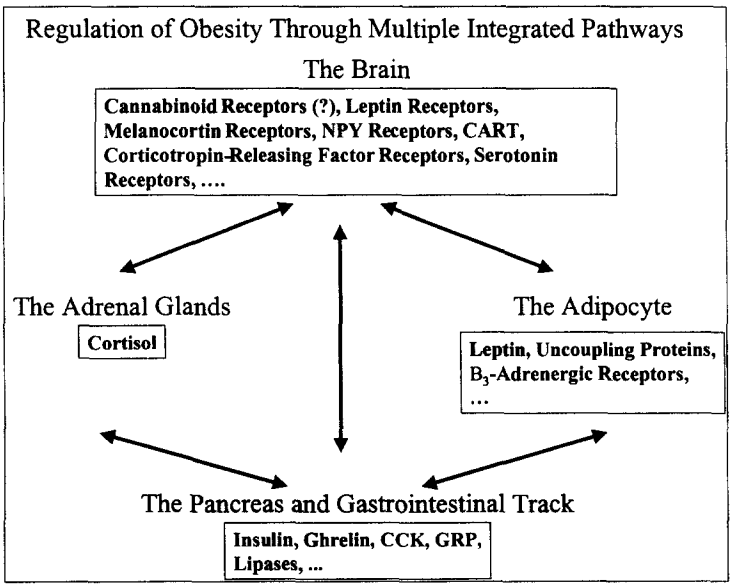
Editor: Janet M. Allen,
Inpharmatica, London, United Kingdom

Chapter 24. Obesity Therapeutics: Prospects and Perspectives

David M. Duhl and Rustum S. Boyce
Chiron Corporation, Emeryville, CA 94608

Introduction – The prevalence of obesity is rising at an alarming rate. When measured by Body Mass Index (BMI = weight in kilograms divided by the square of height in meters), a BMI of 30 is the threshold for obesity. In the United States alone, an analysis of the National Health and Nutrition Examination Survey (NHANES IV) data from 1999-2000 found that the age-adjusted prevalence of obesity was 30.5% and had increased by approximately 8% in just 5 years (NHANES III 1988-1994) (1). This shocking increase in obesity has been mirrored to varying degrees throughout the world.

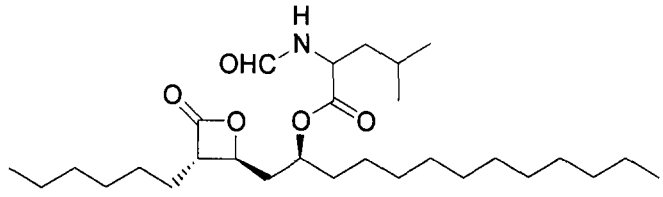
Obesity is often mischaracterized as a cosmetic, or life style issue when in fact it is a devastating disease with tremendous health and financial consequences. In the US alone, it has been estimated that there are greater than 300,000 deaths per year (2). This distressing effect on life expectancy is largely related directly to the life threatening co-morbidities of obesity such as non-insulin-dependent diabetes, hypertension, coronary artery disease, and some forms of cancer (3). The less lethal comorbidities associated with obesity include gallstones, osteoarthritis, degenerative arthritis, and apnea. The financial consequences of obesity can be measured in multiple ways; as a total cost to society (direct and indirect), estimated percent of total medical cost, and direct value of obesity related medications (4-6). Whichever parameter is chosen to measure the financial impact of obesity, the cost is highly significant to our society.



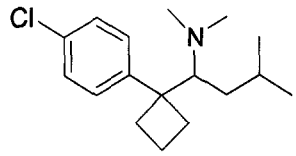
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HISTORICAL APPROACHES TO OBESITY THERAPEUTICS

Historically, the pharmaceutical industry has had little success in finding safe and effective drugs for the treatment of obesity (7-10). Currently the two largest selling anti-obesity drugs are Xenical **1** (Orlistat) and Meridia **2** (Sibutramine).

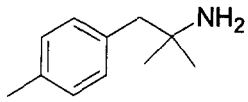


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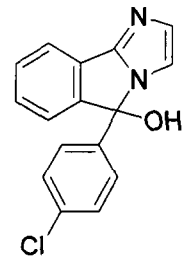


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Both these medications have only moderate long-term efficacy and have prescription limiting side effects. Two other medications that have been widely prescribed for obesity are Phentermine **3** (alone or in combination with fenfluramine), Mazindol **4**, and Diethylpropion.



3



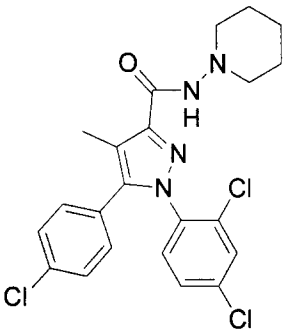
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A comprehensive list of all medications for obesity is outside of the scope of this article (7-10), but as with Meridia and Xenical, all current or past therapeutics have had limited effect on obesity or its co-morbidities, and are associated with a long history of safety concerns. The poor efficacy and safety issues have, no doubt, limited the current size of the obesity therapeutic market.

CURRENT APPROACHES TO OBESITY THERAPEUTICS

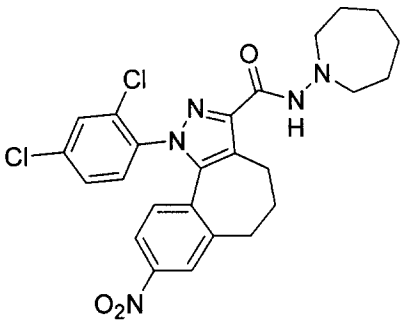
On-going clinical trials: While there are currently a large number of clinical trials reported to be on-going for the treatment of obesity, this review will focus on three that have garnered the most attention recently; SR-141716A (Rimonabant), P-57, and recombinant human cillary neurotrophic factor (rCNTF, Axokine) (11).

SR-141716A - SR-141716A **5** is a cannabinoid CB1 receptor inverse agonist being developed by Synthelabo (Sanofi) and currently reported to be in phase III trials (12).



5

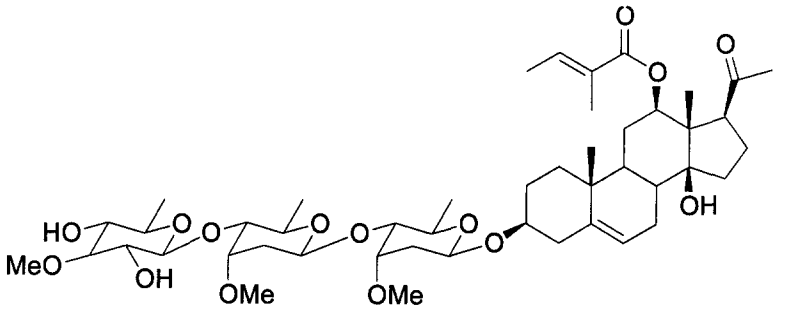
Both central and peripheral mechanisms of action have been suggested for SR-141716A (13,14). Recent publications have described the structure-activity relationships of SR-141716 analogues and on attempts to decrease the reported high lipophilicity of Rimonabant (15,16). Concerns over the potential of SR-141716 tend to center around the lipophilicity of the compound, and ultimate durability of the currently reported, short-term, effects on food consumption. A number of groups have reported some success in developing potent analogues to SR-141716A e.g. **6**, (17,18).



6

P-57 - P-57 **7** is a natural product derived from a succulent of the Hoodia family, initially developed by Phytopharm and recently licensed to Pfizer (19-21). P-57 is currently reported to be in phase II studies as an appetite suppressant and for treatment of Type II diabetes. In a small (60 patients), placebo-controlled, human proof of principle study, P-57 was reported to achieve a 30% reduction in calorie intake (19). As of the time of writing of this review, there is no targeted reported

mechanism of action for P-57 that would help in the optimization of second-generation compounds.



7

Axokine - While not a medicinal chemistry based approach, a discussion of Axokine is necessary for a thorough assessment of the competitive landscape in the area of anti-obesity drug development (22). As stated above, Axokine **8** has been derived from CNTF and is a peptide, or protein based, approach (The composition of Axokine is not explicitly outlined in the patent. Recombinant CNTF Axokine-15 is shown as a single letter amino acid sequence with mutations CYS17 to ALA (bold & Underlined) and GLN63 to ARG (bold & Underlined) and a 15 amino acid deletion of the carboxyl terminal amino acids (not shown)).

MAFTEHSPLTPHRRDL**A**SRSIWLARKIRSDLTALTESYVKHQGLNK
NINLDSADGMPVASTD**R**WSELTEAERLQENLQAYRTFHVLLARLLE
DQQVHFTPTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPRNEAD
GMPINVGDGGLFEKKLWGLKLQELSQWTVRSIHDLRFISSHQTG

8

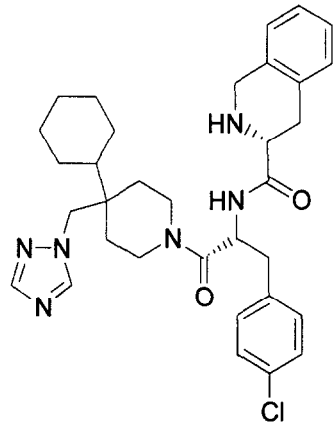
Axokine is currently in phase III clinical trials in an injectable form, and is reported to have shown limited efficacy in man (23). According to a recent announcement of the preliminary results of the phase III trial currently underway, a greater proportion of Axokine(R)-treated patients lost at least 5% of their initial body weight compared with placebo-treated patients (25.1% vs. 17.6%) and showed a greater than average weight loss (6.2 pounds vs. 2.6 pounds) after the first 12 months but approximately 70% developed Axokine specific antibodies.

Pre-Development targets: There are a large number of potential targets for anti-obesity therapeutics that are in various stages of pre-development (24). This review will focus on just a few; Melanocortin 4 receptor (MCR-4), Neuropeptide Y (NPY) receptors, Protein tyrosine phosphatase 1B (PTP1B) and Melanin concentrating hormone receptor.

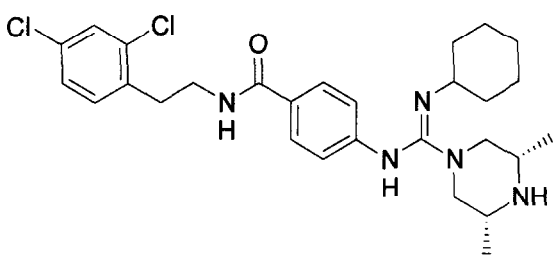
Melanocortin 4 Receptor - Excitement over the potential of the Melanocortin 4 receptor as a target for anti-obesity therapeutics has been quietly growing for some time (25). MCR-4 is an unusual G-protein coupled receptor in that it has a naturally occurring protein agonist (α-MSH) and two protein antagonists (agouti and agouti related protein, AGRP). Investigators studying the obese-yellow phenotype of mice with mutations in the agouti gene initially discovered the role of MCR-4 in weight regulation. Peptide derivatives of α-MSH, which are functional MC-4R agonists,

have been shown to decrease food consumption and weight in rodents. Peptide derivatives of α-MSH, which are functional MC-4R antagonists, as well as agouti and AGRP, have been shown to increase food consumption and weight in rodents. In addition, genetic studies have shown that the melanocortin-4 receptor (MC-4R) has a critical role in the regulation of food consumption and metabolism in humans, as well as mice. Since there are five known melanocortin receptors (MCR-1, MCR-2, MCR-3, MCR-4, and MCR-5), with relatively high similarity at the amino acid level, identification of selective MCR-4 agonist could be difficult.

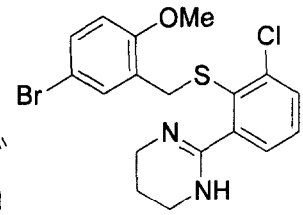
Recently a number of research groups have claimed to have some success in identifying specific small molecule agonists for MCR-4 (26,27). Most of these compounds have evolved from peptide based **9** approaches but some non-peptide based compounds **10** and **11** have also been discovered (28,29). 2,3-Diaryl-5-anilino[1,2,4,]thiadiazoles agonists have been reported to effect feeding behavior when given intraperitoneally, but not orally, in rats (30). Aside from concerns over the pharmacokinetic properties of peptide based therapeutics, and therapeutic liabilities of agonists in general, there have been reports of erectogenic effects in rodents which could conceivably limit the utility of MCR-4 therapeutics (31).



9



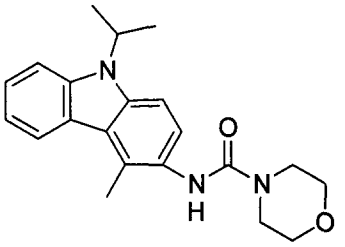
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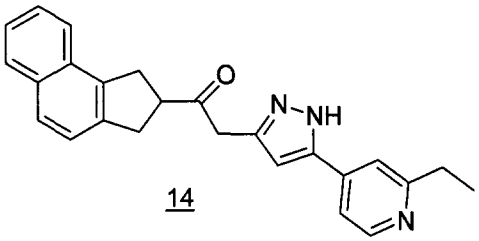
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Neuropeptide Y Receptors - Neuropeptide Y (NPY) is a small (36-amino acid) amidated peptide that has potent orexigenic effects when injected directly (intracerebroventicular administration) into the brains of rats (31). To-date, at least six NPY receptors have been identified. The NPY receptors, Y1 and Y5, are most often identified as the targets for anti-obesity therapeutics. There is much conflicting evidence on the potential utility of NPY receptors, but with the recent demonstration that infusion of the NPY Y2 receptor peptidic agonist PYY3-36 reduced food intake

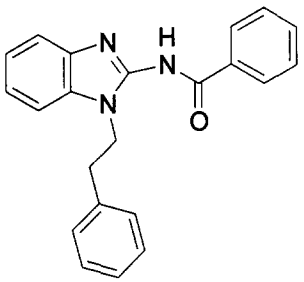
in humans by 33%, has re-invigorated this area of research (32,33). Recent reports describe receptor specific carbazole ureas **13** and orally available, brain-penetrable arylpyrazole **14** for the treatment of obesity. A non-orally available NPY-Y5 specific compound (GW438014A) has been reported to decrease the rate of weight gain and reduce fat mass by (10 mg/kg) intraperitoneal administration **15** BID (34-36).



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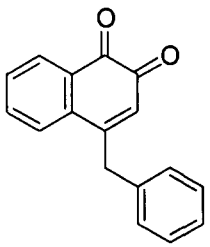


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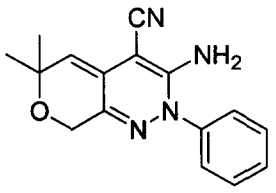


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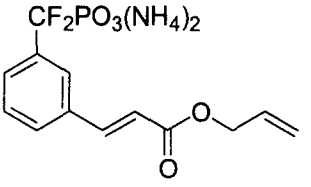
Protein Tyrosine Phosphatase 1B - Another active area for anti-obesity research has been the use of protein tyrosine phosphatase 1B (PTP1B) inhibitors for the treatment of obesity and diabetes. (38). PTP1B is involved in down-regulation of receptor tyrosine kinase activity following stimulation of the insulin or leptin receptors. In theory, inhibition of this pathway should ameliorate the insulin/leptin de-sensitization often seen in obese patents. In addition, increased insulin sensitivity and resistance to diet induce obesity was seen in PTP1B knockout mice (39). Recent reports have described the synthesis of selective 1,2-Napthoquinone inhibitors **16**, non-competitive Pyridazine inhibitors **17**, competitive [Difluoro-(3-alkenylphenyl)-methyl]-phosphonic acids **18** and orally available derivatives of 2-(oxaylamno)benzoic acid **19** as potent PTP1B inhibitors (40-43).



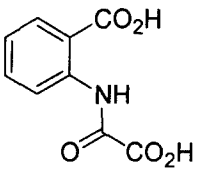
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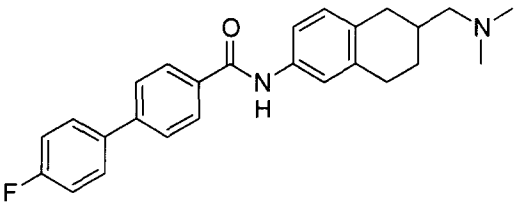


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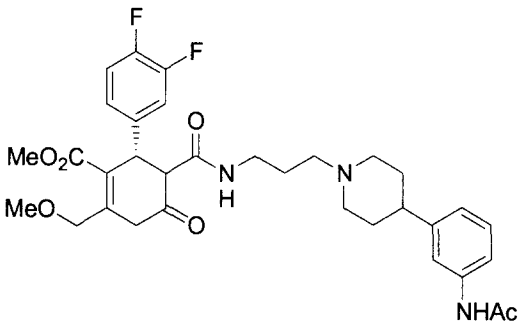


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Melanin-Concentrating Hormone Receptor - While it has been known for some time that the cyclic 19-amino peptide Melanin-concentrating hormone increases food intake when injected intracerebroventricularly, the Melanin-concentrating hormone receptor (MCH1R) responsible for this activity has only recently been identified (44). Mice carrying knockouts of the MCH1R gene have a normal body weight, but are lean (less body fat), hyperphagic and have altered metabolism (45,46). Most importantly, MCH1R knockout mice are resistant to obesity induced by high fat diets. Recent reports describe two selective and potent MCH1R antagonist, T-226296 **20** and SNAP7941 **21** (47, 48).



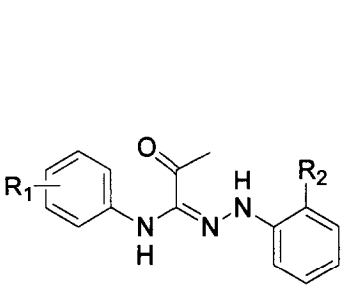
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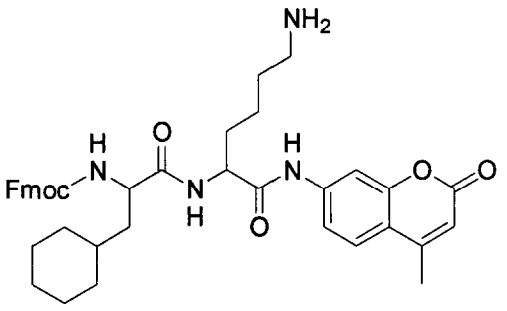
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Both compounds are reported to be potent (K_i =5.5 and 15 nM vs MCH respectively) and are highly selective with respect to the second reported Melanin-concentrating hormone receptor (MCH2R) (44). T-226296 is reported to be orally available and able to inhibit the increase in food intake following intracerebroventricular administration of MCH. Intraperitoneal injections of SNAP7941 are also reported to cause inhibition of food intake following intracerebroventricular administration of MCH. In addition, twice daily intraperitoneal injections of SNAP7941 induced a greater decrease in food intake in diet-induced obese rats then rats treated with D-fenfluramine (3 mg/kg). Surprisingly, oral administration of SNAP7941 demonstrated anxiolytic effects in the rat forced-swim test, rat social interaction test and the quinea pig maternal-separation vocalization test (48).

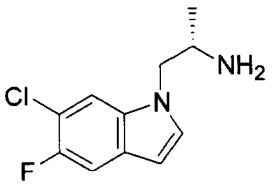
A recent publication was devoted to a more complete review of targets for anti-obesity therapeutic and included discussions on thyroid hormones, B_3-adrenoceptor agonists, leptin, cocaine amphetamine regulated transcript, corticotropin-releasing factor receptors (CFR1), orexin, ACRP30, Ghrelin, Galanin, Glucagon-like peptide 1 (GLP-1), and Bombesin receptors (49-59). While many avenues for new anti-obesity drugs have been investigated, unfortunately for the vast majority of these targets, there has been relatively little, new, information published on small molecule ligands and/or their *in vivo* efficacy in obesity models (8). A peptidic derivative of GLP-1 (NN2211) has been shown to have a dose dependent short term effect on food intake in diabetic mice and its human pharmacokinetics has been described (60,61). In addition, there have been reports on Arylamidrazones as ligands for CRF1 **22**, and a description of a nonpeptide galanin receptor agonist **23** (62,63). Unfortunately there have been no published reports describing effects of either series of compounds on weight gain or eating behavior. Finally, there is a recent publication describing Serotonin compounds **24** and **25**, and there efficacy *in vivo* (64).



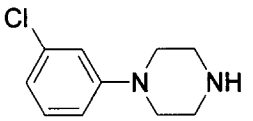
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THE UNTAPPED POTENTIAL OF ANTI-OBESITY DRUGS

The large potential market and the shortage of safe, efficacious compounds is driving many researchers to undertake the difficult task of creating new anti-obesity drugs. Overall the track record of the pharmaceutical industry has been poor in this area, but the recent explosion in our understanding of the biology of obesity has changed the drug discovery landscape. The identification of a multitude of new targets for obesity therapeutics, and a deeper understanding of existing targets, has increased our potential for finding the "right" focus for increasing efficacy. And while there is a higher sensitivity for safety in anti-obesity therapeutics then that for cancer

therapeutics, there is also a greater understanding of the health and financial consequences of this unmet medical need. While the potential of anti-obesity drugs currently remains largely untapped, the industry should be optimistic about the finding solutions to these dual issues in the future.

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Chapter 25. SNPs: A human genetic tool for the new millennium

Albert B. Seymour[1], Poulabi Banerjee[1], Aidan Power[2], and Patrice M. Milos[1]
Discovery[1] and Clinical[2]Pharmacogenomics, Pfizer Global Research and Development

Introduction - Human beings have known for generations that specific characteristics such as eye color and facial features, as well as disease risk, are inherited and that these phenotypes vary considerably throughout the human population. The solving of the DNA structure in 1953 and the completion of the human genome project 50 years later has lead to the identification of extensive variation between individuals at the DNA level. For more than 100 years, human geneticists have been studying how the inheritance of genetic variation contributes to human disease risk. Today, more than 1,200 genes have been identified that cause human disease (1). Comparing the sequence of the human genome between individuals has lead to the identification of millions of human DNA variants (2,3). The vast majority of these variants are in the form of single nucleotide polymorphisms (SNPs) which can now be used by human geneticists, on an unprecedented scale, to characterize human populations, migration patterns, and evolution, and to identify genes predisposing to common diseases that contribute to significant human morbidity and mortality.

HUMAN GENETIC POLYMORPHISMS

DNA variation occurs frequently and consists of various types of changes that exist at differing frequencies. The sequencing of the genome, and the research that has followed, has revealed that human beings vary in their DNA sequence at approximately 0.1% of the nucleotides, resulting in an estimated 3,000,000-15,000,000 polymorphisms. These polymorphisms can be categorized into several different types depending on the type of DNA variation that contributes to the polymorphism (Table 1).

Table 1. Examples of polymorphisms.
Polymorphic bases are underlined for each pair

Single Nucleotide Polymorphisms (SNP)	GAATTTAAG GAATTCAAG
Simple sequence Length Polymorphisms	NCACACACAN NCACACACACACAN
Insertion /Deletion	GAAATTCCAAG GAAACCAG

The very first human genetic studies involved the use of Restriction Fragment Length Polymorphisms (RFLPs), the majority of which were single nucleotide polymorphisms that introduced or abolished the recognition sequence of a restriction endonuclease (4). The use of RFLPs for pedigree-based disease gene hunting was not ideal as the maximum heterozygosity, the frequency at which an individual carries both alleles, could only reach 50%. The higher the heterozygosity for a particular genetic marker, the better the probability of a subject carrying two distinguishable copies that can be tracked from one generation to the next.

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Thus, the discovery of better markers was necessary in order to progress further with efficient human genetic studies.

Subsequent to the discovery of the RFLPs, simple sequence length polymorphisms (SSLPs) were identified at fairly high densities across the genome. These polymorphisms were defined by the number of tandem repeats of nucleotides in a succession (5,6). For example, a person may have inherited 12 CA nucleotide repeats at a specific locus from her mother, but 20 CA repeats in the same locus from her father. Several studies have suggested that the expansion and contraction of these repeats during DNA replication occurs at a frequency much higher than that of single nucleotide mistakes. This results in the introduction of new alleles into the population at a rate higher than that observed at single nucleotides. The advantage of these polymorphisms is the larger number of alleles that can be observed in the population at any one locus. This increase in the number of alleles renders these markers significantly more polymorphic than RFLPs with heterozygosity ranging from 1-90%. With small nuclear families for human genetic studies, the increase in heterozygosity made these markers much more powerful as the probability to detect a heterozygote subject was much higher than with RFLPs. Furthermore, these polymorphisms could be genotyped using standard electrophoresis and did not require the use of restriction endonucleases. SSLPs have been the predominant polymorphism used in human genetic studies since around 1990 and have served as a genetic mapping tool that has resulted in the discovery of hundreds of genes that contribute to rare Mendelian diseases (7). However, their use in population-based genetic association studies has been limited due to their high mutation rate (which introduces new alleles into the population and makes interpretation of specific allelic associations difficult) and their low density across the human genome.

Single nucleotide polymorphisms (SNPs) are the result of a substitution of a nucleotide at a specific location in the genome and have been identified as the cause of many diseases and phenotypes such as sickle cell anemia and differences in blood groups. The recent completion of the human genome sequence and the efforts of the SNP Consortium, a collaboration between 13 pharmaceutical companies and the Wellcome Trust, has resulted in the identification and mapping of more than 3,000,000 SNPs across the human genome.

The need for SNPs - Genome scans to identify genes contributing to common diseases such as schizophrenia, osteoarthritis, diabetes and cardiovascular disease have had limited success due to the fact that these diseases likely result from multiple genes, each contributing a small effect, combined with environmental factors (8). The use of isolated populations resulting from a small number of founders, such as Iceland, has enhanced the power to identify genes contributing to disease based on the smaller number of disease causing mutations introduced into the populations as well as several other population genetic parameters. Recently, the identification of neuregulin, a gene contributing to schizophrenia, was identified using this approach (9). However, the use of traditional family-based studies to identify these genes are generally underpowered due to the small numbers of affected subjects within families, the age of onset for many common diseases restricting the collection of large multi-generational families, the limited number of recombinant events occurring over only a few generations, large variation in the penetrance of disease alleles, to variation in environmental risk exposures, and the expected small effect size of single genes. It has long been recognized that association studies using unrelated subjects has more power to detect genes with smaller effect sizes based on population recombinant rates over hundreds of generations, the ability to evaluate large populations characterized by discordant phenotypes (i.e. inflammatory bowel disease vs. healthy matched controls), and the assumption that common diseases are caused by common variants. However, until

recently, the density of polymorphisms required to scan the genome using an association study design was not available.

The vast majority of human SNPs are biallelic, although there are several examples of triallelic SNPs. SNPs have been described for all possible transitions and transversions, although the most common is the A/G transition due to the 5-methylcytosine deamination reactions that occur frequently, particularly at CpG dinucleotides (10). SNPs range in frequency from 1% to 50% in the general population and for the most part are observed across ethnicities, but a significant proportion of SNPs exhibit differences in allele frequency depending on the ethnicity of the study population (11,12). It was the discovery of the high density of these polymorphisms across the human genome, together with technologies that enable the cost-efficient scoring of SNPs, that sparked a new era in the search for disease genes and the search for genes contributing to other traits such as response to therapeutic intervention. The most recent tally of human SNPs is 3,049,569 (13). These SNPs are currently being used to map disease genes, develop pharmaceutical agents, understand the historical migration patterns of human population and even study the evolution of humans from our primate ancestors (14,15).

SNP Discovery - In the mid to late 1990s the drive towards SNP discovery came from quantitative estimates which consistently showed that whole genome association studies would be more powerful than the traditional family-based linkage analysis for the identification of genes involved in complex diseases (16). However, successfully executing a whole genome association study to identify genes contributing to common diseases required a map of SNPs spanning the human genome at a high density (1 SNP/3-50 kb) as well as technological advances in genotyping to enable a cost-effective experiment.

In 1997, The SNP Consortium (TSC) comprised of 13 pharmaceutical companies and the Wellcome Trust was formed with the goal of re-sequencing a representative portion of the human genome to discover novel SNPs (17). The original goal was to identify 300,000 SNPs and map 170,000 of these, evenly spaced across the genome. SNP identification was performed at three centers, The Whitehead Institute, The Sanger Institute and Washington University. Each center sequenced a reduced representation fraction of the genome based on size selection of genomic DNA after digestion with restriction endonucleases. The source of DNA used was a pool of genomic DNA derived from 24 healthy volunteers, who remain anonymous. This effort ultimately turned out to be far more productive than originally planned and lead to the discovery of more than 1.5 million SNPs mapped across the human genome (18).

In parallel with the efforts from TSC, the International Human Genome Sequencing Consortium was discovering SNPs by analyzing clone overlaps derived from the human genome physical map (19). A publication of the first map of human SNPs was published in February 2001, describing the mapping of 1.42 million SNPs (2). These efforts have resulted in the delivery of a SNP map at a density for which whole genome association studies are now feasible. As of early 2003, SNP data (build 111) from the public SNP map at the National Center of Biotechnology contains 3,736,344 SNPs. The density of SNPs across the human genome is currently estimated at 1 SNP every 943 base pairs (20).

The total number of common SNPs (>10% frequency) existing in the human population is still unknown. Estimates have ranged from 3,000,000 to 15,000,000 based on empirical and theoretical modeling (7). The markers necessary for large-scale human genetic association studies have been identified. The next hurdle is to

characterize these SNPs with respect to allele frequencies, organization within the genome,segregation patterns from one generation to the next, and ultimately their functional role in contributing to common diseases.

SNP Typing - Methodological advances in the genotyping of SNPs is leading to the development of accurate, cost-effective approaches to the analysis of SNP data on a very large scale (21). Current approaches can be divided into two broad categories, sequence extension and allelic discrimination by hybridization.

The majority of methods involve the amplification of the SNP-containing region of genomic DNA using the polymerase chain reaction (PCR). After amplification, the SNP can be interrogated using one of several methods currently available. The most widely used approach employs a second primer that anneals directly adjacent to the variable base. Taking advantage of dideoxy nucleotides labeled with a fluorescent dye, the reaction can extend one base and then terminate, resulting in a population of primers specific for the SNP at the extended nucleotide. Measurement of the specific fluorescent intensity, fluorescence polarization, or mass of the extended product can be used to directly score the SNP (21,22). This method of genotyping is amenable to multiplexing, where several SNPs can be scored simultaneously by modifying the number of fluorescent dyes, or the size of the specific primers.

Hybridization-based approaches rely on the kinetics of DNA annealing as the basis for the specificity of the genotyping. This methodology has been developed for use in single homogeneous assays utilizing both hybridization kinetics and fluorescence resonance energy transfer, as well as array-based approaches (23-25). Using an array, millions of short DNA segments, oligonucleotides, which are complementary to the specific region of genomic DNA being investigated, can be placed on a surface, generally silica glass (26). The genomic DNA region containing the SNP is amplified using the polymerase chain reaction, labeled with a specific fluorescent dye and hybridized to the array under conditions that only enable hybridization if 100% of the sequence is complementary. In a similar fashion, the arrays can serve as solid templates for capturing specific sequences and enabling primer extension reactions to occur directly on the array (27,28). This enables the manufacturing of universal arrays that are independent of the SNPs being scored.

There are several technologies that have expanded on the array-based approach and have incorporated microsphere beads with specific labels and probes that enable the execution of primer extension or hybridization-based assays directly on the beads (29). This has increased the extent of multiplexing to several hundred-fold, which decreases the cost of the reactions even further. While these developments in genotyping have definitely increased the capacity to genotype up to 1,000,000 genotypes in a day, there is still no technology available that can economically provide individual genotyping data on the scale necessary to perform a whole genome association study. Estimates of SNP requirements for a whole genome association study range from 60,000 SNPs to over 1,000,000 SNPs bringing the number of individual SNPs needed to as many as 1 billion. Assuming a genotyping rate of 1 million SNPs/day, that study would take more than 3 years of genotyping, 24 hours a day, seven days a week. Therefore, the advantages gained by having individual genotype data for a whole genome association scan may not be attainable based on current technical limitations.

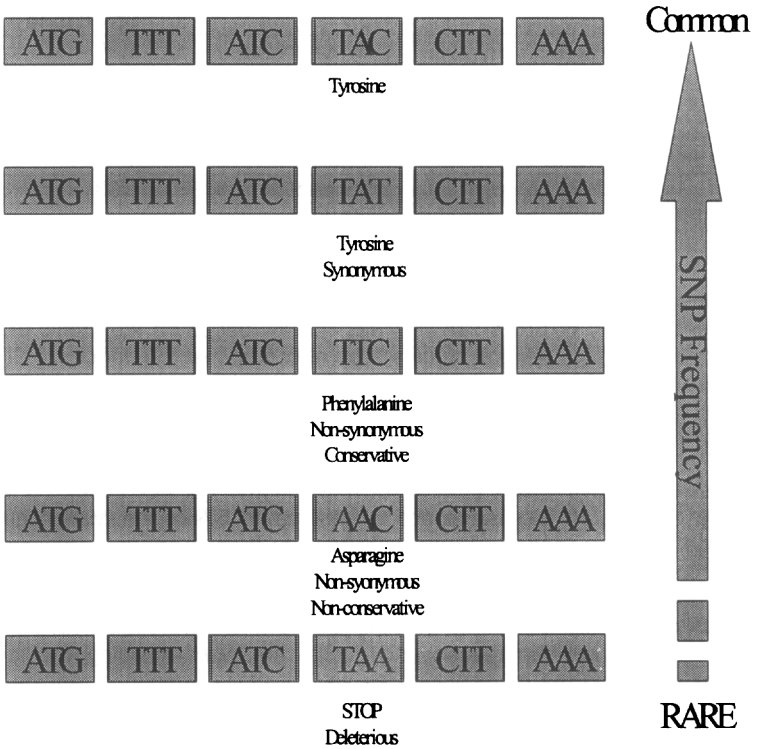
DNA pooling approaches to enable genome scans have been proposed as an alternative method to enable a single pass genome scan while reducing the total number of individual genotypes by several orders of magnitude (30). Using this approach, equal concentrations of genomic DNA from each subject within a

particular population of study are pooled into a single tube and subsequent genotyping is performed using DNA from these pools. This method requires a genotyping methodology that can accurately quantitate signal frequencies in a population of alleles. It is also dependent on the accurate quantification of DNA from each individual. Several groups have reported on the utility of this approach in large scale human genetic association studies and it is likely to be the method of choice for the first whole genome scans (30,31).

SNP CHARACTERIZATION AND IMPLICATIONS FOR STUDY DESIGN

Several studies investigating specific regions of the genome suggest that the distribution of SNPs across the genome is uneven, and may be driven by factors such as regional recombination rates and shared genealogical history. In addition to these natural forces, there are several reports that describe the density of SNPs within genes and differences based upon coding and non-coding regions (32-36). The effect SNPs have on the final translated protein can vary based on the location of the SNP within the gene (37). Figure 1 shows examples of different types of SNPs and how they can affect the protein product.

Figure 1



Empirical data suggest that regions of genes specifically coding for proteins are more conserved than other regions. Coding region polymorphisms that do not result

in a change of amino acid (synonymous) are observed more frequently than would be expected by chance. In two large studies, 44-52% of SNPs identified were synonymous, compared to 47-55%, which were non-synonymous (34,35). Since approximately two-thirds of the coding nucleotides are critical in determining the codon for an amino acid, the expected number of non-synonymous SNPs is closer to 66%. This observed reduction in non-synonymous SNPs suggests evolutionary conservation of these regions and that selection may be responsible for the reduction in nucleotide diversity within these regions (38). This is also evident when comparing gene sequences across species where the conservation of exonic regions is significantly higher than intronic and regulatory regions.

Table 2 shows a summary of the distribution of SNPs based on coding and non-conding regions as estimated from the SNPs in dbSNP. These data are similar to those previously reported in that the number of synonymous and non-synonymous SNPs within coding regions is approximately a 1:1 ratio, again suggesting selection against non-synonymous. The density of noncoding, including untranslated regions of exons and intronic regions, suggest less selective pressures as evidenced by the 11 and 50-fold increase in SNP density within these regions, respectively.

Table 2: SNP density in the genome

Number of SNPs	Number of Genes	Function	Ration of SNP/Gene
16175	8101	Synonymous	1.99
19271	8808	Non-synonymous	2.1
162301	14139	Untranslated Region	11.5
805,096	16215	Intron	49.7
241	233	Splice Site	1.03
Data obtained from dbSNP summary build 111 (20)			

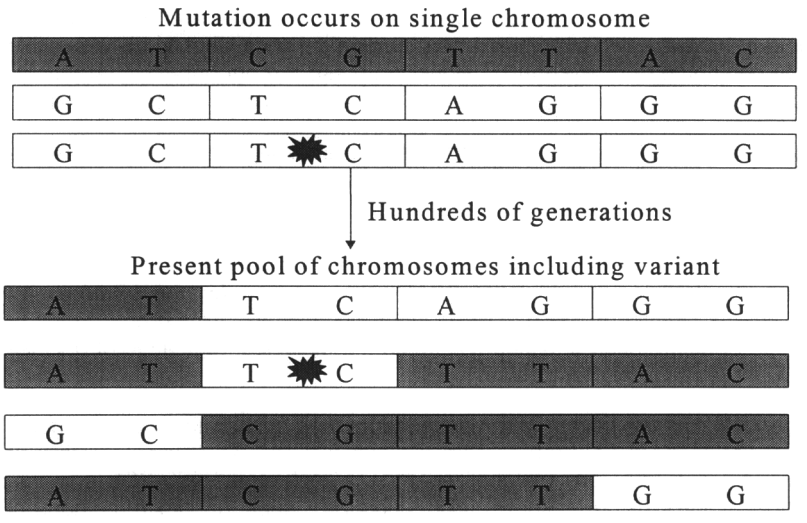
SNP density not only differs according to the region being investigated, but the frequency and density of SNPs can vary significantly based on the ethnic origin of human populations (11). Several studies have observed that the density of SNPs is higher in populations of African descent compared to other races around the world. Knowledge of these differences in SNP frequency and density needs to be considered when executing a case control genetic association study.

The vast majority of SNPs throughout the genome will not affect the coding sequence of genes. In fact, since only an estimated 5% of the human genome codes for genes, most SNPs will not even map to human genes. However, the effect of these non-genic SNPs on the regulation of gene transcription, DNA replication, and stability is far from known and is a primary research question that the genetics community is actively seeking to answer. The sequencing of other organisms, for example the mouse and gorilla, are now providing the tools to characterize cross-species conservation as an indicator of genomic regions under evolutionary conversation pressure. This comparative genomics approach is being to used to identify functional regions within these non-genic regions of the human genome.

The identification of SNPs that are causative of disease as opposed to those that have no functional effect is a tremendous effort. The economic cost of scoring every SNP in every subject is prohibitive today. Thus, geneticists must select a panel of SNPs that has the density necessary to identify genetic associations. However, the density needed is currently unknown. Estimates of SNP density needed for a whole

genome scan are based upon a principle of inheritance in which SNPs in close proximity tend to segregate together more frequently than SNPs that are far apart. This principle is defined as linkage disequilibrium, the non-random association of two SNPs (Figure 2). Since all SNPs originated at one point during human evolution as mutations, they occurred on one specific chromosome. At the time of mutation, the SNP is tightly associated with very large regions of neighboring DNA. However, for each generation, recombination between the SNP and neighboring DNA occurs. This process, over thousands of generations, has resulted in blocks of SNPs that segregate from one generation to the next as a single unit. These blocks of SNPs are defined as haplotypes. An understanding of the size of these haplotypes within the human population can be used to estimate how many SNPs are needed to represent the unique haplotypes across the genome. Haplotypes can be used to identify disease relevant SNPs even if the actual causative SNP is not directly scored. This is due to the fact that the causative SNP segregates through generations on a specific haplotype background that can be observed using only a small set of SNPs (39,40). Several recent studies have estimated the length of these haplotypes across various regions of the genome (41-43). Empirical data suggest that these haplotypes extend from only a few thousand base-pairs to more than a million base pairs depending on the region of the genome being evaluated. The average distance is in the range of 20,000 base-pairs for Caucasians, but is less for humans of African ancestory, being in the range of 15,000 base-pairs.

Figure 2: Linkage Disequilibrium



UTILITY OF SNPS IN MEDICINE AND THE PHARMACEUTICAL INDUSTRY

Understanding the link between genetic variation and phenotype variation is the foundation for the science of human genetics. Human genetic research has lead to the discovery of more than 1,200 genes that cause rare Mendelian diseases. The genetic effect in these diseases is very strong and in the majority of them contributes to 100% of the disease. However, the majority of human mortality and morbidity is due to diseases that do not fit a clear Mendelian inheritance pattern. These diseases have been defined as complex and multifactorial based on genetic and epidemiological data suggesting that multiple genes and environmental risk factors contribute to the disease. Therefore, it can be predicted that any single gene may only contribute a small amount of risk, but the combination of several genes together with environmental risk factors leads to the expression of the disease phenotype. Capturing this information and showing utility in medical practice and the pharmaceutical industry is the current focus of much research.

The application of human genetics in the pharmaceutical industry can cover the full spectrum of the drug discovery and development process. Human genetics can identify and validate novel targets that contribute to the disease, predict the activity of relevant drug metabolism enzymes and transporters, as well as predict subsets of subjects that may harbor an increased risk to develop adverse events and identify subjects that may elicit an enhanced clinical response to a particular therapy (44).

The identification of novel genes that contribute to the pathogenesis of disease has long been recognized as a powerful utility of human genetics. The challenge with many of these studies in the pharmaceutical industry is the identification of genes that are critical to the disease pathogenesis and overlap with a family of genes that encode proteins which are amenable to modification with small molecules or biological agents. Numerous studies have identified associations between genes and disease. APOE4 and Alzheimers disease and HLA-DR4 and rheumatoid arthritis are two examples where human genetics identified SNPs within genes that contribute to disease (45). However, these are not attractive targets for pharmacological intervention despite the roles they play in disease pathogenesis.

A recent example of where human genetics has increased the attractiveness of a particular target is in diabetes mellitus. Thiazolidinediones are ligands with high-affinity to peroxisome proliferator-activated receptor-γ (PPAR-γ) that modify insulin sensitivity and plasma glucose levels in type 2 diabetics (46). In 1999, mutations within PPAR-γ were identified in subjects with severe insulin resistance (47). This human genetic data further validated PPAR-γ as a target for the treatment of diabetes mellitus. Although the mutations were identified in rare extreme forms of the disease, the information provided a link into the biological regulation of glucose in humans.

The foundation of the field of pharmacogenetics was built around understanding intersubject variability in drug metabolism enzyme activity. Approximately 25% of the drugs on the market are metabolized by a cytochrome P450 2D6 enzyme. Phenotyping studies using a known 2D6 substrate, such as dextramethorpan, measure the ratio of unchanged drug to its metabolite to identify subjects who have reductions in their 2D6 activity. These subjects with reduced or absent enzyme activity are defined as "poor metabolizers." Studies investigating the 2D6 gene for novel sequence variations identified that the subjects with reduced activity, as measured by phenotyping, carried polymorphisms that abolish or reduce the activity of the enzyme. It has been established that genotyping subjects for these variants could identify "poor-metabolizers" with a greater than 98% sensitivity (48). Genetic screening of subjects enrolling in clinical development trials studying a known 2D6

substrate is now feasible and can be used in the interpretation of pharmacokinetic, safety and efficacy variabilities.

More recently, studies have identified SNPs within key drug transporters that can be used to better interpret drug responses. The multi-drug resistance 1 gene (MDR-1) harbors at least 17 SNPs, 5 of which result in amino acid changes (49). The function of this gene is an efflux pump that serves as a barrier for drug transport into the cells being treated. A SNP within exon 26 of this gene has been associated with reduced levels of expression and subsequently an effect on plasma levels of digoxin, a known P-glycoprotein substrate. This SNP is observed in the general population at a frequency of approximately 50%, thus its impact on population kinetics of drug transport could be significant.

Based on the density of SNPs across the genome, and recent studies evaluating SNPs in genes, it can be estimated that every gene in the human genome contains at least one SNP within its genomic structure. Not all genes will contain functionally relevant SNPs and the frequency of the SNPs may vary from rare (1%) to common (>10%). This variation in the human genome contributes not only to disease susceptibility, but also to variation in drug response. This has been recently exemplified by several reports associating SNPs in genes with efficacy and safety risks.

Abacavir is a reverse transcriptase inhibitor for the treatment of human immunodeficiency virus (HIV). Approximately 4% of patients receiving this therapy develop a hypersensitivity reaction (HSR) (50). Based on the clinical symptoms of the reaction, candidate genes were selected to execute a SNP-based association study to identify markers that associate with HSR. Two independent reports identified a significant association between HLA-B57, a polymorphism within the major histocompatability complex, and HSR (51,52). The frequency of the marker was greater than 50% in the subjects with HSR, compared to only 5% in the subjects without. This single marker would constitute a diagnostic accuracy of 30-70% (53). While this marker does explain a large portion of the risk, it does not reach the accuracy needed for a predictor of serious adverse events. This marker was identified by focusing on biologically relevant candidate genes, so the probability that other genes contributing to this risk were missed, is high. A whole genome scan exploiting the mapped SNPs may provide, in this example, the identification of subsequent genes contributing to this risk and ultimately a set of markers that, when combined, achieve adequate predictive value to enable the development of a diagnostic.

Safety diagnostics require very accurate and predictive markers whereas the utility of efficacy markers may not necessitate the same stringent criteria. The use of a SNP-based genetic marker to increase the probability of response in a clinical development setting could have direct impact on the sample size required for a clinical trial. However, while the marker may predict an increased probability of response within a population of subjects, the predictability may be lower for an individual. The treatment of major depression with selective serotonin reuptake inhibitors (SSRIs) is well established. The SSRI class of compounds all have high affinity for the serotonin transporter. This transporter contains a polymorphism in a regulatory region upstream from the gene that influences expression levels of the protein. The polymorphism consists of a long and short form, based on the number of a 44 basepair repetitive element. The long form is associated with increased expression. Subjects carrying two copies of the long form respond more rapidly to SSRIs than do subjects carrying only one or no copy (54,55). Incorporation of this marker into a clinical development trial, where response time may be a differentiating endpoint, may strengthen the interpretation of relative efficacy.

Conclusions - Although, the mapping of SNPs to the human genome has occurred very recently, multiple examples of the utility of SNP-based human genetics already exist. Advances in clinical tools to characterize disease phenotypes more objectively coupled with advances in genotyping technologies, will enable geneticists to identify and characterize genes involved with common diseases that inflict significant morbidity and mortality. Over the next two years the output of several whole genome association studies using a pooled DNA approach will be available to evaluate for their utility in providing large-scale identification of disease and pharmacologically relevant genes. These enhancements will provide novel genes with the potential for developing better therapies targeting the molecular etiology of common diseases. In addition, a better understanding of human disease through human genetics may provide the opportunity to enhance the efficiency of clinical development of new drugs and identify patient populations that will have maximum benefit of particular therapies.

It can be envisaged that the genes and SNPs that contribute to common disease susceptibility will be increasingly used for preventive medicine. Subjects at increased risk will be identified through the use of genetic diagnostics and prescribed lifestyle modifications and/or pharmacological intervention to reduce known environmental risks and decrease the risk of disease expression. While these utilities are still some time in the future, the tools to begin identifying and characterizing the genes are here today.

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Chapter 26. RNAi: When Interfering Is a Plus

Blanche-Marie Shamoon and Christoph Reinhard
Chiron Corporation, Emeryville, CA 94608

Introduction - A group working on polarity in C.*elegans* embryos reported eight years ago the puzzling fact that injection of either sense or antisense RNA into *C. elegans* was sufficient to interfere with specific gene expression (1). It took another 3 years to show that the gene silencing effect didn't actually result from the activity of either single-stranded antisense or sense RNA but rather from that of double-stranded RNA contaminants (dsRNA) (2). The authors coined the term "RNA interference" (RNAi) to describe the use of small inhibitory double-stranded RNA (siRNA) to target for degradation sequence-specific cellular mRNAs, and as a result to silence gene expression. As presented in this review, RNAi is a gene silencing mechanism widespread among eukaryotes, that possesses very unexpected features and shows remarkable potency (2); indeed a few molecules of injected dsRNA were shown to be sufficient, and ten times more efficient than either sense or antisense RNAs alone, to allow for complete gene silencing. In addition, in some phyla, silencing can spread across cellular boundaries (3,4). The initial dsRNA amount injected is unable to account for such long lasting and/or widespread effects and suggested that part of the RNAi process involved an amplification and maintenance mechanism *via* catalysis and/or synthesis. A striking example is seen during embryogenesis where RNAi remained effective even after the huge dilution effect of a 50 to 100-fold increase in cell mass in *C.elegans*, *Drosophila*, and mouse (2,5,6). However, despite feverish investigation, such spreading capacity has not yet been found in human cells. It should be noted, however that even in *C.elegans,* transmission is not stably inherited. Indeed, F2 progeny from RNAi-treated worms generally revert to normal phenotype (7,8). Since its emergence, the field of RNA interference has revolutionized reverse genetic approaches because it has allowed for rapid surveys of a large number of gene functions in nematodes in particular, but also in insects, fungi, parasites, zebrafish, mouse, and cultured human cells (9). With the more recent development of RNAi in mammalian systems, investigators are not only dissecting gene function but also attempting the development of new anti-viral therapeutics. In this rapidly evolving field, the goal of this article is to familiarize the reader with our current state of knowledge about siRNA structure, biogenesis, mechanisms of action, biological role, model systems, and potential applications as a tool for target validation or as a therapeutic approach (9-16).

POST-TRANSCRIPTIONAL GENE SILENCING

The key processes of gene silencing can vary widely not only in timing but also in location - nucleus or cytoplasm - and they can be either reversible or permanent. Clearly processes affecting DNA, such as methylation, imprinting and paramutation, occur in the nucleus (17). Such mechanisms are for the most distinct from RNAi even though RNAi-like mechanisms in the nucleus have been recently uncovered. Indeed a series of remarkable observation in plants and yeast implicates an RNAi-like mechanism also in chromosomal phenomena that involve suppression of transposable elements and repetitive sequences, as well as chromosome X inactivation and silencing of imprinted genes (17,18). There is evidence to support RNAi sharing common features with RNA-directed DNA methylation, a silencing mechanism whereby a target gene is silenced by the addition of a high proportion of non-canonical methylations. During such processes dsRNAs arise either from transposons, from centromeric repeats, or from transgene expression and through an RNAi-like mechanism targets chromatin structure or formation and maintenance of heterochromatin (17,19). A current intriguing model suggests that RNA-directed

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DNA methylation enforces silencing by increasing the proportion of aberrant RNA transcribed. Indeed methylation does not seem to occur in gene promoters, which would block transcription initiation, but rather seems to occur directly in the coding sequence of the target gene, blocking transcription elongation but not initiation and producing prematurely terminated aberrant mRNAs (20).

In contrast, a group of post-transcriptional processes believed to occur in the cytoplasm encompasses RNAi, post-transcriptional gene silencing (PTGS) and cosuppression in plants, as well as quelling in fungi (except *S.cerevisiae*) and algae (21,22). PTGS in plants either infected with viruses or injected with a transgene, leads to rapid (within 24h) and effective silencing through a mechanism similar to RNAi. This is particularly true when the triggering RNA is present at a high copy number, a signature feature of foreign gene expression (21).During PTGS the eventual decrease in transgene expression or viral growth induced by RNAi is initiated by the production of dsRNA replicative intermediates. Indeed the accumulation of these foreign dsRNAs triggers the production of the actual silencing siRNA agent. In some cases, the silencing will affect not only the transgene or virus but also the endogenous gene to which the dsRNA carries sequence homology (23). Cosuppression in plants, and quelling in fungi are the terms to describe this phenomenon. In some cases however no silencing affects the endogenous gene, even though the silencing agent passes through cells during RNAi spreading (4,24).

There are also other post-transcriptional mechanisms at play in eukaryotic cells. Small non-translated RNAs known as small temporal RNAs (stRNAs) are similar in size to siRNAs. They are conserved in worm, fly and human but have not yet been identified in plants (25). Uncovered a decade ago stRNAs can repress translation of a specific endogenous mRNA but only after translation has been initiated and most importantly without affecting the mRNA stability (26). Both stRNA and siRNA appear to use a common biogenesis pathway. However, while the stRNA role in developmental timing of *C.elegans* has been well established, its mode of action is still largely obscure. By contrast, knowledge about siRNAs mechanism of action is advancing in strides but its biological role is still quite unclear.

Biological function of RNAi - It is now clear that RNAi is a highly conserved general mechanism involved in gene regulation, development, and antiviral response (11). RNAi is such a powerful method for probing gene function that the initial focus went to the technical applications and uses. For this reason, investigation on the biological role of RNAi has started to be appreciated only in the last couple of years. However, there is already enough evidence to suggest that RNAi originated as a way for cells to perform genome surveillance monitoring invasion by foreign genes but also by endogenous mobile genetic elements. For example, *C.elegans* RNAi-deficient strains are prone to high frequency of mutations due to increased mobility of transposons (27). Also, in various systems, transposons tend to be silenced by their packaging into the heterochromatin. It is therefore tempting to speculate that RNAi plays a role in stabilizing the genome possibly by influencing heterochromatin, and in preventing recombination of mobile genetic elements that could otherwise cause a genomic rearrangement. However, whether RNAi regulates transposons directly at the genomic level or by targeting mRNAs encoding for transposases or other key molecules, still needs to be determined. *C.elegans* mutants show that not all RNAi-deficient worms mobilize transposons suggesting common elements but not complete overlap between RNAi and transposon silencing pathways. A striking argument in favor of a protective role of RNAi against foreign genes is the fact that for numerous plant viruses their virulence is determined by encoding suppressors of PTGS (28). Conversely, mutations in the host have been shown to be critical in restoring an anti-viral response by developing ways to counteract PTGS viral suppressors. Plants use RNAi not only to recover from viral infection but also to protect themselves from future challenges with homologous viruses. In a current model the capability of PTGS to shut down transgene expression reflects the fact

that RNAs transcribed from it are perceived as viruses. Therefore, PTGS would function as an ancient form of anti-viral defense in eukaryotes aimed at infection recovery (16).

SiRNAs structure - In 1999, seminal work identified siRNAs by showing the presence in plants undergoing viral or transgene-induced RNAi, of short double stranded RNAs fragments of uniform length. Their size was estimated at 25 nucleotides, complementary to both strands of the targeted mRNA and resulting from the processing of a longer dsRNA precursor of approximately 70 nucleotides (29). We have since gained tremendous insight into the RNAi process. One key feature is the processing of the long hairpin-structured dsRNA precursor into a siRNA of defined structure. Exon but not intron sequence can efficiently trigger RNAi and cloning and sequencing of the siRNAs revealed their very specific structure: two 21 nt single-stranded RNA with 5' monophosphate and 3' hydroxyl termini, able to form a 19bp duplex with 2-3 nt overhang (30). Long hairpin precursors sometimes form a perfect hairpin-loop structure but they often carry multiple partially complementary areas within the hairpin sequence. Both siRNAs and stRNAs are processed in a similar manner but stRNAs seem to bare imperfect complementarity to their target mRNA, while siRNAs appear to carry no mismatch at all with their target (31). Several authors speculate that this difference might be relevant to their different silencing mechanisms. Neither siRNAs nor stRNAs appear to be degradation products from the target mRNA but authentic reaction intermediates because their production from the precursor does not require the presence of the target RNA (32). It was therefore suggested that these small RNAs are the specificity determinant in RNAi. Conveniently synthetic siRNA appear to be as active as the ones produced enzymatically *in vitro*. However because they can be fairly expensive several groups have established alternative *in vitro* strategies to produce siRNA using T7 promoter or using constructs to express hairpin structures under the control of various mammalian promoters (33,34).

MECHANISM OF ACTION

Biochemical and molecular evidence supports a 4-step model for the RNAi pathway: initiation step, effector step, target recognition step, and target cleavage step.

Initiation Step - In the current model, the initiation step is an ATP-dependent cleavage of the silent trigger or dsRNA precursor into 21-25 nucleotides siRNAs (Fig.1) (32,35). The multidomain enzyme named Dicer in *Drosophila*, member of the RNAse III family, produces the siRNAs with the features of other RNAse III products, i.e. two single-stranded nucleotides on their 3' ends and a 5' monophosphate (30,35). However, in experiments on mammalian cells, when siRNAs is produced using T7 transcription the duplexes obtained contain 5' triphosphate termini but are nonetheless effective. It is therefore possible that either the 5' triphosphates are efficiently converted into 5' monophosphates or that, in mammalian cells, the requirement for 5' monophosphate ends is not as stringent as it is in *Drosophila* (36). Dicer contains several domains: an ATP-dependent RNA helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain, two RNAse III domains in tandem, and a dsRNA-binding domain. This enzyme acts as a dimer and each pseudo monomer, due to its two RNAse III domains, is able to cleave dsRNA twice, therefore each Dicer protein can generate one siRNA by introducing 4 cuts into the dsRNA precursor (15). The requirement of Dicer for ATP is surprising in light of the lack of requirement for high-energy cofactors by other RNAse III. Even though no conclusion has been reached yet, it has been suggested that it is related to either the need to unwind RNA to create bulges prior to cleavage or the need to translocate the enzyme along the precursor. Other suggestions include regulating binding of Dicer to dsRNA, or modulating its enzymatic activity. The function of the 130 amino acid PAZ-domain is unknown but is surmised to allow both homo and heterodimerization (37). Proteins carrying this domain have been shown to be

crucial in RNAi or PTGS. In worms, flies and humans, some proteins of the Argonaute family link the RNAi machinery with the ribosomal machinery. For example, the translation elongation initiation factor-2c, eIF2C also carries both a PAZ-domain as well as a 300 C-terminus amino acid Piwi-domain equally of unknown function (37,38). Spacing differences between the two RNAse III domains in tandem, in the various Dicer homologs has led to speculation that it is a reason for species-specific size differences in siRNAs (35).

Even though it is still unclear if Dicer acts alone, in a larger complex or both, it is has been established that Dicer is also involved with the processing of developmental stRNAs from dsRNA stable hairpin precursors (39). Indeed, deletion of dcr-1, the *C.elegans* Dicer homolog, not only abrogates RNAi but it also causes misregulation of developmental timing as well as defects in oogenesis (39). A similar deletion in plants leads among others to the disruption of embryo development (10). In worms the severe consequences of DCR-1 deletion, i.e. the heterochronic phenotype, are due to its role in cleaving dsRNA precursors into stRNAs from the genes lin-4 and let-7 which functions ensure the orderly progression of development, respectively from larva stage L1 to L2 and from stage L4 to adult (40).

Small non-translated RNAs increasingly appear to perform a multitude of functions some of which are components of conserved pathways. Only recently, over one hundred potential small regulatory RNAs called microRNAs or miRNAs were identified in fly embryos, worms, and cultured human cells (31). Many developmental switches are still unknown and it is possible that some of the newly discovered miRNAs will also act as stRNAs at various developmental stages as previously seen with lin-4 and let-7 regulation. Nonetheless, given the sheer number of miRNAs identified, alternative roles are possible. These molecules may be involved in spatial development, stress response, cell cycle regulation or in as yet uncovered specialized functions. Some miRNAs could also turn out to be new siRNAs. Indeed in plants newly identified miRNAs appear to regulate genes in a manner similar to siRNAs by targeting open reading frames (ORFs) and silencing genes through mRNA degradation processes (41).

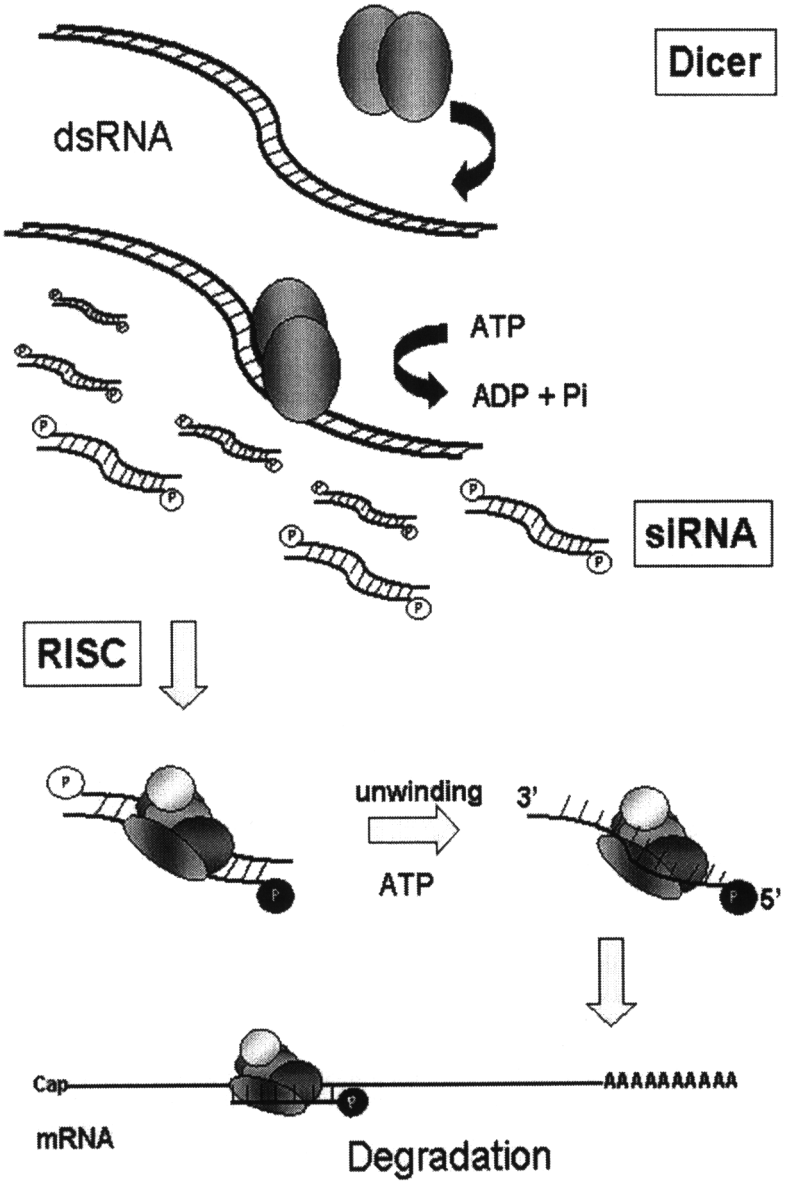
The production of siRNAs by Dicer results in symmetric duplexes while processing of dsRNAs hairpin precursor into stRNAs results in the stabilization of only one branch of the stem (10). What mechanism Dicer uses to determine which part of the precursor needs to be excised is yet another fascinating mystery. In this function a role is likely to be played by accessory factors associated with Dicer activity, a number of which has been identified in genetic screens. Worm mutants lacking Rde-1 cannot initiate RNAi but are otherwise normal, while mutants of the PAZ-domain-containing proteins ALG-1/ALG-2 (Argonaute-like gene) can carry out RNAi but suffer severe developmental defect due to a failure to generate appropriate levels of lin-4 and let-7 stRNAs (27). In *Drosophila*, two Argonaute homologues (dAgo-1 and dAgo-2) are necessary for RNAi, and in human cells, the Argonaute homologue eIF2C co-purifies as a component of the RNAi-induced silencing complex (RISC; see below). Baring similarities to human eIF2C, the homologues to Rde-1 in fungi and plants, respectively qde-2 and ago-1, are both necessary for gene silencing in somatic tissues of these organisms (42,43).

After cleavage of the dsRNA precursor by Dicer, the siRNA bound to the enzyme triggers the formation of the RNAi-induced silencing complex or RISC that was purified in *Drosophila* (13). A human RISC has not yet been characterized with certainty. However, eIF2C is associated with an RNA-protein complex, the miRNA ribonucleoprotein particle (miRNP) of similar size as the *Drosophila* RISC (13). This complex in HeLa cells also contains the proteins Gemin4 and the putative DEAD-box RNA helicase Gemin3. Both proteins are already known to associate with the survival of motor neurons protein (SMN) in a complex that restructures nuclear RNPs. However such complex does not contain eIF2C and Dicer. The role of

Gemin3/4 in the RNAi is unclear but the presence of helicase motifs in Gemin 3 raises the possibility of a role in the unwinding of miRNAs and siRNAs (44).

Amplification of Silencing - In order to explain the strong potency of RNAi, a model was suggested involving a number of rounds of physical amplification of the aberrant population of RNA triggers by an RNA-directed RNA polymerase (RdRP) (45). Indeed, initial siRNAs are apparently either not in sufficient amount or do not have the proper structure to trigger efficient RNAi *in vivo*. Experiments carried out in *C.elegans* and in *Drosophila* have shown that the way amplification of the initial population of siRNAs occurs requires hybridization of the target mRNA transcript

Figure 1 : Model for RNAi interference in mammalian cells. Role of Dicer and RISC.



with the antisense strand of the siRNA once a threshold concentration of siRNAs is reached (46). A secondary population of siRNAs would then result from the RdRP-mediated synthesis of a duplex followed by its cleavage by Dicer. The resulting increase in siRNA present would in turn intensify the target mRNA degradation. Further, a fraction of the secondary siRNAs could be unwound by a helicase activity or by RdRP itself, allowing then both strands of siRNA to be used as template for yet more amplification (46). However, such model where secondary elongation of siRNAs is derived from original siRNAs used as primer accurately predicted that secondary siRNAs derived from sequences upstream of the initial siRNA would appear and accumulate. This phenomenon termed transitive RNAi could lead to silencing any related RNA carrying sequences with sufficient homology to these secondary siRNAs. Unfortunately this could lead to degradation of alternative splice variants or homologues of the original target gene. The consequence of this unexpected twist for a reverse genetic approach, but also for target validation, in worms and plants is that, during a specific gene knock out experiment, there is a risk of inadvertently affecting other related genes. From a practical point of view, whenever possible, this clearly implies a careful design of the dsRNA trigger that takes into account possible transitive RNAi. From a biological point of view, it points towards the existence of a tight control mechanism of the amplification/transitivity processes, which could otherwise spread out of control any time RNAi is triggered and therefore jeopardize normal cell transcription. The existence of this phenomenon in human cells is still unclear because transitive RNAi occurrence in higher eukaryotes is not established. In fact, RdRP activity has been clearly reported in *Drosophila* embryo extracts but no sequence homologue has yet been identified in the *Drosophila* or human genome and no transitive RNAi has yet been observed in flies (46,47).

Spreading of Silencing - The nature of the systemic transmission signal in plants and animals is still unknown. Suggested candidates are the siRNAs themselves, the silent dsRNAs trigger or the dsRNA formed *via* RdRP-dependent amplification. In plants, the data supports two distinct mechanisms; one short-range cell-to-cell transmission and one long-range transmission through the plant vasculature (4). Movement of RNAs and proteins between plant cells using intimate connections such as plasmodesmata are well known and can account for short-range transmission of silencing agent. However, viral silencing inhibitors blocking siRNA production have failed to prevent systemic silencing in plants arguing against siRNAs being critical in long-range transmission (48).

In *C.elegans*, a new transmembrane protein *sid-1* was shown recently to be required for systemic silencing (49). This protein would function as a channel for the import of the silencing trigger; it is mainly absent from neuronal cells consistent with the original observation of their resistance to RNAi spreading. No homologues to *sid-1* were identified in *Drosophila* consistent with the lack of RNAi spreading in flies but they were surprisingly identified in mammals despite their resistance to RNAi spreading (50). This raises the possibility that some aspects of RNAi in mammals are not exclusively cell-autonomous.

The Effector Step - A determinant insight into the mechanism of RNAi emerged from *in vitro* studies showing that it was enforced by mRNA degradation (2,43). The process was shown in *Drosophila* to be initiated by the assembly of a nuclease complex, the RNA-induced silencing complex or RISC, able to target a specific mRNA (36,43). The discovery of the siRNAs prompted the search for their association with the RISC with the underlying assumption that the identification of specific mRNA substrates is dictated by base pairing. In fly embryo extracts, the RISC can be isolated as an inactive multiprotein and siRNA complex of 250KD that can be activated by addition of ATP into a 100KD complex RISC* able to cleave its specific mRNA target (36). Double-stranded siRNAs are incorporated in the RISC

even though single-stranded one would seem more effective at seeking their homologous target. The need to unwind siRNAs in the RISC may explain the need for ATP to obtain activation (51). Genetic searches for RNAi deficient-mutants in *Drosophila* have identified a number of helicases (qde-3, mut-6, and mut-14) that could in fact play a role of RISC activators (15,52).

Recent data in HeLa cells suggest that even though the RISC is involved in the RNAi pathway, it contains the single-stranded branch of the let-7 stRNA despite the absence of mRNAs in human cells that could be let-7 RNAi targets, (13,53). It now appears likely that this human miRNP complex is in fact the human RISC and that such complex can carry out both target cleavage in the RNAi pathway and translational control in the miRNA pathway. In HeLa cell extracts once RISC is formed, the incorporated siRNA can no longer exchange with free siRNAs. However, the complex can be assembled by directly providing single-stranded siRNAs instead of duplexes (53).

Target recognition and cleavage step - The high efficiency and timing of RNAi suggests an active mechanism to find the specific mRNA target among the transcriptome pool. Little is known about this except that, in *Drosophila,* most RISC might be associated with the ribosomes (43). The lack of 5' phosphate on siRNAs will prevent formation of the RISC and cleavage of mRNA target in *Drosophila* embryo lysates. The action of a kinase either adding or maintaining the siRNA 5' phosphate has been suggested to play a role in creating a molecular reference point from which the target mRNA cleavage site is measured. It also allows monitoring the authenticity of siRNAs and ensures licensing only *bona fide* siRNAs to trigger target decay (36). RISC* cleaves its target endonucleolytically at a specific position within the sequence hybridized by the siRNA antisense strand near the middle of the duplex using the siRNA 5'end but not the 3'end as a guide. Intermediate cleavage products are never observed arguing in favor of the presence within the RISC* of an exonuclease. In *C.elegans*, mut-7 an essential component of RNAi has nuclease homology but no *Drosophila* relative is known yet as part of the RISC (9,15,54).

RNAi Model Systems - RNAi has been studied extensively in invertebrates such as nematodes and insects, in vertebrates such as zebrafish, mouse and human, in plants such as *Arabidopsis*, and *Nicotiana*, and in fungi such as *Neurospora*, mainly due to the availability for these models of synergistic genetics and biochemical strengths. However many other organisms selected for their medical importance have seen their study hampered by experimental difficulties until RNAi appeared as a tool. Such models include parasitic protozoa such as *Trypanosoma brucei*, *Plasmodium falciparum*, or *Toxoplasma gondii* and pathogenic fungi such as the encapsulated *Cryptococcus neoformans, Candida albicans*, *Aspergillus fumigatus* as well as *Histoplasma capsulatum* (55). The use of RNAi in these organisms brought the possibility of specific gene disruption. For many of these organisms classical genetic approaches are often out of reach because the sexual cycle is unknown, complex or experimentally difficult. Most of these obstacles have been overcome to date by the use of RNAi in certain pathogens (56). The case of *T. brucei*, the causative agent of African sleeping sickness, provides an illustration of such a successful application (57). Reported in 1998, the first demonstration of successful RNAi in this organism came from siRNAs directed against α-tubulin, which blocked cytokenisis and led to the formation of multinucleated cells (58). Multiple genes have been targeted since, allowing the study of various aspects of trypanosomes biology. Induction of cell death by targeting degradation of the RNA encoding either topoisomerase II or FLA1, a protein required for flagellum attachment, shows that RNAi in parasites can be used for target validation (59,60). This technology could provide the *in vivo* solution to the challenge of ensuring the specificity needed to destroy parasitic invaders while leaving the host unharmed. Clearly success will depend among others upon selecting the appropriate targets and the availability of delivery systems capable to reach the host compartment infected with the parasite. RNAi is now an important tool for the advancement of

basic biology of eukaryotic pathogens as well as for the possible identification of new antimicrobial targets (55).

APPLICATIONS OF RNAi TECHNOLOGY

RNAi As A Genomic Tool - RNAi has become the dominant reverse genetic method in nematodes where silencing can be initiated by either injecting dsRNA or simply soaking worms in dsRNA containing solution or even by feeding them with dsRNA-producing *E.coli*. RNAi in *C.elegans* is one of the most robust genetic methods to understand the function of each predicted gene and its role in the overall organism biology (61). Being a fast, efficient and high throughput technique, it has allowed the analysis of nearly all of the 19,000 predicted worm genes (62,63). The relative efficiency of RNAi in producing mutant phenotypes was tested by comparing the results of 50 genes known to have a loss of function phenotype with the result obtained using RNAi. A loss-of-function phenotype was observed for 62% of the genes and 81% of these phenocopied the corresponding mutant (64). An estimate indicates that over three quarters of human disease-genes have a *C.elegans* homolog. Using *C.elegans to screen* drugs to 1) validate target candidates, 2) determine compound activity could therefore help to validate homolog targets in humans and advance screening by defining at least some of the active scaffolds (62).

RNAi can also play a crucial role in drug discovery by functionally validating potential drug targets. It also allows for identifying compounds that can mimic, improve or worsen the biological role of validated or uncharacterized targets. The combined availability of the entire *C.elegans* genome sequence with the possibility to design and synthesize dsRNAs against any sequence has made possible the generation of bacterial libraries expressing dsRNAs directed against every predicted gene in *C.elegans* (65). Growing worms on these bacteria while treating them simultaneously with a compound inducing a known phenotype allows for the identification of genes involved in or regulating the pathway and therefore that enhance or suppress the phenotype (62).

RNAi may also facilitate drug screening and development by identifying genes involved in drug resistance or sensitization thereby providing information about new compounds mode of action; it can also allow identification of compounds that improve or worsen response to a treatment-phenotype. For example, dsRNA directed against *C.elegans* RAD51 homolog results in increased germline apoptosis. Mutations in the DNA checkpoint/repair enzyme mrt-2 prevent *C.elegans* rad-51-induced apoptosis (66). By screening compound libraries, it is possible to identify small molecules able to antagonize the repair pathway in worms treated with rad51 siRNA (62).

Until the last couple of years, gene function in higher eukaryotes was assessed by methods such as disruption by both constitutive and conditional genome knock out, introduction of a transgene, molecular cloning of genes involved in genetic diseases by either linkage studies or expression cloning from cDNA libraries. However, all of these reverse genetics approaches are lengthy and therefore incompatible with large-scale gene function studies. Faster methods such as gene targeting by antisense or ribozyme have been applied with limited success *in vivo,* in particular, in oncology. This is mainly due to either lack of stability or high toxicity of antisense depending on the chemistry used, poor delivery to the desired area, accumulation in others organs such as kidney, lung and liver, and short term knock out effects due to both degradation and clearance. Despite the high efficacy of antisense shown *in vitro* by many investigators, the reality of antisense efficacy *in vivo* is still to come for cancer treatment. It is therefore not surprising that RNAi has become very popular as the new alternative for reverse genetics both in lower eukaryotes as well as in mouse and human. It remains an open question as to whether RNAi will fulfill the promise of being a viable therapeutic approach for

specific and long-term *in vivo* gene down-regulation. Meanwhile, RNAi has certainly become the preferred method for gene expression down-regulation in cultured mammalian cells since the critical discovery in 2001 that transfection of dsRNAs shorter than 30 base pairs including siRNA duplexes of similar length and structure as the natural processing products from precursors, fail to activate non specific dsRNA-triggered pathways that mediate suppression of gene expression and trigger apoptosis (67). One of those non specific pathways is the RNA-dependant protein kinase (PKR) pathway activated by type I interferon, itself strongly induced by the presence of dsRNAs; this pathway responds to dsRNA by phosphorylating EIF-2α leading to non-specific translation arrest. The other pathway activates 2', 5'-oligoadenylate synthetases whose products in turn activate the latent RNase L, a non-specific enzyme that targets all mRNAs for degradation (13,68).

RNAi In Mammalian Cells - An RNAi methods review was published in 2002 detailing approaches for the analysis of mammalian gene function in a number of aspects of biology such as cell cycle, metabolic pathways, gene expression or cytoskeleton but also for high throughput analysis of gene function (69). A number of suppliers of RNA reagents are able to provide RNA nucleotides synthesized with the appropriately protected ribonucleosides compatible with *in vitro* uses. So far, RPI is the only company that has developed duplexes claimed to be stable *in vivo* and that could be delivered systemically. Indeed, recently presented data showed enhanced *in vivo* stability of a panel of chemically modified siRNAs targeted against the Hepatitis B Virus (HBV) (70). The modifications conferred the siRNAs significantly prolonged serum stability with a level of inhibition of viral envelope protein synthesis similar to that seen with unmodified siRNAs. Besides, the inhibition was achieved at doses approximately ten-fold lower than those needed for optimal anti-HBV ribozymes activity (70). Methods for generating *in vitro* double-stranded RNA are also available and they mainly use as templates PCR fragments either containing both T7 and T3 promoters or cloned in a vector providing both promoters oriented to transcribe the complementary strands (61). An additional interesting report has shown the ability to specifically silence among several, a particular splice variant in *Drosophila* cells. This method may prove to be a powerful tool for higher eukaryotes in addressing this problem i.e. the elucidation of the distinct and sometimes opposing functions of specific protein isoforms (71). Finally, concomitant treatment of cells with more than one siRNA has been reported as a success in one case but as difficult to achieve in another due to competition between the 2 siRNAs, which brings credence to the idea that the RNAi machinery is limiting or can be titrated in mammalian cells and in *Drosophila* (2,65,72).

Gene Silencing Using D-RNAi - D-RNAi (Messenger RNA-antisense DNA interference, termed mRNA-aDNA) is a novel posttranscriptional phenomenon of silencing gene expression by transfection of a duplex of mRNA-aDNA and was first observed in 2001 when silencing of bcl-2 using D-RNAi in human prostate cancer cells treated with phorbol ester, induced apoptosis rather than proliferation (73). It was also shown to be effective *in vivo* using D-RNAi directed against β-catenin in chicken embryos. D-RNAi was found to have long lasting gene knockout effects due to posttranscriptional gene silencing mechanism involving homologous recombination between intracellular mRNA and the mRNA components of a D-RNAi construct (74). This method however is not currently widely used possibly because it has not been shown to work in many different cell types and more work is required to prepare a D-RNAi hybrid than a dsRNA.

A drawback of the methods introducing duplexes in cells to trigger RNAi is that they produce only transient phenotypes because siRNAs are not stably inherited and because mammals apparently lack the robust amplification mechanism found in plants and worms. An additional problem is that RNAi of late-acting genes is not as consistent as that of embryonically expressed genes possibly due to excessive siRNAs dilution as cell division proceeds. However several methods have been developed with the aim of circumventing these limitations and broaden the spectrum

of potential applications. In *C.elegans* expression of hairpin dsRNAs from transgenes carrying inverted repeats of a gene-specific sequence, driven by a heat-shock inducible promoter proved to be advantageous in providing heritable and conditional specific gene inactivation (50). Similar *in vivo* enforced expression of a hairpin structure as silencing trigger has been established in *Drosophila*, plants and trypanosomes (75-78).

Early in 2002, stable expression of a long 500-base-pair dsRNA leading to gene suppression by RNAi was reported in murine normal embryonic stem cells, in murine embryonic carcinoma cell lines and in a few murine somatic cell lines (79). However this approach was limited to transfectable cell lines deficient in the generic dsRNA response. A few months later, several groups showed that short hairpin RNAs (shRNAs), modeled on miRNAs, placed downstream from an RNA-polymerase II or III dependent promoter (CMV or H1 and U6 promoters respectively), can be stably expressed *in vivo* to induce RNAi in mammalian cells without triggering the non-specific dsRNA response (33,80-82). The use of such constructs placing shRNAs under the control of inducible-promoters will most likely constitute the next generation of RNAi stable transgenes (83,84). The availability of stable RNAi in mammalian cells is a major breakthrough that allows monitoring both *in vivo* and *in vitro,* over long periods of time gene silencing-induced phenotypes. For *in vitro* target validation it allows assay in the longer-term of RNAi-induced phenotypes, for example, proliferation, apoptosis or soft agar growth, of stably engineered cancer cells in which a gene encoding a very stable protein is targeted. Recently, a study was published that used either synthetic siRNAs or vector-based expression of siRNAs to interfere with the PI (3)-kinase signal transduction pathway (85). PI (3)-kinase activity in HeLa cells was successfully inhibited and results were similar to those achieved by use of chemical inhibitors or after transfection with conventional antisense molecules.

Although no systematic study of RNAi potency has been performed in mammalian cells, a highly expressed gene such as nuclear lamin in HeLa cells was knocked out to undetectable levels with siRNA transfection (67,86). In the absence of intense amplification of the silencing agent in these cells, it suggests that each RNAi complex can perform several rounds of mRNA cleavage (53). Reports flourished last year, showing effective RNAi-induced knock out of middle to low expressed genes in human, mouse and African Green monkey cells (13). Considering the pace of this field and the genomic approach already undertaken by some groups, it is possible that in a few years the loss-of-function phenotype of most human genes will have been tested in cultured cells using shRNA-induced RNAi.

RNAi And *In Vivo* Validation - In the case of *in vivo* validation, specific stable knockout cells can be assayed for their properties such as growth, angiogenic or metastatic potential as tumors in xenograft models. It is also likely that inducible-RNAi transgenic mice to virtually any mouse gene will soon be available. RNAi-transgenic mice carrying hypomorphic alleles would provide endless sources of cells, some of which could be established as lines, carrying a specific gene knock out within a stable background. Furthermore, using classical genetics, i.e. crossing different lines of transgenic mice would further our understanding of biological pathways and of genes and pathways interactions.

Individual siRNA designed against different regions of the same mRNA can have dramatic silencing efficiency differences that can be influenced by shifting the siRNA by as little as three base pairs (87). The reason for such positional effects is unclear and may be related to sites of accessibility, as it is the case with antisense oligonucleotides or with ribozymes. Site accessibility using folding programs cannot yet be well predicted. Assays have, therefore, been developed for this purpose; they involve the incubation of the site-specific oligonucleotides with cellular extracts containing the target mRNA followed by cleavage with the endogenous RNase H, an RNase able to recognize RNA-DNA hybrids, and finally the amount of cleaved

mRNA is monitored by RT-PCR (88). From a practical point of view however, when siRNA fails, the simplest approach is to try one that targets a different region of the mRNA.

In terms of target validation and biological studies, RNAi offers a concrete way to benefit from the wealth of information contained in the sequenced human genome. However, it is likely that for some genes and/or for some specific gene regions, it will only lead to partial or lack of phenotype due to gene redundancy, or to compensation by alternative pathways. This seems to be particularly true in mammalian cells compared to *Drosophila* cells and, similar to existing antisense technology, success depends on the cell type, on the level of expression the target gene and on the gene redundancy.

RNAi AS A THERAPEUTIC APPROACH

RNAi *in vivo* delivery - *In vivo* delivery of dsRNA for therapeutic purposes will require high-efficiency delivery and appropriate biodistribution, stability of the silencing agent and lack of toxicity. A number of groups have already disclosed successful *in vivo* delivery attempts using viral vectors to transduce cells. Replication-defective retroviruses were used successfully to transduce human stem cells raising the possibility that along a similar principle, *ex-vivo* treatments could be attempted, for example, by engineering cells to resist HIV infection (15). This could be accomplished by targeting directly HIV RNAs or the host's viral receptors. Proof-of-principle experiments were done in mice showing that siRNA mediated silencing *in vivo* using systemic recombinant adenoviral vectors injection to reduce expression of a highly expressed endogenous liver gene (89). They also showed GFP silencing in the brain localized to the treated hemisphere, in GFP-transgenic mice after intra-cranial injection. In the current atmosphere of distrust of viral-based therapies, this data provide at least conceptual support for RNAi-targeted suppression using as delivery vehicles viral vectors such as adenovirus, adeno-associated virus, lentirus or herpesvirus. Considering RNAi remarkable specificity dependent on nucleotide interactions, it is also conceivable that the *in vivo* knock out of a dominant disease-causing allele could be achieved without affecting the normal allele or other genes therefore without triggering apparent adverse effects.

Fas receptor engagement is involved in a broad spectrum of liver diseases where massive apoptosis, fibrosis, inflammation and secondary-necrosis occur that can lead to lethal fulminant hepatitis. Recently RNAi was used successfully to silence Fas and to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis, one being chronic and the other fulminant (90). This data provides support for the therapeutic prospects of RNAi to prevent cytotoxic liver injury.

Inhibition of viral infection in mammalian cells - RNAi technology has provided a new approach to combat viral infections besides vaccines and antiviral drugs. RNAi can be applied not only to cellular genes but also to viral genes and it appears to do so efficiently at least in cell culture. HeLa cells infected with the rapidly multiplying poliovirus can be protected by siRNAs added directly to the media as a result of direct interference with viral replication and eventually as a result of viral clearance from the human cells (91). In the case of the respiratory syncytial virus (RSV), the causative agent of severe respiratory disease siRNA treatment of infected cells led to decreased viral mRNA expression but the full length viral genome was not affected, possibly as a result of its encapsidation rendering it inaccessible (92).

Several groups were able to modulate HIV1 replication at both early and late steps, as well as inhibit viral production in human primary lymphocytes (93). The use of siRNAs or plasmid-derived siRNA directed against the viral long terminal repeats (LTR) or the accessory factors *vif* and *nef* reduced 30-50 fold the viral production as a result of interrupting early cycle events thereby preventing establishment of the provirus (94). When transfection of siRNA was done four days post-infection with

HIV1, a 50% reduction in the steady-state viral production was observed. Targeting of the viral receptor CD4 with siRNAs led to an impressive inhibition of viral entry measured by β-galactosidase assay and syncytial formation, a decrease in free viral titers and a decrease in re-infection. Due to the key role of CD4 in the normal immune function, targeting the coreceptor, CCR5 might be a better choice, as homozygous mutation of this molecule confers protection against HIV1 infection without severe immune consequences in humans (95).

The potential success of the siRNAs as anti-infectious therapeutics may be based on the fact that, unlike antisense oligonucleotides, they tap into cellular pre-existing silencing mechanisms. In the case of cells infected with poliovirus, the authors detected the emergence of a siRNA-resistant viral population due to sequence mutations of the viral genome at the site of interaction with the siRNA. That population however was present prior to siRNA treatment and was simply given a selective advantage (95). In the case of HIV siRNAs, a single mismatch compared to the viral genome significantly decreased the RNAi efficacy and four mismatches in the siRNA against *vif* abolished the silencing effect (94). However, it should be expected with viruses and in particular RNA viruses that sequence variability will occur and therefore multiple conserved sites should optimally be targeted at once in order to avoid rendering the virus immune to siRNA-mediated silencing.

Conclusion - RNAi is a very good example of a serendipitous discovery which has proven of value not only in revealing a new regulatory paradigm in cell biology but also a terrific tool for manipulating gene expression to tease out gene function on a genomic scale. It also holds the potential for new therapeutic approaches in human genetic and/or infectious diseases. There are still many unanswered questions, in particular, about the biological role of RNAi in the world of eukaryotes. However, it is certain that the quest for the answers is on its way and in the coming years it will provide surprises and excitement to investigators, as well as reshape the way future biological studies in mammalian cell systems are designed.

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Chapter 27. Lipid Rafts in Immune Cell Signaling

Susan K. Pierce
DHHS/NIH/NIAID/Twinbrook II
12441 Parklawn Drive, Room 200B, MSC 8180
Rockville, MD 20852

Introduction – The cells of the immune system express a variety of receptors that allow the system to recognize and respond to the universe of foreign pathogenic organisms including viruses, bacteria and parasites. An important class of immune receptors are the multi-chain immune recognition receptors or MIRRs that include the antigen receptors on the two major classes of lymphocytes, namely, B cells (the B cell receptor or BCR) and T cells (the T cell receptor or TCR) (1). Another important MIRR family member, the high affinity receptor for IgE, or FcεR1, is expressed on mast cells and basophils and plays a key role in allergic immune responses. The engagement of the MIRRs by their multivalent ligands, either soluble antigens for the BCR, peptide fragments of antigens bound to Major Histocompatibility Complex (MHC) molecules for the TCR and complexes of antigen bound to IgE antibodies for the FcεRI, leads to the initiation of tyrosine-kinase based signaling cascades that ultimately results in the activation of the cells to provide their immune functions. The members of the MIRR family are multi-chain complexes that share several common structural features. Each contains ligand binding, transmembrane chains with short cytoplasmic tails that themselves have no ability to connect to intracellular signaling machinery. Signaling is mediated by associated transmembrane proteins with large cytoplasmic domains that contain tyrosine-based motifs termed immunoreceptor tyrosine-based activation motifs or ITAMs. For the TCR, this signaling complex is composed of five proteins termed CD3; for the BCR, it is a two protein complex referred to as Igα/Igβ and for for FcγR1 it is a homodimer of what are termed γ chains. The MIRRs have no inherent tyrosine kinase activity but upon multivalent ligand binding they associate with a member of the Src-family kinase that phosphorylates tyrosines within the ITAMS of the cytoplasmic domains of the MIRRs resulting in the initiation of signaling cascades. Although the molecular details of the signaling cascades are now known in some detail, the nature of the events that trigger the association of the MIRRs with the Src family kinases and initiate the response is less well understood. The events that induce the initial association of the MIRRs with and phosphorylation by tyrosine kinases is of significant interest as they represent potential targets for therapies to block immune cell activation in allergy, autoimmune disease and transplantation.

Recent evidence indicates that cholesterol- and sphingolipid-rich membrane microdomains, termed lipid rafts, play an important role in the initiation of MIRR signaling by segregating the MIRRs from the Src-family kinases and other key signaling components in resting cells and facilitating the association of these upon MIRR ligand binding (2). Here the evidence that lipid rafts function as platforms for MIRR signaling will be reviewed as will the evidence that lipid rafts play a key role in the regulation of immune cell signaling imposed by the developmental state of the cell, the engagement of coreceptors and by infection of cells by pathogens. Lastly, the potential of lipid rafts as therapeutic targets will be discussed.

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THE PROPERTIES OF LIPID RAFTS

Recent evidence indicates that the plasma membrane is not a uniform lipid bilayer but rather contains within it discrete microdomains rich in cholesterol and sphingolipids termed lipid rafts. Several excellent reviews have been published recently describing both the biochemistry and biological function of lipid rafts (1,3-6). Briefly, the outer leaflet of the plasma membrane contains both sphingolipids with highly saturated acyl chains and glycerophospholipids with unsaturated, kinked acyl chains. In model membranes composed of mixtures of these two lipids, the sphingolipids spontaneously partition out of the glycerophospholipids and form highly ordered gel-like microdomains due to the tight packing of the saturated acyl chains. In biological membranes the binding of cholesterol by the sphingolipids dramatically alters the properties of the microdomains promoting the formation of liquid ordered domains. Thus, the sphingolipid- and cholesterol-rich microdomains are relatively ordered domains that can be envisioned as floating in the disordered glycerophospholipid bilayer and consequently were coined lipid rafts. The inner leaflet of the lipid rafts is less well characterized but is believed to be composed of saturated phospholipids. The inner and outer leaflets are coupled, although the nature of the coupling is not known.

In terms of their role in immune cell signaling, the central feature of lipid rafts is that they provide a mechanism for the lateral segregation of proteins within the plasma membrane (3). This ability to segregate proteins provides a means of concentrating and compartmentalizing certain components of signaling pathway and excluding others. At present, the rules that govern the inclusion and exclusion of proteins from lipid rafts have not been fully delineated, however, analyses of the composition of rafts allows some generalizations to be made. For their characterization, lipid rafts have been separated from the glycerolphospholipid membranes in cells based on their differential solubility in nonionic detergents (3,7). Thus, for example, lipid rafts are isolated from B lymphocytes by sucrose density gradient sedimentation of cell lysates prepared in 1% Triton x 100 at 4°C, conditions under which lipid rafts are insoluble. There are a number of potential pitfalls associated with the use of detergent solubility to isolate lipid rafts, not the least of which is the potential for the detergent itself to induce the formation of lipid rafts. However, independent evidence for the existence of lipid rafts in the membranes of living cells has come from studies using chemical crosslinking and fluorescence energy transfer (FRET) to detect the proximity of two proteins in the membrane; photonic force microscopy to measure the local diffusion of single proteins within the membrane and single fluorophore tracking microscopy to monitor the diffusion and dynamics of individual proteins and lipids in the plasma membrane (8-12). When tested, the identification of proteins in the lipid rafts in membranes of living cells has correlated well with their detergent solubility.

Results of the analyses of lipid rafts using such techniques have provided some general features of proteins that partition into lipid rafts. Current evidence indicates that most transmembrane proteins are excluded from rafts, however, a small number constitutively reside in rafts but for these S-palmitoylation is generally required (3,7). Presumably the saturated acyl chain of the palmitate contributes to the stabilization of the transmembrane regions of these proteins in the rafts. An important example of such proteins in immune cell signaling is the linker for activation in T cells (LAT), a transmembrane protein with a large cytoplasmic domain that acts as a molecular adapter facilitating the association of a number of signaling components with the TCR (13). A second class of proteins that partition into lipid rafts are those that associate with the outer leaflet of the plasma membrane through a glycosylphosphatidylinositol (GPI)-linkage (14). This observation is of interest as there are several examples of immune cell receptors that are GPI-linked and when

bound to their ligand induce signaling, including for example, CD14, the receptor for the bacterial mitogen LPS. Although the mechanism by which GPI-linked receptors signal is not well understood, their segregation into lipid rafts has been postulated to be critical to this function (14). A number of soluble, cytoplasmic proteins that play key roles in immune cell signaling associate with the plasma membrane by virtue of their acylation. Significantly, the nature of the acyl chains appears to dictate raft partitioning (7,15). For example, proteins that are acylated by N-myristoylation and S-palmitoylation partition into rafts while proteins modified by unsaturated fatty acids or prenyl groups alone are excluded. However, the palmitoylation of a prenlyated protein can redirect it into rafts. For example, the Src-family kinases, Lyn in B cells and mast cells and Lck in T cells, are myristoylated and palmitoylated and localized to rafts as is the GTPase H-ras which is palmitoylated and farnesylated (16-19). In contrast, K-ras which is farnesylated but not palmitoylated is excluded from rafts (19). Significantly, palmitoylation is a post translational, readily reversible process providing a mechanism for cells to control the association of the signaling components with lipid rafts (20,21).

Lipid rafts are estimated by detergent solubility and fluorescence anisotropy measurements to represent greater than 40% of immune cell membranes (22, 23). The lipid rafts in resting cells appear to be highly dynamic submicroscopic structures (50nm in diameter) that contain only thousands of lipids and a small number of proteins (3,9,11). These are often referred to as elemental rafts. The crosslinking of signaling receptors associated with rafts appears to result in the clustering of rafts forming larger more stable domains that are often associated with the actin cytoskeleton (3,24,25).

LIPID RAFTS IN IMMUNE CELL SIGNALING

Recently, evidence has accumulated that supports a critical role for lipid rafts in the earliest events in the initiation of signaling by the MIRRs in B cells, T cells and mast cells (2). In each case the MIRRs in resting cells are excluded from lipid rafts as are several receptors with the potential to negatively regulate MIRR signaling including the phosphatase CD45 and the phosphatase associating, CD22. Significantly, as described above, the lipid rafts concentrate the Src family kinases, Lyn and Lck, that play critical roles in the initiation of signaling by the MIRRs by phosphorylating ITAM tyrosine residues within their cytoplasmic domains. It has been observed that the ligation of the MIRRs by multivalent ligands induces the partitioning of the ligated MIRR into lipid rafts where the receptors are phosphorylated by the Src-family kinases and signaling is initiated.

A description of the evidence for a role of lipid rafts in TCR signaling in T cells illustrates the principles of the relationship of the MIRR family of receptors with lipid rafts. Studies using either detergent solubilization to isolate rafts or confocal microscopy to colocalize the TCR with raft glycosphingolipids have provided evidence that in resting mature T cell the TCR is excluded from lipid rafts (26,27). The rafts in resting T cells concentrate several important components of the TCR signaling pathway including the Src-family kinase, Lck, the TCR molecular adaptor LAT, Cbp and Csk, two proteins that play a role in the regulation of Lck activity, PIP2 a substrate for the lipid kinase, PI3K, that is activated upon TCR ligation and the TCR coreceptors CD4 and CD8 that are required to co-engage the MHC-antigenic peptide complex along with the TCR (26-32). CD45, a phosphatase that can negatively regulate TCR signaling is excluded from lipid rafts in T cells (26,27,33). The apparent exclusion of the TCR from lipid rafts can be viewed as representing an equilibrium distribution of the TCR in the raft- and nonraft-regions of the membrane in which partitioning out of the rafts is favored. Upon binding to antigenic peptide-MHC complexes the equilibrium is shifted and the TCR partitions into lipid rafts

where the ζ chain of the TCR's CD3 complex is phosphorylated by Lck (26,27). Several additional raft components are also phosphorylated upon TCR engagement including LAT and PI3K (26,27). Subsequently, a number of activated signaling components are recruited to the lipid rafts including ZAP-70, a central kinase in TCR signaling, PLC-γ1 a phospholipase that triggers events leading to Ca^{2+} fluxes and the molecular adaptor, Slp-76, that plays a key role in organizing and facilitating the interactions of a number of the components of the TCR signaling pathway (26,27,30,34). The TCR in rafts subsequently becomes attached to the actin cytoskeleton.

The components of the lipid rafts from resting and activated T cells have been identified, for the most part, by the use of specific antibodies and consequently may represent only a small portion of rafts associated proteins, namely those for which reagents are available. Recently the techniques of protein identification termed proteomics have been applied to the characterization of lipid rafts resulting in identification of over 70 different proteins associated with the rafts of resting T cells (35). Significantly, most of these were either proteins involved in signaling or components of the cytoskeleton.

The estimated small size of elemental rafts and the number of signaling proteins that may be predicted to associate with them raises the question, are all rafts identical or is there heterogeneity among lipid rafts? At present there is little experimental data that speaks to this issue but two studies in T cells suggest that functionally distinct heterogeneous rafts may exist. Polarization of the T cell membrane during chemotaxis resulted in the asymmetric distribution of membrane receptors involved in the chemotaxic response that colocalized with rafts containing either the gangliosides, GM1 or GM3. Thus, the leading edge receptors partitioned into rafts composed of GM3 and the uropod or trailing edge components partitioned into rafts composed of GM1 (36). In separate experiments, Lck and LAT, both constitutive components of T cell rafts, showed differential detergent solubility suggesting that the microenvironment in which they reside are not identical (37). The issue of the heterogeneity of rafts will be an important one to resolve in order to understand how individual rafts function.

The results reviewed thus far provide a correlation between the partitioning of the TCR into lipid rafts and the initiation of signaling. Several additional observations provide evidence that lipid rafts are necessary for TCR signaling. Cholesterol is essential for the integrity of lipid rafts and disruption of lipid rafts by cholesterol depletion of T cell membranes using drugs such as methl-β-cyclodextrine, filipin and nystatin inhibit T cell signaling through the PLC-γ dependent pathway (26). Because lipid rafts segregate negative regulators of signaling, as well as positive regulators, it is also possible that raft disruption would have the effect of inducing the activation of certain TCR signaling pathways, a phenomenon which has also been observed (38). TCR signaling is also defective in T cells from mice deficient in sphingomyelinase the activity of which is necessary for raft integrity (39). T cells that express mutants of the T cell adaptor LAT that lack the cysteine residue for palmitoylation and consequently are excluded from lipid rafts, are deficient in signaling (13,40,41). Conversely, the targeting of the negative regulator of TCR signaling, the protein phosphatase SHP-1, to lipid rafts blocks TCR signaling (42). Taken together these results provide reasonable evidence that functional rafts are necessary for TCR signaling.

At present the mechanism by which any of the MIRRs partition into lipid rafts is only poorly understood. Evidence from studies of both the FcεR1 and the BCR indicate that partitioning into rafts does not require active movement of the receptor by the actin cytoskeleton (43-45). Studies of signaling inactive mutants of the BCR

indicate that signaling is not a prerequisite for partitioning into rafts (44). Thus, the partitioning of the MIRRs into elemental rafts following multivalent antigen binding may simply be a function of the crosslinking or oligomerization of the receptor by antigen binding that may result in an oligomeric conformation of the transmembrane regions of the BCR that prefers the microenvironment of the lipid rafts. In B cells it was observed that the association of the BCR with lipid rafts was weakened if signaling or association of the BCR with the actin cytoskeleton were disrupted (44). Thus, it may be that for vigorous signaling to proceed the elemental rafts must be clustered by the signaling adaptors recruited to the rafts or by attachment to the cytoskeleton.

Lipid Raft-Mediated Regulation of Immune Cell Function – The results described above provide evidence for a role for lipid rafts in the initiation of immune cell signaling through the MIRRs. If rafts play an essential role in signaling it might be anticipated that factors that affect the outcome of signaling would function at least in part by influencing the partitioning of the MIRRs into rafts. Immune cell signaling is known to be influenced by the developmental state of the cells, by the engagement of coreceptors that both enhance and dampen signaling, and by infection with intracellular pathogens. Recently, the effect of such factors on the relationship between lipid rafts has been explored and in several cases the ability of the MIRRs to partition into rafts has been observed to be affected indicating that the function of rafts can be regulated (2). Understanding the molecular mechanisms underlying such regulation will likely provide fundamental information about the function of lipid rafts in immune cell signaling.

The partitioning of the MIRRs into rafts, both the TCR in T cell and the BCR in B cells, appears to be developmentally regulated. Early in development B and T cells express MIRRs that contain surrogate ligand binding chains and these pre-TCRs, and pre-BCRs function in a ligand-independent fashion to signal for cell survival. At this developmental stage a significant portion of the pre-TCR and pre-BCR were observed to constitutively partition into rafts and engage in signaling (46,47). For the pre-TCR it has been determined that the surrogate TCR chain is palmitoylated which may be important to promote raft association (47). As T and B cell development proceeds the TCR and BCR function in a process termed selection that results in the elimination of self-reactive cells and the expansion of cells that have the potential to participate in an immune response. In both immature T and B cells the MIRRs have been shown to be unable to partition stably into lipid rafts and signaling for cell death during negative selection appears to proceed outside of rafts (48-50). The relationships between the MIRRs in lipid rafts in developing T and B cells is in contrast to that described above for mature cells. The powerful modern tools of lipid and protein identification should provide the means to determine if there are developmentally determined differences in the lipid rafts that account for their different behavior.

Coreceptors that have been demonstrated to both enhance and dampen immune cell signaling have also been shown to influence the ability of MIRRs to partition into lipid rafts in both T cells and B cells. One such example is that of the coreceptor complex, CD19/CD21, in B cells. This coreceptor is composed of CD21, a receptor for a fragment of a complement protein, C3d, that becomes covalently coupled to antigens during infections and CD19, an adaptor protein that interacts with several components of the BCR signaling cascade including Lyn, the activity of which it amplifies (51). When coligated to the BCR through the binding of C3d tagged antigens CD19/CD21 greatly enhances BCR signaling such that maximal activation is achieved by engagement of $1/1000^{th}$ of the BCRs that would be required to activate the B cells by antigen alone. It was determined that the CD19/CD21 complex like the BCR is excluded from lipid rafts in resting cells (52). Coligation

results in the partitioning of both the BCR and the CD19/CD21 complex into lipid rafts. As compared to BCR crosslinking alone, co-activation of the BCR and the CD19/CD21 complex leads to near complete partitioning of the BCR and prolonged signaling from the lipid rafts. BCR crosslinking alone results in the association of ~40% of the BCR with rafts and signaling persist for ~15 min in contrast to near complete partitioning of the coligated BCR and signaling that persists for well over an hour. The mechanisms by which the CD19/CD21 complex prolongs residency of the BCR in lipid rafts are not known but are of significant interest. There are also examples of coreceptors that function to limit the residency of MIRRs in lipid rafts. In B cells the receptor for Fcγ, FcγRIIB1, functions to block BCR signaling when coligated to the BCR through the binding of complexes of IgG bound to antigen by recruiting the inositol phosphatase, SHIP (53). SHIP dephosphorylates PIP3, the product of PI3 kinase activity, which dampens BCR signaling resulting in only a transient association of the BCR with lipid rafts (54). The ability of coreceptors to prolong or limit the residency of MIRRs in lipid rafts represents a novel mechanism by which coreceptors function to regulate MIRR signaling.

The Relationship of Lipid Rafts and immune cell pathogens - Evidence has accumulated that lipid rafts play essential roles in the life cycles of a number of pathogenic viruses, bacteria and parasites (55). Lipid rafts appear to function to facilitate pathogen entry, replication and assembly by concentrating cellular surface receptors and cytoplasmic components critical for these processes. In addition, pathogens encode gene products that coop the function of lipid rafts in infected cells including immune cells and in doing so block their ability to function.

HIV-1 infection of T cells illustrated the functions of rafts in the life cycles of viruses. During initial infection HIV-1 binds sequentially to the T cell glycoproteins CD4 and to raft associated coreceptors including CCR5 and CXCR4, leading to raft clustering (56-58). The association of HIV with the raft associated receptors appears critical as disruption of rafts by cholesterol depletion or by blocking sphingolipid synthesis inhibits HIV-1 entry into T cells (57-59). HIV-1 assembly and budding from infected T cells is also dependent on lipid rafts. Two important HIV-1 proteins, GAG that mediates multiple steps in viral assembly (60,61), and the envelope protein ENV which is palmitoylated (62) associate with rafts during viral assembly (63) and disruption of rafts blocks virus assembly and budding (61).

Epstein Barr Virus (EBV) infection of B cells illustrated how pathogens can coopt the function of rafts and in so doing block immune cell activation. During latent EBV infections two viral proteins are expressed, latent membrane proteins 1 and 2A (LMP1 and LMP2A). LMP1 is constitutively present in lipid rafts where it generates signals that mimic those of a potent B cell coreceptor CD40, promoting the growth transformation of the B cells (64-66). LMP2A is also constitutively present in rafts where it generates signals that promote the survival of the infected B cell and block BCR induced signaling (67). The BCR in LMP2A-expressing B cells does not partition into lipid rafts and does not initiate signals that would end viral latency by inducing activation of the virus (65,68). Thus, EBV co-opts the function of the rafts in infected B cells to promote long-term growth of the cell necessary to maintain latency and to block the B cell activation.

RAFTS AS POTENTIAL TARGETS OF THERAPIES

The observations that lipid rafts play an important role in immune cell signaling suggests that they may provide new targets for treatment of autoimmune diseases and allergy and to block organ rejection in transplantation. To provide such opportunities the molecular mechanisms by which rafts function to facilitate MIRR signaling will need to be delineated in detail. Nonetheless, even in the absence of

such critical information there are emerging links between existing clinical therapies and their effects on lipid rafts that suggest that new classes of drugs may be developed to modify immune responsiveness based on the ability to modulate the function of lipid rafts.

Glucocorticoids (GCs) are a class of cholesterol-derived steroids produced by the hypothalamic-pituitary-adrenal axis that have significant immunosuppressive and anti-inflammatory effects on the immune system (69). GCs inhibit T cell responses and consequently, GCs have been widely prescribed in the treatment of autoimmunity, allergy and inflammatory disease and in the prevention of graft rejection. The primary targets for the action of GCs appear to be intracellular receptors that alter nuclear gene transcription. However, recent evidence indicates that GCs alter both the lipid composition of the inner leaflet of the lipid rafts and the palmitoylation of cellular proteins (70). Thus, in GC-treated T cells the raft associated proteins LAT, Cbp and Lck fail to be compartmentalized. Knowledge of the mechanism by which GCs alter membrane composition may provide new targets for immunosuppression that can be further exploited.

Polyunsaturated fatty acids (PUFA) such as those abundant in marine fish oils modulate immune responses and consequently have been used clinically as immunosuppressants and in the treatment of inflammatory diseases (71). Recently PUFAs have been shown to block TCR signaling by modifying the inner leaflet of lipid rafts and by being incorporated directly into proteins through S-acylation, including the Src-family kinases, disrupting their raft localization (20,21,72). The understanding that PUFAs can misdirect proteins and block raft functions may provide new opportunities for treatments of autoimmune and inflammatory diseases.

The cholesterol reducing drugs, the statins, already in widespread use in the clinics have been shown to modulate T cell responses (73). It is possible that drugs that effect membrane cholesterol levels and consequently raft functions could be effective immunosuppressants.

Conclusions – The recent appreciation that the spatial organization at the plasma membrane of immune cell signaling receptors, MIRRs, and the component of their signaling cascades is critical to their function provides new opportunities for therapeutic intervention in the signaling process. The studies reviewed here provide evidence the lipid rafts may be a key component in achieving the optimal spatial organization for MIRR signaling. As a more complete understanding of the molecular mechanisms by which rafts effect the organization of the membrane is achieved, it should become clearer if these processes can be regulated to alter the outcome of MIRR ligand binding.

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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

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Chapter 28. Recent development in Cheminformatics and Chemogenomics

A.W. Edith Chan and John P. Overington
Inpharmatica, 60 Charlotte Street, London W1T 2NU, UK

Introduction – During the past 5 years, the global effort of sequencing the human genome has provided us with an enormous number of potential protein drug targets (1). Some of these are completely novel targets, with little known biology, while many are new sequences belonging to known gene families, such as the kinases, GPCRs, and nuclear hormone receptors (2,3). To make effective use of these post genomic efforts, new drug research and development strategies need to be devised such that multiple targets can be addressed simultaneously. Much existing knowledge and experience, gathered over the years on the known target families provides a good starting point for these new research discovery paradigms. The approaches we review here involve the use of chemical information and expertise, or cheminformatics, together with the technologies of chemogenomics and chemical genomics for making the transition 'from gene to drug'. While cheminformatics refers to a computational analysis, chemogenomics and chemical genomics employs a broad array of experimental techniques from genomics, proteomics, biology, and chemistry. This review will report the recent chemistry developments in chemogenomics, emphasizing the *in silico* side of these technologies, and provide examples of what they have delivered so far.

CHEMINFORMATICS

Cheminformatics, sometimes spelt chemoinformatics, encompasses the design, creation, organization, management, retrieval, analysis, dissemination, visualization and use of chemical information (4). It encompasses a number of well-established computational areas that have existed separately for many years. Cheminformatics can be roughly divided into two areas: data creation and analysis.

Data creation covers the creation (or compound/library registration), storage, and display of chemical information. The technology has a long and well-recorded pedigree and will only be mentioned briefly. For small molecule database creation and storage, the most commonly used systems are from MDL (5), Daylight (6), Accelrys (7), and Tripos (8). All have their own proprietary way of storing chemical structures, their activities and properties and other related information, using relational database (Oracle) technology. These 4 database systems have their own interface for viewing and displaying molecular structures, either provided as part of the commercial system used and modified or designed internally by pharmaceutical/biotech companies to suit internal business practices.

Data analysis consists of a large spectrum of computational chemistry techniques (4), such as chemometrics, statistical methods for molecular similarity or diversity analysis and QSAR. Typically, chemical structures are represented by a connection table or in an equivalent linear notation, such as SMILES strings (9) or SLN (10) (Sybyl Line Notation). Structural keys or fingerprints, that describe either the presence or absence of sub-structures or fragments, are used in 2D/3D structural searches, the most commonly used being MDL's 2D structure MACCS

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keys, Daylight, and Unity fingerprints (7,11,12). Chemical descriptors, based on chemical, physical, and topological properties are also commonly used (13). For 3D descriptors, most common used are 3 or 4-points pharmacophores (14) derived either from a set of ligands or from the binding sites of the protein structures. These might be determined experimentally by X-ray or nuclear magnetic resonance (NMR) techniques or from homology modeling techniques. There are numerous compound-clustering techniques , which group compounds by molecular similarity (15). This is measured most commonly using the Tanimoto coefficient, although there are other measures (16,17). Molecular search (or compound selection) techniques employ substructure, 2D similarity or 3D pharmacophore searches. For 3D searching when there is no target structural information, 3D QSAR, based on statistical analyses of the molecular fields or pharmacophore features of aligned molecules with similar biological activity, can sometimes identify the 3D interactions of each molecule that are important for ligand binding.

Cheminformatics plays an important role in both lead generation and optimization. For example, if the structure of the substrate or an existing ligand is known, 2D/3D similarity searches can be conducted using the template structure to select compounds for testing. Diversity analysis and molecular clustering can be employed to select a representative subset of compounds for biological screening, especially high-throughput screening (HTS), from a large set of compounds, either from a commercial source or from a corporate database. Library design, QSAR, computational chemistry, molecular modeling and diversity analysis all contribute to the design of focused and diverse libraries, split/mix combinatorial chemistry (combi-chem), parallel synthesis, and further virtual screening.

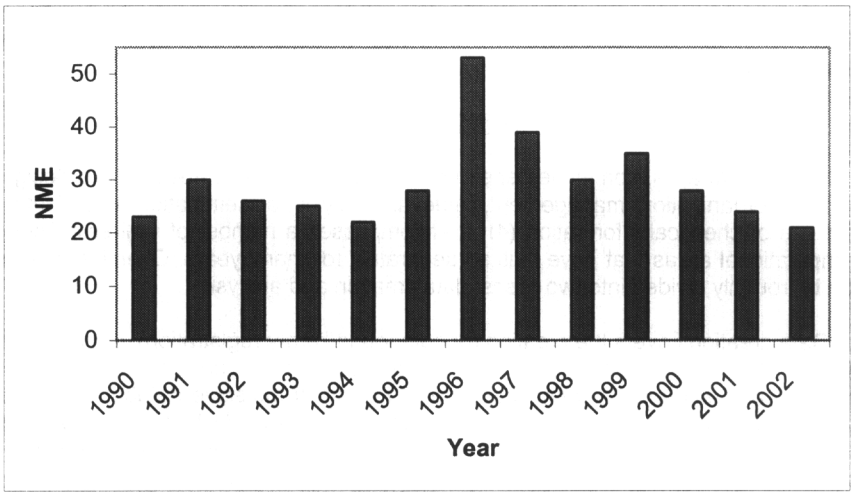


Figure 1. Number of New Molecular Entities (NME) reported from the FDA for the years 1990-2002.

However, in the past five years, a major emphasis of cheminformatics has been in the area of *in silico* ADMET (drug Absorption, Distribution, Metabolism, Excretion, and Toxicity). Figure 1 represents a plot of the number of new molecular entities (NME) reported by the FDA for the years 1990-2002. It can be seen that the role of new NME introduction jumped in 1996, but has since steadily tapered, which suggests that producing drugs with sufficient therapeutic efficacy, selectivity and ADMET properties to satisfy the regulatory approval framework is becoming recently more difficult.

Many drug discovery programs fail largely because the drug candidate fails clinical trials in humans, resulting in a great deal of lost time, expenditure and effort. From an analysis conducted here at Inpharmatica on the IDdb3 database (18), among the approximate 25,000 entries of drug candidate records for the past 10 years, 42% of them are now inactive and 60% of these compounds failed before entering phase I. A study conducted by Edwards presented a similar observation. They have collected data from 9 leading pharmaceutical and biotechnology companies during the year of 2001 and the attrition rate from hit compounds to pre-clinical candidates is 57% (19).

With increased pressure for better products faster, the concept of "Fast in man" is being actively pursued by the industry and ADMET properties of a compound have become the important parameters when designing and optimizing lead-like compounds, especially in combi-chem and focused library design. The quest for most potent receptor binding, formerly the primary goal by medicinal chemists, is not the only parameter considered these days. Due to the difficulties of ADMET studies, this strategy will require a shift from a 'screening' based approach to a knowledge-based compound selection and modification paradigm. In addition, *in vitro* high-throughput assays are increasingly employed to approximate the ADMET characteristics of potential leads at earlier stages of development.

The molecular physiochemical properties such as molecular weight, H-bond donor and acceptor, logP, number of heteroatoms and rotatable bonds, PSA (polar surface areas), toxic and reactive fragments in the molecules can all affect the ADMET properties in general. The best known analyses, the 'rule of 5' was performed by Lipinski and his colleagues in Pfizer (20). His data mining approach showed that good oral absorbed drug typically have a MW less than 500, fewer than 5 hydrogen bond donors, fewer than 10 hydrogen bond acceptors (approximated by number of heteroatoms less than 10), and calculated logP less than 5. Additional findings have emerged recently, which added new rules, such as the number of rotatable bonds being less than 10, the ring count less than 5, etc (21, 22).

Many computational methods are used for ADMET prediction. The most common ones are basic statistics (which is how the rule-of 5 was derived), SAR, and more artificial learning approaches such as genetic algorithms and neural networks.

All these molecular parameters are used in analyzing compound data and building models to predict ADMET properties (23), such as absorption studies in Caco-2 (24) (a human intestinal epithelial cell line derived from a colorectal carcinoma); MDCK (25) (madin-Darby canine kidney) cell monolayers; susceptibility to metabolic degradation using liver microsomes or hepatocytes (26); prediction of logBB in the blood-brain barrier system whose purpose is to maintain the homeostasis of the CNS (central nervous system) by separating the brain from the systemic blood circulation (27). The data sources for analysis usually come from large commercial database, such as ACD (5) for non-drug like, and MDDR (5) and WDI (28) for drug-like compounds.

Most pharmaceutical companies have put such concepts and rules into practice, most commonly as filters for library design, warning flags during compound registration, and in compound selections for high-throughput or virtual screening. These filters include the physical and chemical parameters mentioned above (such as filtering out MW less than 100 and more than 700). In addition, toxic or reactive groups or fragments can be also included in the filters. Training such filters sometimes totally relies on expert knowledge provided by medicinal chemists.

Although this kind of expert knowledge is not widely publicized, some companies have published portions of their findings (29,30).

There is an urgent need to analyze the large amount of chemical data that we have to generate new knowledge and aid decision-making. With the advances in computing and related technologies, such as data storage, web and programming advances, it has become easier to integrate all the information together under the umbrella of cheminformatics. The success of this integration will speed up the traditional R&D process relative to the traditional drug discovery process.

The goal of cheminformatics is to generate knowledge through data integration. The ultimate goal would be to identify the development compound that can inhibit the target protein and have the perfect ADMET data making it safe for use in humans. Currently a more realistic goal is to identify which compounds are 'lead-like', what kind of target libraries a chemist should make, etc.

The pharmaceutical, biotechnology and related industries have been quite successful in achieving these goals. Many companies are now taking the further step of integrating bioinformatics, proteomics, target discovery, target validation, and chemistry, which, together with cheminformatics, form the basis of chemogenomics.

CHEMOGENOMICS

Chemogenomics represents a new approach to target identification and drug development with the potential for dramatically accelerating the process. No longer is a single drug designed for a single protein target; rather, through chemogenomics, multiple drugs can be designed to target multiple-gene families. With this approach, biophysical and chemical information gained on one protein can be applied to structurally similar targets in the same family (31). On the other hand, small molecule leads, identified for one member of a gene family, can also be used to elucidate the function and biological role of another member of that family whose function is not known, as well as enabling simultaneous measurement of their potency and selectivity. This process can also identify problematic, non-selective compounds early in the drug discovery process.

This approach has the potential to cover a broad range of therapeutic areas, because while gene families code for structurally similar proteins, each protein in a gene family can have a very different biological and potential therapeutic function. Different targets within a gene family may be implicated in widely different diseases. Therefore, families of related proteins can be considered together as potential drug targets, rather than any single member. For example, the 50 or more proteins in the nuclear receptor family such as PPAR, CAR, LXR, FXR, are implicated in a broad range of diseases, including diabetes, obesity, and cardiovascular disease (32). There are at least 500 human kinases, several of which are implicated in neurobiology, immunology, and related areas (33,34).

Figure 2 shows one scenario of the workflow in chemogenomics. Various bioinformatics techniques can be applied to the genome for annotation of all possible protein targets (known or novel) (35). These protein targets are then subjected to further *in silico* cheminformatics and bioinformatics analyses, such as homology modeling, domain analysis, drugablility, and selectivity calculations. This informatics driven strategy for target discovery prioritizes the targets, which are most drugable to go through to the experimental stage of target validation, such as screening with a targeted library of chemical structures.

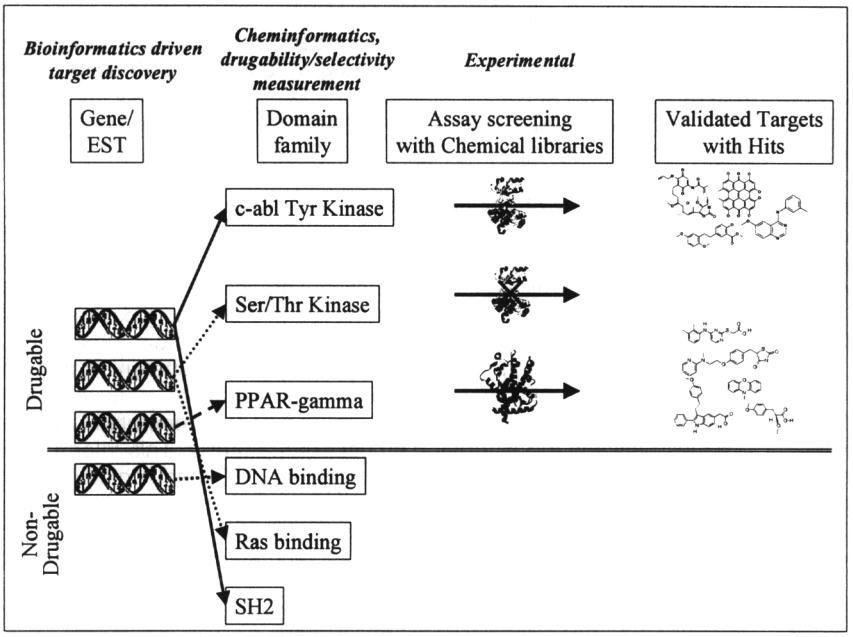


Figure 2. One scenario in Chemogenomics.

Using the example of protein kinases, most research effort has concentrated on the ATP-binding site of the kinase catalytic domain, bearing in mind that some of the individual domains from a protein kinase, such as the SH2 and SH3 domains, might not be 'drugable', *i.e.* amendable to the targeting of therapeutics. These ATP binding sites can be quite similar, despite the fact that each family member can participate in a different biological pathway. Chemogenomics techniques are particularly suited for families such as this, although many issues need to be addressed at the same time. For example, if the inhibitors for many family members are similar, selectivity becomes an issue. Among all the family members, how can one prioritize which target kinase to work on? Some of the recent developments in *in silico* chemogenomics attempt to answer these questions.

In general, there two approaches to the *in silico* analysis of data for potential drugable targets: 1) assignment to precedented domain families which contain protein binding sites of a type that have been previously drugged (36,37), or 2) detection of protein binding sites with structural properties that will predispose them to drug binding (38). The former gives about 120 structural families, mainly of GPCRs, ion channels, proteases, kinases and nuclear hormone receptors. The latter extends the set of drugable domains to novel families where a binding site, inferred by structural homology and binding site analysis to have general properties consistent with binding a drug-like molecule, gives cause to promote it for screening. In this way, cheminformatics binding site analysis will be important when defining novel targets.

The factors governing specificity and selectivity can be deduced by comparing the similarities and differences across the protein's family members (38), both within the same species and across other genomes, especially the pharmacological model organisms of mice and rats. When the structures of these proteins are known from X-ray crystallography, NMR, or through homology modeling, then computational techniques can be applied to study their binding sites. It is known that residues at the surface of a protein are typically less well conserved than internal residues, as

they tend not to be so important for stability or folding (39). However, surface residues in functionally important binding sites, have been shown to have extremely high conservation relative to other residues, evolving at rates up to 50 times more slowly (40). This pattern of conservation within a gene family is extremely important, since experimentally, it has been demonstrated that single amino acid changes are sufficient to generate specificity in protein kinases (41).

The chemogenomics properties of a target protein's binding site can thus be derived by comparing homologous sequences within the family. Primary function and pocket regions present in the protein can be identified by regions having high conservation scores (38). This can suggest which chemical functional groups are needed to target the activity of the protein. Regions of lower conservation in the binding pocket might indicate where selectivity can be introduced, either for a single protein target, or in the design of multi-gene targeted drugs. Substitutents with different chemical functional groups and physical properties might be introduced onto a small molecular scaffold to target different binding environments generated by the variation of residues between the different members of the family. This information provides the starting point of the design of a focused library. Experimentally, many companies have also set up programs to target the specific binding problem, such as PhotoMics in ComGenex (42), where they use photolabile ligands that allow and detect conditional and specific covalent binding to high-abundance proteins.

This kind of *in silico* analysis can provide information on how similar or dissimilar the binding sites are among the members of a family. If some of the family members have very similar or identical binding sites there may be a selectivity issue, because it will imply similar chemical interactions between ligands and the protein. However, for some therapeutic areas where selectivity is not a requirement, the same drug could be designed to target more than one sub-family.

Where there is no structural information about the target protein, and the area of selectivity cannot be identified, then molecular diversity can be applied to a series of known compounds with proven affinity towards one family member. SAR data derived from the known compounds will provide the basis for library design. Usually this kind of diversity is termed "biological" rather than "chemical" diversity and the corresponding chemical synthesis techniques are called retrosynthetic analysis in target-oriented synthesis (TOS) (43) and chemical diversity or diversity-oriented synthesis (DOS), respectively. The use of assay data to identify targets with similar SAR patterns, and of family-based screening strategies, suggest that chemistry efforts can be focused toward related gene families (44,45). In common practice, chemistry effort is usually directed to more controlled parallel synthesis rather than split/mix combinatorial libraries (46).

The original combinatorial library design approach of applying general chemical diversity and screening attempted, somewhat ambitiously, to sample all possible drug compounds, that could be directed at all possible drug targets. Currently, diversity-oriented synthesis draws on the technical developments in combinatorial synthesis, which is more often now applied in TOS.

Traditionally, diverse combi-chem libraries tended to be peptide-like and produced on solid phase resins. These kinds of libraries could be better used for target validation rather than lead finding: first because it is difficult to optimize non-drug like molecules to a drug like lead, and second because combi-chem compounds tended to have high molecular weight, high lipophilicity, too many rotatable bonds and too many amide groups, thus decreasing the chances of good ADMET properties. However, recent developments in combi-chem have moved to solution phase, significantly increasing the number of chemical reactions possible.

They now include more drug-like scaffolds with substitutants (R-groups) having generally lower molecular weight. It is important to ensure that the libraries have a good ADMET profile to avoid downstream attrition. Matthew Bogyo's group has used focused combi-chem libraries in competition experiments to distinguish specifically bound ligands selective for cathepsin B (47). All in all, chemical genomics uses drug-like chemical compounds to probe the proteome so as to identify hits, their potential targets, and biological insight in one single step.

FIRST CAME CHEMICAL GENETICS AND THEN CHEMICAL GENOMICS

In the past decade, chemical genetics has been an approach that systematically uses small molecules to explore biology, rather than to discover new medicine (48,49,50). It also involves the use of small synthetic molecules which induce phenotypic changes by directly perturbing a protein interaction, rather than indirectly by genetic manipulation, such as by mutagenesis studies, to identify key proteins involved in the specific biological pathway of interest (51,52). In many cases the chemical probes are existing drugs whose overall effect is well established, but whose mode of action is not well understood. Coupled with post genome data, this approach, usually termed Chemical Genomics, promises to deliver small molecule drug leads, as well as validation of functions of the proteins derived from the human genome, especially by finding ligands for orphan proteins.

Chemical genetic experiments, therefore, present an opportunity to clarify the specific mode of action of well-known therapeutics. The current wealth of gene sequence information available, in conjunction with recent advances in array-based technologies, has facilitated this chemical genomic approach. High-throughput screening with small, highly specific, synthetic molecules, against multiple protein targets, results in measurable phenotypic changes and presents an opportunity to do gene functional analysis and create new therapeutic leads at the same time.

Small molecules can be used to probe global biology; this is an especially fertile area for organic chemistry. To facilitate such studies, most pharmaceutical and biotechnology companies are engaging in their own in-house screening projects. In public, for example, the Harvard Institute of Chemistry & Cell Biology (ICCB), most recently sponsored by the National Cancer Institute's (NCI) Initiative for Chemical Genetics (ICG), has introduced the concept of small-molecule annotation and profiling (53,54). An additional key element is the ChemBank whose goal is to provide scientists with tools and federated databases to explore biology with small molecules (55). Besides literature data, additional data come from their provision of small molecules for rapid screening for other laboratories. Another example is illustrated by harvesting GPCRs by screening a large panel of orphan receptors (56,57). Another good public source is the ChemID*Plus* database provided by the National Library of Medical (58). It has over 350,000 chemical records that provide links to chemical, biological indication and clinical information.

However the chemical library is made, or the compounds collected, there are three purposes of screening a chemical library: target identification, validation, and lead generation. The target identification library may include a collection of known reference substrates or ligands together with chemically similar compounds, and probably a sampling of novel drug-like compounds. A lead generation library, which is designed based on the information known about a specific target, can also be used in target validation. These are mostly used in cell-based assays or to de-orphan novel targets. De-orphaning GPCR and nuclear receptors are some of the examples (59,60). As nuclear receptors are involved in many core metabolic pathways, a compound collection of lipids, bile acids, steroids, hormones, etc is often used to de-orphanize nuclear receptors (61,62,63). In any case, all the

compounds should possess high affinity and specificity for the target protein, and reasonable membrane permeability, to maximize the probability of significant biological activity in whole cell assays. For focused libraries, it is important to include as much of the information about the target active site as possible, together with a systematic filling in of property space to assure a thorough coverage (64,65).

There have been quite a few success stories, for example, in the fields of ion channels and signaling in the neurosciences. Natural products have always played a particularly prominent role in neurobiology. Scientists in Pfizer recently developed a fruitful effort in both the isolation of channel-blocking natural products from spiders and the synthesis of optimized variants (66). Their collection is used to classify channel subtypes and to probe their functions in neurobiology. Another example to determine biological function using small molecules is the insight gained from human genetics. Pioglitazone, the archetype of the "glitazones", was discovered for treating type 2 diabetes during the 1970s. The glitazones bind and activate the nuclear receptor PPARγ, which is known to play a role in metabolic pathways involving diabetes (67). Small molecules have also been identified that selectively bind and activate five members (paralogs) of the somatostatin receptor family. Having predetermined the selectively of these probes toward all 5 individual paralogs, distinctive functions of the paralogs have been discovered (68).

In summary, the chemogenomics approach, combining chemistry, biology, genomics, bioinformatics, and proteomics, along with cheminformatics techniques such as gene sequence comparison, protein structural analyses and chemical inhibitor design across a whole gene family, enables improvement of drug design for single protein targets, or more ambitiously, consideration of multiple drugs for multiple targets at once. This not only resolves specificity and selectivity issues, but also improves chances of success,. The challenges in utilizing this approach lie in the proper planning and construction of a seamless informatics pipeline, incorporating information as well as experimental data, to harness the promise of the concept.

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Chapter 29. Disruption of Protein-Protein Interactions

Daniel Yohannes
Infinity Pharmaceuticals, Inc.
780 Memorial Drive, Cambridge, MA 02139

Introduction - The association of proteins with other proteins is one of the most common interactions in biology. Such interactions play a central role in the regulation of numerous cellular functions. While many of these processes are mediated by enzymes including kinases, proteases, or glycosylases, the pathways that regulate these cellular functions are initiated or inhibited via specific protein-protein interactions. The precise regulation of these pathways often involves the assembly of multiple proteins. The resultant oligomeric protein complexes comprise many enzymes, viral proteins, and receptor-ligand partners (1). The assembly of protein complexes is critical for allosteric control, formation and conformational maintenance of active sites of oligomeric enzymes, regulatory processes such as signal transduction, cell-cell contacts, electron transport systems, and antigen-antibody interactions (2-12). Specific intervention of these partners may lead to an increased understanding of which components to target in pathological situations.

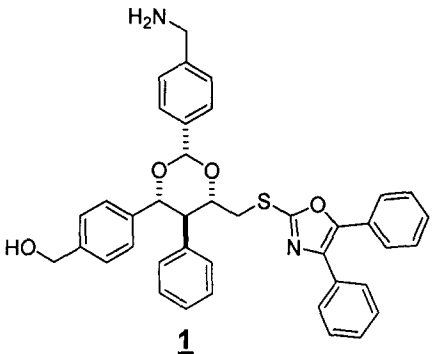
The wealth of information regarding protein-protein interactions as well as many descriptions of aberrant protein-protein interactions in disease has made the disruption of protein-protein interactions a focus of recent research activity in the pharmaceutical and academic scientific communities. The quest for biochemical probes and therapeutic agents which intervene at protein-protein interfaces provided the impetus for the discovery of antibodies, dominant-negative proteins, or short peptides which inhibit protein assembly in the literature. Modulation of protein-protein interactions by small molecules has proved to be very challenging and there have been a paucity of successes reported in the literature. However, this chapter will cover recent advances in the identification and detection of protein-protein interactions, as well as the identification of protein complexes which have become recent targets of pharmaceutical intervention. The associated molecular tools and potential therapeutics which have emerged in the past year will also be described.

Identification and Detection of Protein-Protein Interactions - Before the advent of genomics, standard methods for the detection of protein-protein interactions were crosslinking, co-fractionation by chromatography and co-immunoprecipitation. In 1989, Fields and Song reported the "Yeast Two-Hybrid Assay" (13). This breakthrough genetic system studied protein-protein interactions by taking advantage of the properties of the GAL4 protein of yeast saccharomyces cerevisiae. GAL4 is a transcription factor made up of an N-terminal DNA binding domain and a C-terminal activation domain. A 'bait' protein was fused to the DNA binding domain and used to identify protein partners from a library of c-DNAs cloned into a vector encoding the GAL4 transactivation domain. Interaction of bait partners enhances the transcription of the GAL4 promoter. The binding partners were then sequenced. This unbiased process allows identification of direct protein-protein interactions.

The advancement of proteomics has allowed protein microarrays to complement gene expression profiling. Initially, the technology involved the arraying of cDNA expression libraries on PVDF membranes (14). Early methods in which proteins were arrayed at low density have been superceded by well defined high density

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protein arrays on glass surfaces in the past several years. In a seminal paper, the identification of protein-protein interactions has been disclosed by using fluorophore tagged proteins to probe proteins arrayed on aldehyde slides (15). In this system, the authors were able to elucidate the protein-protein interactions of FKBP12 using commercially available instrumentation. The current state of protein array methodologies has been recently reviewed (16). Further recent advances include a report describing a protein-domain chip used to identify novel protein-protein interactions (17). In this work, GST fusion proteins were arrayed onto nitrocellulose-coated slides to generate a protein domain chip on which the proteins maintained their binding integrity. These immobilized proteins interacted with proteins from a cell lysate and the interaction was detected with a specific antibody to provide an intracellular interaction map for a cytosolic protein of interest. From this work, the domain-binding profiles were determined for Sam68 (Src-associated during mitosis 68) and a core small nuclear ribonucleoprotein called SmB'. The use of red and green fluorescent proteins (RFP and GFP) has gained popularity in protein microarrays. This technique permits the investigation of protein-protein interactions without the need for additional labeling steps (fluorescent dye labelling and purification) of probe proteins. Such an approach was reported whereby recombinant proteins labeled with RFP and GFP were used in protein microarrays as tags to investigate antigen-antibody interactions and other protein-protein interactions (18). Although membrane protein-protein interactions are not commonly found by protein array technology, a recent report documents the immobilization of a membrane which provides for lateral fluidity of proteins, and which houses functional GPCRs as determined by their binding profiles (19).



1

While protein microarrays provide, in principle, potential for full access of the proteome on a solid phase, in a recent review on protein microarray technology, it has been suggested that it is fundamentally more challenging to work with proteins than with nucleic acids (20). Thus a newer technique has emerged: the immobilization of small molecules on a surface which permits the use of tagged proteins in the mobile phase for detection. The potential for an ultra-high-throughput full-proteome analysis with this approach is very high. An early report of this approach coupled this novel array methodology with large numbers of molecules derived from diversity-oriented synthesis (DOS) (21). By probing a high density microarray of DOS small molecules with fluorescently labeled yeast protein Ure2p (which suppresses transcription factors Gln3p and Nil1p), a highly specific compound called uretupamine (1) was identified which activates a glucose-sensitive transcriptional pathway downstream of Ure2p (22). Cell based mechanistic assays with the identified small molecules allows this process to become a powerful tool, and it is currently being industrialized in a drug discovery setting. An alternative approach to small molecule microarrays has been taken wherein small libraries of designed (mechanism-based) covalently-modifying inhibitors conjugated to sequence-encoded peptide nucleic acids were able to identify activity-based profiles

of cysteine proteases in crude cell lysates (23). Both techniques use only very small quantities of proteins as well as whole cell lysates, which permit the screening of small molecules across the soluble proteome.

Catalytic RNAs are also being used for small molecule screening to identify protein-protein disruptors. A novel screen described last year employs protein-dependent RNA catalysts (ribozymes) that can monitor protein-protein, protein-RNA, and protein-small molecule interactions in real time using changes in fluorescence as a readout (24). Ribozymes have been engineered with fluorescent tags and nucleic acid-protein binding domains. Binding of a protein target to the reporter ribozyme alters the ribozyme's self-cleavage activity, which can be monitored by changes in the amount of fluorescence detected. In this way, a library of antibiotics was screened by monitoring fluorescence changes to inhibit the interaction between the HIV-1 Rev protein and Rev-binding element (RBE). An inhibitor was identified which attenuated HIV-1 replication in cells. In a second example, it was possible to monitor interactions between the blood-clotting factor thrombin and its protein partners. The approach appears to be applicable to many targets, and particularly suitable for protein-protein interactions.

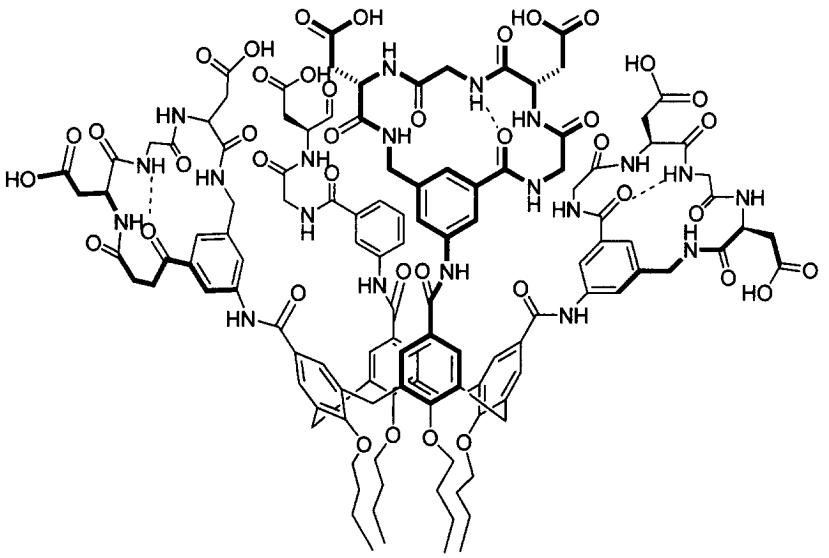
A novel application of FTIR to study protein-protein interactions has recently been reported. This approach combines FTIR with principle components analysis and protein titration experiments to identify association-induced changes in protein structure (25). The system studied was the complex formation between bacterial monooxygenase Cytochrome P450BM-3 Heme Domain and Flavin Mononucleotide (FMN) Reductase Domain. The solution phase secondary structure for each protein was established and compared both with the crystal structure and the solution-phase changes in structure on formation of the complex. Indeed, the structures for the BMD-FMND complex were different in solution relative to the X-ray.

New Chemical Approaches to Small Molecule Disruptors of Protein-Protein Interactions – A number of approaches to evaluate the ability of small molecules to disrupt protein-protein interactions have emerged recently from diversity oriented synthesis to designed and targeted systems. They are discussed in a thorough recent review (26).

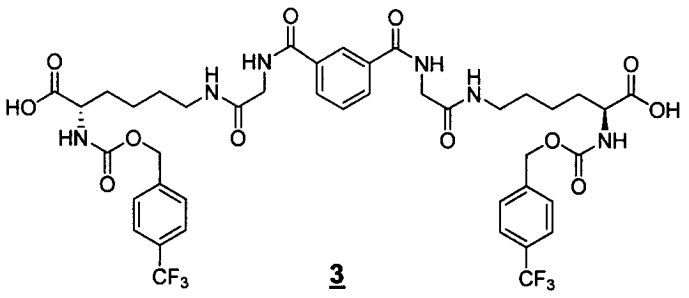
Recently, a synthetic molecule (**2**) has been designed to display a large, functionalized and variable interaction exterior which bound to the surface of chymotrypsin and disrupted its interaction with some of its protein inhibitors (PI). In the case of chymotrypsin - soybean trypsin inhibitor, the mechanism appears to involve the formation of an initial ternary complex with time-dependent displacement of the PI. This represents the first example of a synthetic agent that blocks the interaction of a protease and its PI. As such, it is an unusual illustration of a strategy which seeks to prevent natural association of proteins by blocking large surface areas of that interaction.

This type of synthetic design has been adapted to a combinatorial approach (27). The approach is to generate libraries of compounds with two or more binding groups separated by variable linkers which can interact with one or more protein targets. In this case, 600 symmetrical dimeric structures with three diversity subunits were used to identify a prototypical inhibitor (**3**) of the association of MMP2 with integrin $\alpha_v\beta_3$ in an in vitro binding assay. Interestingly, the inhibitor did not directly inhibit MMP2 activity or disrupt the binding of integrin $\alpha_v\beta_3$ to its natural extracellular matrix target, vitronectin, but bound integrin $\alpha_v\beta_3$ in a dose-dependent manner. Such a mechanism suggests that this agent and others that are similar may have both direct and indirect effects mediated by perturbations of smaller, more specific protein-protein interactions than that of **2**. Compound **3** possesses in vivo activity (suppression of angiogenesis in a chick CAM model and near complete reduction of

solid tumors in xenograph models), making it an impressive example of small molecule intervention at protein-protein interaction sites (28). The design of solution-phase combinatiorial libraries for the modulation of both protein-protein and protein-DNA interactions has been successfully generalized (29).



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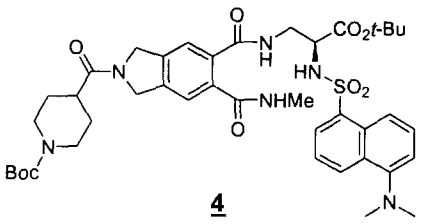


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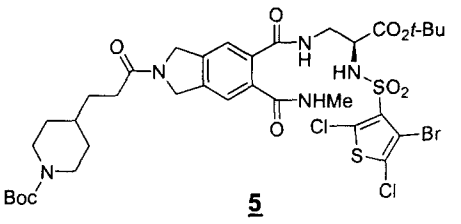
Targets for Protein-Protein Intervention – Recently available methods for probing, detecting and identifying specific functional activities of proteins have allowed evaluation of opportunities where intervention may lead to the understanding of fundamental processes in diseases as well as to drug therapy. For instance, apoptosis, an essential process in both normal development and in the disease state, is initiated and regulated by protein-protein interactions. The use of small molecule ligands targeting the Bcl-2 or Bcl-xL systems as well as the interactions of IAPs with caspases and Smac/DIABLO have been highlighted (30).

Examples in the past year of new protein-protein targets which have been the subject of small molecule disruption include the dimerization of the strongly oncogenic Myc transcription factor with Max, a basic helix-loop-helix leucine zipper protein (31). The library of small molecules utilized in this study emerged from a rigid bicyclic core resembling an Arg-Gly-Asp (RGD) mimic with substituents inspired by

recent advances which identified nonclassical peptide-like compounds as high affinity ligands for integrin $\alpha_v\beta_3$ receptors. From this peptidomimetic library, candidate inhibitors **4** and **5** emerged as inhibitors of this dimerization in FRET, Elisa and EMSA assays. While their in vitro inhibition was measured in the 20uM range, these compounds also interfered with Myc-induced oncogenic transformation in chicken embryo fibroblast cultures.



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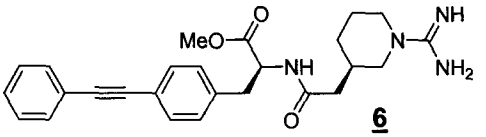
An exhaustive and unbiased yeast two hybrid screen has been carried out to identify interaction partners of two human Raf kinase isoforms, A-Raf and C-Raf. Using the N-terminal regulatory domain as bait, 20 different proteins that were isolated in this screen included three which were previously found : Ha-Ras, R-Ras, and 14-3-3 proteins (32). In addition, another 17 new Raf-interacting proteins were also found, and these were not detected by high throughput two-hybrid systems which use multiple 'baits' that compete with each other. The novel interactors included TOPK/PBK kinase (a signalosome component) and two new putative protein phosphatases. The cysteine-rich zinc binding domain (CRD) within the N-terminal domain was found to interact with all 20 proteins.

The yeast two-hybrid system has been a widely used tool to identify protein-protein interactions; however, its utility is not applicable to all proteins. One class which meets this limitation is that of membrane bound proteins, which do not enter the nucleus of a cell, a requirement for the two-hybrid system. Therefore, membrane proteins, are not available for study using this method in its current format. A new two-hybrid system has now been developed which makes it possible to detect protein partners that interact with membrane proteins, and this technology has been adapted into a high-throughput screening format (33). The method utilizes the properties of ubiquitin, a small, conserved protein which is used to tag the N-termini of proteins for proteosomal degradation. Ubiquitin can be split into N-terminal (Nub) and C-terminal (Cub) halves, which can reconstitute spontaneously to from a 'split ubiquitin' that is recognized by ubiquitin-specific proteases (UBPs). A reporter protein can be fused to the Cub moiety, which can itself be fused to a membrane protein. When such a modified membrane protein (or a cDNA library) interacts with another protein which has been fused to the Nub moiety, this interaction promotes the reconstitution of 'split ubiquitin'. This reassembly event triggers a proteolysis that releases the reporter protein, which enters the nucleus and activates reporter genes. Such a membrane-based two-hybrid system enables detection of interactions between membrane and cytosolic proteins, as well as weak and transient protein-protein interactions in vivo and in situ. Small molecule inhibitors of these interactions identified in this cell-based manner may be less likely to carry cytotoxicity liabilities.

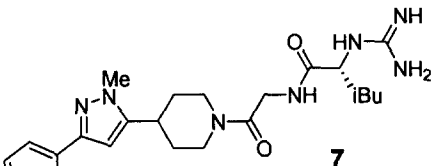
Viral replication and assembly in host cells involves the formation of macromolecular structures and enzyme complexes, both of which inherently constitute protein-protein interactions. Examples of viral assemblies include HSV, HIV, HPV, RSV and their associated proteins, all of which are targets for drug discovery. Recent developments in antiviral chemotherapy based on these protein-protein interaction targets have been summarized (34). A common theme in the

review is the development of peptides and peptidomimetics which mimic one of the faces within the viral protein-protein complexes to generate requisite specificity.

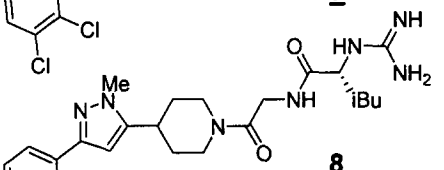
Novel Small Molecule Inhibitors – The opportunities and challenges of small molecules as inhibitors of protein-protein targets have been outlined (35). Protein-protein interfaces are typically large, flat surfaces which make it difficult for small molecules to access. However, by examining the effect of alanine scanning on binding, it has been demonstrated that only a small set of "hot spot" residues at protein interfaces contribute significantly to the binding free energy of human growth hormone to its receptor (36). This may be a general feature of other protein-protein interactions.

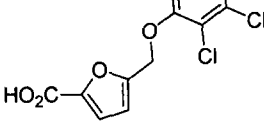


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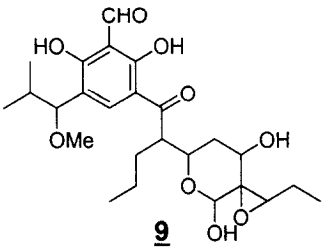




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This understanding has been applied towards drug discovery by use of a fragment assembly process called tethering, a site directed screening method for rapid identification of low-affinity fragments that bind to specific sites on a target protein (37). These fragments with affinity for the target are then connected to yield more potent molecules. By this methodology, a potent (IC50 = 60nM) small molecule inhibitor (**8**) of the IL-2 / IL-2Rα was recently identified (38). The medicinal chemistry efforts initiated on compound **6** (a known low micromolar binder of IL-2) using structure-based design approaches generated a novel lead series exemplified by **7**. Tethering methods which used 10 individual cysteine mutations of IL-2 to screen a library of 7000 disulfide-containing fragments focused on one region of the mutants. This region selected a defined set of ca. ten structurally related small aromatic carboxylic acid fragments. The SAR of aromatic carboxylic acids which emerged form this study led to **8**.

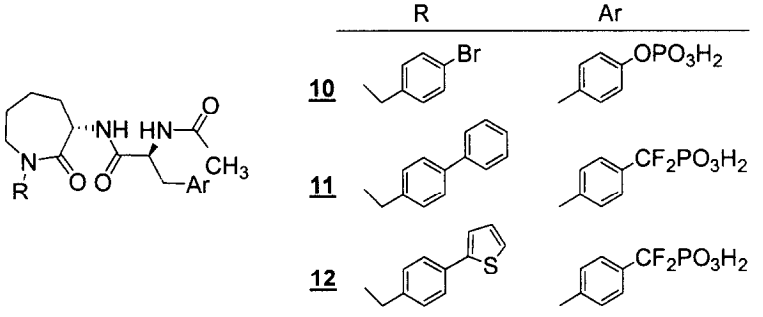
A recent example of a natural product inhibitor of a protein-protein interaction is UCS15A (**9**), a small molecule non-kinase inhibitor of Src signal transduction (39). Src tyrosine kinase is important in signal transduction following growth factor stimulation and integrin-mediated cell-substrate adhesion. As Src-signal transduction defects are implicated in a number of disease states, this protein could be a target for small molecules that either block the kinase activity or the interactions between Src and other signaling molecules.



9

Compound **9** inhibits the Src-specific tyrosine phosphorylation of many proteins in v- Src-transformed cells, including Src substrates cortactin and Sam68. Despite the loss of tyrosine phosphorylation of Src substrates, UCS15A differs from conventional Src-inhibitors in that it does not inhibit the tyrosine kinase activity of Src. UCS15A does not destabilize Src, like the herbimycin and radicicol class of compounds that act by interfering with HSP90 binding. Studies by Sharma suggest that UCS15A exerted its Src-inhibitory effects by a novel mechanism that involves disruption of protein-protein interactions of Src with its substrates such as Sam68 and an unidentified protein of 62 kDa through Src-SH3-mediated protein-protein interactions (40).

Src-SH2 domain interactions with phosphotyrosine containing proteins have been the target of some recently disclosed ligands. Of note, osteoclast-mediated bone resorption is one of the many important cellular functions in which Src participates (41, 42). A recent report identified non-peptidic ligands capaple of mimicking a pYEEI tetrapeptide which is recognized by the SH2 domain of c-Src (43). Recently, non-peptidic ligands **10**, **11**, and **12**, unlike their peptide counterparts, can inhibit the interaction of protein partners of the SH2 domain of c-Src without altering the enzyme activity. These high-affinity SH2 binders disrupt the signaling cascade necessary for bone resorption by inhibiting the protein-protein interactions.



Conclusion – Protein-protein interactions have been historically difficult for small molecules to access. The examples above serve to illustrate that systematic approaches are yielding an increasing number of small molecule modulators of these processes. As proteomics research moves towards the mapping of all constitutive and dynamic protein-protein interactions, global approaches are focusing on functional networks (interactomes) (44). These new improved proteomic technologies should reveal more protein-protein interaction targets for future therapeutic intervention.

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Chapter 30. Recent Advances in Virtual Ligand Screening

James F. Blake and Ellen R. Laird
Array BioPharma Inc
3200 Walnut Street, Boulder CO 80301

Introduction – Random high-throughput screening as a means for finding novel lead compounds against a variety of potential therapeutic targets is a widely accepted technique in modern drug discovery (1). Virtual ligand screening based on high-throughput protein-ligand docking and three-dimensional pharmacophore queries for the identification of compounds from databases provides a computational approach for the identification of novel compounds. These two techniques are often complementary. Pharmacophore searching methods are most often employed when the 3D structure of a target enzyme or receptor is not available, but a small number of active compounds are known. On the other hand, database docking methods are routinely used when an X-ray structure of the target protein is available.

In this review, we will focus on recent advances in the use of two main 3D virtual ligand screening techniques: pharmacophore derived and protein-ligand docking methods, with an emphasis on results, rather than methodology development. We have focused our review on selected cases where a pharmacophore model or receptor structural information has been used in a search for novel lead matter, and the virtual hits have been verified by one or more biological tests.

PHARMACOPHORE-BASED VIRTUAL SCREENS

For our purposes, a pharmacophore is defined as the spatial arrangement of a minimal set of discriminating molecular features necessary to characterize the biological activity of a given system. Molecular fragments or macro classes such as hydrogen-bond acceptor/donor sites, ionized, or hydrophobic groups, can represent each of these features in three-dimensional space. A variety of techniques can be used to develop a pharmacophore model ranging from simple hypothesis generation *via* manual molecular overlays to completely automated procedures. References 2-5 provide good overviews of the strategies that will be discussed here.

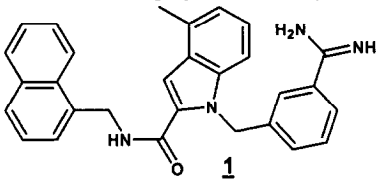
Among the more popular commercially available automated procedures are programs such as DISCO (6), GASP (7) and Catalyst (8-9). Each of these programs attempts to determine the minimal pharmacophore given a small set of compounds, typically 5-10 diverse structures that span a range of biological activities. The underlying assumption in many of these methods is that most of the compounds in the training set will share a similar set of pharmacophore features (*eg.*, hydrogen-bond acceptor or aromatic ring), though individual compounds need not necessarily contain all of the hypothetical pharmacophore elements. Once a pharmacophore is determined, it can be used to search compound databases, aid in virtual library design, or be used directly in lead optimization.

Recently, the performance of each of these pharmacophore generation programs was assessed based on their ability to reproduce target pharmacophores that were derived from various protein-ligand X-ray complexes (10). In that study, the authors considered five protein targets – Thrombin, CDK-2, DHFR, HIV-RT, and Thermolysin. The target pharmacophores were defined based on the observed protein-ligand interactions that were common to all molecules in each set. Generally, only low molecular weight non-peptidic compounds were considered, and were chosen to represent diverse structural classes; an average of 7 ligands were used for each protein target. Overall, the authors rank GASP ≈ Catalyst > DISCO

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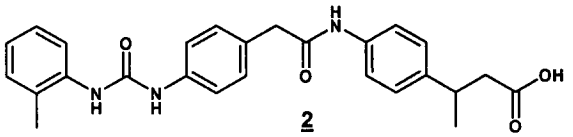
based on their ability to reproduce the target pharmacophores. Though automated procedures for pharmacophore generation are very useful and efficient, they are by no means necessary to enable the searching of compound databases.

Structure-Based Pharmacophore Identification – Researchers at Aventis used a combination of peptide mutation data, NMR structural studies on the urotensin II (U-II) peptide, and a number of analogs to derive a three point pharmacophore model (11). The peptide mutation experiments identified Trp-7, Lys-8, and Tyr-9 in U-II as important contributors to the recognition and activation activity of the peptide, while the NMR studies defined the overall three-dimensional arrangement of the peptide. By assuming the solution-based NMR structure resembles the bioactive conformation, a pharmacophore model consisting of two hydrophobic–aromatic groups and one positive ionizable group was constructed by centering the aromatic-hydrophobic groups on Trp-7 and Tyr-9 rings, and the positive ionizable feature centered on the N_ε of Lys-8 (Catalyst definitions). The Catalyst query was then used to carry out a virtual screen of the Aventis compound collection. Biological testing of the 500 compounds selected resulted in identification of 10 highly active compounds with IC_{50} values between 400 nM (1) and 7 μM, and belonging to six different structural classes. This pharmacophore-based search resulted in a nearly 20x increase in hit rate compared to conventional GPCR high-throughput screens as well as validation of the pharmacophore model (11).



1

A similar strategy was used to discover novel non-peptidic inhibitors of α4β1 (VLA-4) (12). The structure of a lead compound, 4-[N-(2-methylphenyl)-ureido]phenylacetyl-Leu-Asp-Val], was modeled based on the X-ray conformation of the Ile39-Asp40-Ser41 region of VCAM-1. A Catalyst query was generated based on the position of the carboxylate COO- in the Asp of the lead compound. A virtual library of possible replacements for Leu-Asp-Val was constructed from reagents in the ACD (13) and PAPU (4-[N-(2-methylphenyl)-ureido]phenylacetyl). The resulting collection of 8894 compounds was searched using the pharmacophore, and 12 compounds were selected based on fit, availability, and ease of synthesis. Of these compound 2 displayed inhibitory potency (1.3 nM) nearly equal to that of the original lead (0.6 nM).

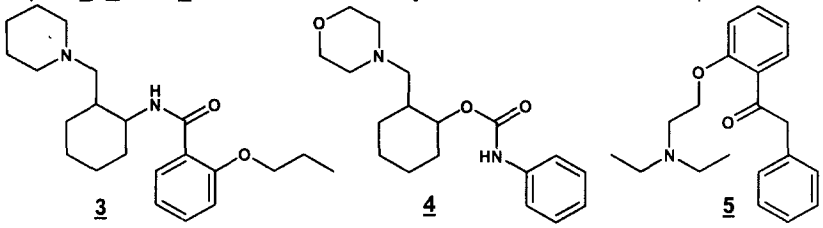


2

Structure-Activity Based Pharmacophore Identification – Transporters represent an important class of targets for a variety of therapeutic indications such as depression, anxiety, Parkinson's disease, and substance abuse. Specifically, the dopamine transporter (DAT) has been implicated in cocaine addition, and the search for novel inhibitors has benefited from pharmacophore queries based on known antagonists (14-17). From extensive structure-activity relationship (SAR) studies on cocaine and related analogs, a pharmacophore was constructed, which consists of sp^3 nitrogen required to be part of a ring, an aromatic ring, and a carbonyl group (14). A 3D search of the NCI database (18) of 206,876 compounds using the Chem-X program (19) found 4096 hits. Elimination of compounds with molecular weight > 1000, inappropriate ring N, and structural analogs resulted in the selection of 70 compounds for testing. Of these, 44 (63%) showed good activity in the primary binding assay. Further studies by these researchers (14-17) resulted in the development of a modified pharmacophore where the carbonyl group is replaced

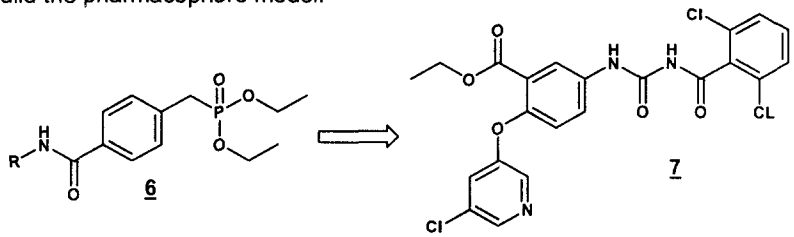
with an aromatic ring. Using a similar searching methodology, several novel classes of compounds based on 3,4-disubstituted pyrrolidines (15), substituted pyridines (16), and 2,3-disubstituted quinuclidines (17) have been reported for DAT inhibition.

GPCRs are an important class of targets for which no 3D structures currently exist. Novel leads are almost exclusively based on either modifications of natural ligands, or HTS of large databases. Based on a series of known muscarinic M_3 antagonists, two pharmacophore models were constructed using DISCO, and consists of a tertiary nitrogen, a hydrogen-bond acceptor, and two hydrogen-bond donor sites (20). The pharmacophore models were used to query the Astra corporate database using UNITY (21). These queries produced 177 unique hits. Subsequent testing of 172 available compounds yielded the three most potent examples **3**, **4**, and **5**, which are structurally distinct from the lead compounds.



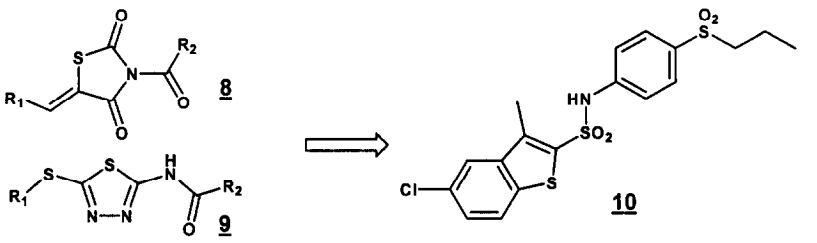
3 **4** **5**

Inhibitors of mesangial cell proliferation (MCP) are thought to be useful in the treatment of glomerular diseases such as diabetic nephropathy and lupus (22). Kurogi and co-workers (22) discovered a novel series of MCP inhibitors through construction of a pharmacophore from four benzylphosphonate compounds (represented by **6**) with significant MCP inhibitory potency. The Catalyst pharmacophore consisted of two aromatic rings, two hydrophobic sites, and three hydrogen-bond acceptor sites. A search of the Maybridge database (23) of 47,045 compounds gave rise to 41 hits. Four of the best fitting compounds were tested for MCP inhibitory activity and demonstrated potency comparable to compounds in the original training set (exemplified by **7**). Additionally, the new lead compounds were devoid of the cell toxicity present in the original series of compounds that were used to build the pharmacophore model.

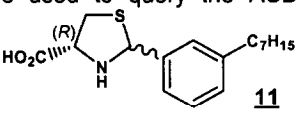


6 **7**

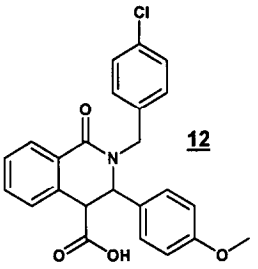
Nearly all known chymase inhibitors suffer from poor stability *in vivo*, which will tend to lessen their potential usefulness as therapeutic agents (24). Efforts to improve the stability of a series of thiazolidinedione (**8**) and thiadiazole (**9**) chymase inhibitors proved difficult without sacrificing potency (24). Identification of a new class of inhibitors followed construction of a pharmacophore based on 26 compounds. The pharmacophore consisted of two hydrogen-bond acceptors flanked on each end by hydrophobic groups. A search of the ACD collection of compounds with Catalyst identified a number of hits, 45 of which were selected for testing. Three of the selected compounds showed potency >30% at 1 μM in the primary assay. Compound **10** showed 100% stability and an IC_{50} of 909 nM against chymase.



A common theme in the development of novel therapeutic agents is the replacement of problematic functionality. Sphingosine-1-phosphate (S1P) is a phosphoric acid containing lipid that is involved in signaling endothelial differentiation gene (EDG) receptors (25). Koide and co-workers (25) created a pharmacophore using Catalyst that was based on a series of conformers of S1P. The features consisted of a negative and a positive ionizable group, hydrogen-bond acceptor, hydrophobic group, and a shape-based constraint designed to mimic the lipid tail of S1P. The twenty pharmacophores were used to query the ACD database, resulting in 58 hits. Testing of 32 samples revealed two compounds based on a thiazolidine carboxylic acid motif with greater than 30% inhibition at 10 μM. Further optimization of this series afforded compound **11**, which is selective for the EDG-3 receptor subtype.



The discovery of novel noncompetitive AMPA antagonists may provide treatment for patients with severe epilepsy (26). Currently, there are three major classes of noncompetitive AMPA antagonists known, represented by 2,3-benzodiazepines, phthalazine, and quinazolines. Barreca and co-workers used published data on fourteen compounds to construct a pharmacophore with Catalyst that contains two hydrophobic groups, one hydrogen-bond acceptor, and one aromatic ring feature (26). The pharmacophore was used to query the Maybridge database, which, after filtering, resulted in 200 compounds for further consideration. A total of eight compounds were ultimately selected for testing in an anticonvulsant assay. Of these, compound **12** displayed activity comparable to that of the compounds used to construct the pharmacophore model, and represents a novel class of noncompetitive AMPA antagonists that would be suitable for lead optimization.



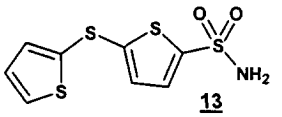
USE OF RECEPTOR SITE INFORMATION

The availability of crystallographic or NMR information regarding the active site of interest introduces the possibility of using fast docking methods for compound selection or filtering of virtual combinatorial libraries. The various algorithms for generating possible binding modes have been reviewed recently (27), and the technical details will not be repeated here. Currently, the most popular programs for high-throughput ligand docking are DOCK (28), FlexX (29), and Gold (30). These tools apply diverse technologies to the problem, *i.e.*, rapid shape matching, incremental construction, and genetic algorithms, respectively. While most of the prominent tools for docking are adept at finding appropriate poses for a given ligand, considerable issues remain for rank-ordering a variety of possible ligands (31,32). Many researchers have come to rely upon consensus scoring methods to aid in the actual compound selection (33-36). Alternatively, one may derive 3D database

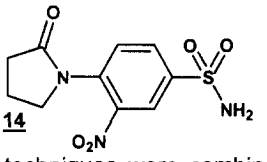
queries from active-site features and steric constraints for use with programs such as Unity.

Sequential filters – Most virtual screening experiments that utilize active site information involve several stages of increasing complexity; large compound collections or virtual combinatorial libraries can be pre-filtered for desirable molecular properties or pharmacophoric elements prior to the application of sophisticated docking techniques.

Inhibition of carbonic anhydrase (CA) remains a target for the treatment of glaucoma, and several X-ray structures of potent inhibitors are available. The consistency in the binding site features attracted Grüneberg *et al.* to apply sequential filtering techniques to find novel leads in this mature therapeutic area (37). Significant pre-evaluation of the active site features included consideration of displaceable waters and receptor site probing for favorable areas of interaction. The search database consisted of *ca.* 100,000 compounds from the Maybridge and LeadQuest (38) compound collections; known CA inhibitors were included for validation. The database was first filtered for rule-of-five compliance (39) and the presence of precedented zinc-binding groups, using a 2D Unity search, with 5904 compounds passing these filters. The results of the receptor site probing were then combined with features of known inhibitors to define pharmacophoric centers. The derived Unity 3D query included two acceptors, one donor, and adjacent hydrophobe spheres to approximate an elliptical shape. The flexible database search retrieved 3314 compounds, which were ranked vs. two known inhibitors using computed similarity and volume superimposition to approximate active site volume. The 100 best-ranked hits were then docked into the active site using FlexX. Visual inspection and consideration of the various scores were used to select 13 compounds for biological testing *via* a photometric assay. Three of the compounds were subnanomolar (*e.g.*, **13** and **14**), one is nanomolar, and seven are micromolar inhibitors; although all of the hits are of the well-precedented sulfonamide class, they are not covered by existing patents.

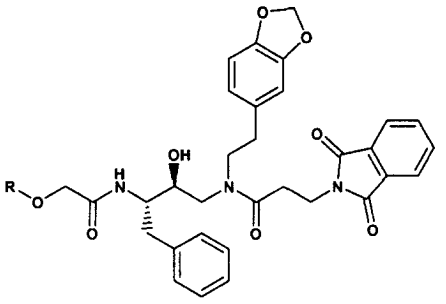


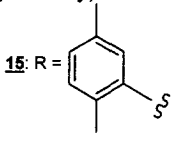
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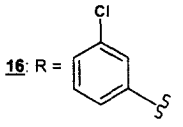


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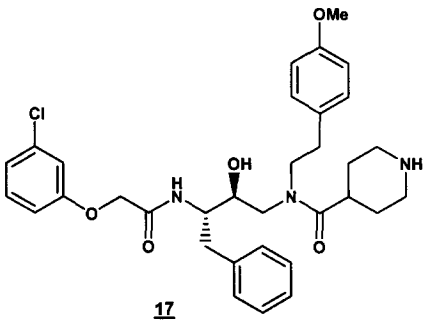
Structure-aided library design – A variety of techniques were combined in library design efforts to identify leads for the malarial aspartyl protease plasmepsin II (Plm II) (40). Owing to a 35% sequence similarity to Cathepsin D, a library previously designed for Cathepsin D was screened for Plm II activity. Of 1039 compounds, 13 showed >50% inhibition at 1μM in a fluorogenic peptide substrate assay. Compounds **15** and **16** were resynthesized in purer form and were shown to be submicromolar inhibitors of Plm II (300 and 220 nM, respectively).







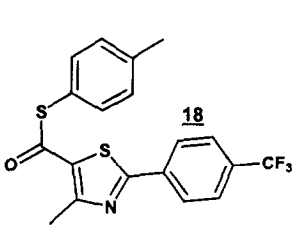
Six iterations of library design were conducted to optimize these leads, using three sidechain variation sites and two methods of monomer selection. For each optimization site, the selected reagents from the ACD collection were first filtered for synthetic considerations, molecular weight, and acceptable functionalities. One mode of sidechain selection was based on diversity metrics and hierarchical clustering. Alternatively, the scaffold was anchored in the active site of the X-ray crystal structure of Plm II (from a complex with pepstatin), and the monomers were "grown" into available space by iterative attachment of their constitutive fragments, with consideration of preferred torsions during the growth process. At each layer of growth, the existing portions were minimized and ranked, with the 25 top-scoring pieces advancing to the next growth stage. At the conclusion of growth, the best-scoring pose of each molecule was saved for comparison to others. The best compounds were evaluated for conformational accessibility and hydrogen-bonding potential; a subset of these were selected for hierachical clustering, and the best-scoring compound from each cluster was selected. Improvements in activity were observed throughout the library iterations, with SAR that could be rationalized in terms of the modeled structures. This iterative procedure eventually produced **17**, with a K_i of 4.3 nM, 15-fold selectivity over Cathepsin D, and significant improvements in molecular weight and ClogP. One notable complication was the difficulty in modeling sidechains in the tight S1' and S2' subsites. The authors presumed some induced fit capability on the part of the enzyme, which was substantiated by a subsequent X-ray structure of Plm II with one of the potent analogues.



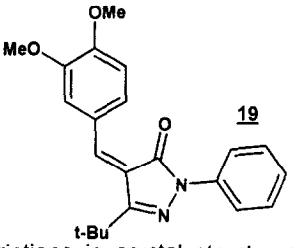
17

Incorporating active site flexibility – The previous two examples highlight the value of using multiple active sites. In the case of carbonic anhydrase, multiple structures were available to validate the consistency of the active site, while library design for Plm II was complicated by the lack of sufficient information at the outset. In the following examples, investigators were able to use structural information to effectively increase the number of valid binding possibilities.

Schapira, *et al.* incorporated a generalized hypothesis for nuclear hormone receptor antagonism as a starting point for the identification of novel ligands for retinoic acid receptor-α (RARα) (41). Using the X-ray structure of the ligand-binding domain of the estrogen receptor-α (ERα) complexed with the antagonist tamoxifen as a guide, the authors repositioned the C-terminal helix of the RARα structure to create an antagonist-appropriate active site. A grid potential representation of the binding site was then used for a flexible ligand search of the ACD (153,000 compounds) using the program MolSoft (42). A generous scoring cutoff was used to select over 700 of these hits for minimization within the intact active site, with receptor side-chain and ligand flexibility included. Of the 500 top-scoring hits from this optimization step, 32 were selected *via* inspection for biological testing. Two novel antagonists, **18** and **19**, showed inhibitory activity of 55% and 33% at 20μM in an *in vitro* screen. The authors relied upon intuitive inspection of the results (*i.e.*, retention of a particular hydrogen-bonding pattern and quality of fit) to guide their selection rather than reliance purely upon scoring functions.

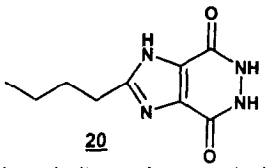


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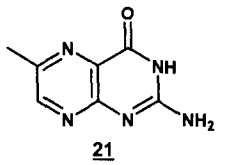


19

Researchers at Novo Nordisk used variations in crystal structures to identify novel inhibitors of tRNA-guanine transglycosylase (TGT) (43). A previously designed series of pyridazinedione compounds was used in traditional 2D substructure searches of the corporate database. All five compounds selected and screened for TGT inhibition were active; two showed K_is under 10μM. Attempts to dock these compounds into the high-resolution X-ray structure suggested that an important donor to the backbone carbonyl of Leu231 was absent. One of the analogs (**20**) was subsequently soaked into TGT crystals, and showed an unexpected backbone inversion wherein the NH of Ala232 is oriented into the binding site and the carbonyl of Leu231 is rotated out. A previously unobserved water molecule bridges the inhibitor to Ala232. The investigators devised a Unity database query that includes a donor to Leu231 or a donor/acceptor to the bridging water. Other consistently observed hydrogen bonding features were also included. Spatial tolerances for the features were derived from the results of active site mapping with the DrugScore program. Virtual screening was conducted on a combination of eight databases (totaling over 800,000 compounds), and was performed in stages of increasing complexity. Compounds with more than seven rotatable bonds and molecular weights over 450 were first eliminated. The remaining compounds were then filtered to assure the presence of the hydrogen-bonding features; the spatial requirements were subsequently introduced, and finally combined with excluded volume features to approximate the steric requirements of the active site. The resulting 856 hits comprised six chemical classes and represented the alternate binding features; 726 of the hits utilize the bridging water. Appropriate compounds were selected by inspection for docking using FlexX, followed by minimization. Only nine compounds were submitted for enzyme inhibition assays; five of these exhibited activities under 10μM. The most potent of these, **21**, has an inhibition constant of 250μM. Importantly, the predicted binding modes for the inhibitors have been used to rationalize the relative activities and are being applied in an optimization process.

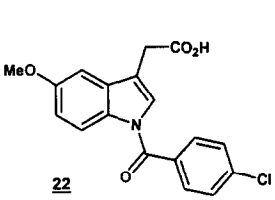


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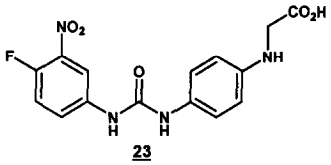


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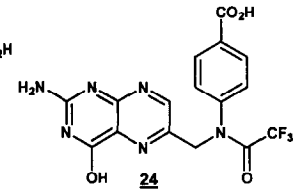
Exploiting multiple subsites – Iwata, *et al.* opted to use virtual screening to identify novel inhibitors of aldose reductase (AR) (44). The ternary complex of AR with NADPH and glucose-6-phosphate was used to calculate an active site potential energy grid, the output of which was used to search the ACD (*ca.* 120,000 compounds) with a proprietary program. Of 718 virtual hits, 179 were selected by inspection, and 36 were eventually purchased. Ten of the purchased compounds were considered active; three had IC50s below 10μM, and all comprise novel series. The three most active compounds are illustrated below (**22**,**23**, and **24**). Details of the proposed binding modes for the three series suggests non-overlapping features that could be merged in future designs. Several analogues were synthesized based on compound **22**, with SAR that is consistent with the predicted binding mode and included two analogues with IC_{50}s of 210 and 310 nM.



22

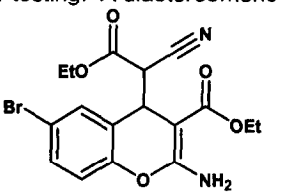


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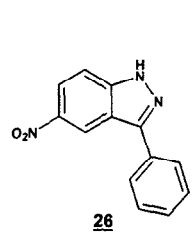
Virtual screening using homology models – Bcl-2 is a key regulator of apoptosis; in their search for cell-permeable chemical probes, Wang, *et al.* constructed a homology model of human Bcl-2 based on the X-ray and NMR structures of the related Bcl-x_L (47% sequence identity), and utilized the features of the essential BakBH3 binding pocket in a virtual screen of the ACD (45). The DOCK program was applied in a rigid ligand-docking mode, and the 1000 highest scoring results were minimized in the active site. Although interaction energies were computed, the investigators relied upon manual inspection for compounds with favorable shape complementarity and hydrogen bonding features. Of 53 compounds that met the selection criteria, 28 diverse compounds were acquired for testing. A diastereomeric mixture of **25** was identified in a fluorescence polarization assay as a competitive inhibitor with an IC50 of *ca.* 9 μM. Additional functional experiments demonstrated that **25** also induced cell death in HL-60 tumor cells in a dose dependent manner, with DNA fragmentation patterns characteristic of apoptotic cells. Apoptosis was shown to involve activation of caspases–9 and 3 in a pathway distinct from that of Fas/TNFr, and in a fashion dependent on Apaf-1.



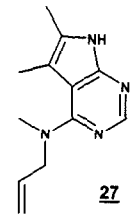
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Comparison to HTS – Truly direct comparisons between the results of high-throughput screening and virtual screening have not been published. In the final two examples, HTS was conducted on corporate collections, while the virtual screening experiments were carried out using databases of commercially available compounds.

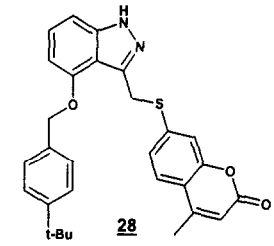
After HTS failed to produce non-quinolone or coumarin lead structures for inhibition of DNA gyrase, researchers at Hoffman-La Roche opted for virtual screening to identify alternatives (46). As the known antibacterial agents bind to the ATP site on the B subunit, the authors opted to use "needle screening" for small fragments (MW < 300). X-ray structures of the known inhibitors showed partially overlapping features, particularly two hydrogen bonds that were retained in the pharmacophore hypothesis. The programs LUDI (47) and Catalyst were used to search the ACD and part of the Roche collection (*ca.* 350,000 compounds). The LUDI search combined with the molecular weight requirement pared the list to about 200 compounds. The Catalyst search was alternatively used to increase the precision of the required pharmacophore, and resulted in selection of 400 compounds. Initially, these 600 compounds were tested in an assay configured to detect weak binding, and the results were used to select analogues of the first hits. A total of 3000 compounds were screened to find 150 hits that represented 14 structural classes. These hits were 2-3 orders of magnitude less effective than known inhibitors, but were validated by several biophysical methods, including X-ray crystal structures of DNA gyrase with hits such as **26** and **27**. These X-ray structures were vital in the optimization process in generating compounds such as **28**, which is 10-fold more potent than the known inhibitor novobiocin and is substantially less complex.



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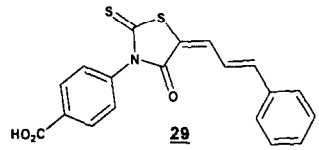


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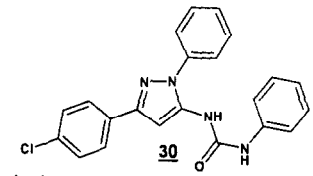


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Researchers at Pharmacia compared the results of an HTS of their corporate collection (400,000 molecules) against PTP-1B with 365 compounds that were found *via* virtual screening of 235,000 commercially available compounds (48). The DOCK program was used to select 1000 high-scoring compounds from the ACD, BioSpecs, and Maybridge collections. Selection for testing included compounds that spanned the two phosphotyrosine binding sites that are observed in X-ray crystal structures (49) and a comparable number of non-spanners. Of the 127 active compounds (IC_{50}s < 100μM), 21 compounds were < 10μM; of these there were 10 spanners and 11 non-spanners. Both charged (*e.g.*, **29**; 4.4 μM) and neutral (**30**; 12.0 μM) hits were represented.



29



30

In comparing the results of the HTS and virtual screens, the investigators note that not only was the hit-rate substantially higher for the virtual screen (of 400,000 compounds screened *via* HTS, 85 had IC50s < 100 μM; 6 compounds were < 10 μM), a significantly higher proportion passed a variety of filters for "druglikeness" (70% vs. 30%). The authors do emphasize that differences in the assay conditions may have been detrimental to the HTS results, although this does not diminish the value of the compounds found by virtual screening.

Conclusions – Virtual ligand screening with tools such as Catalyst, Unity, GOLD, FlexX, and DOCK provide the ability to select a relatively small number of compounds from large databases for testing. It is notable that most researchers rely heavily upon inspection and intuitive evaluation in addition to software results; in the case of active sites, most try to incorporate knowledge of site flexibility into their selection criteria. In many of the cited examples, the investigators chose a very small proportion of potential ligands for biological testing – frequently fewer than 50. It is likely that many of the best examples of the application of virtual screening have not yet appeared in the literature as they have spurred an optimization program of considerable proprietary value.

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Chapter 31. Enzyme Induction – Mechanisms, Assays, and Relevance to Drug Discovery and Development

David C. Evans*, Dylan P. Hartley, and Raymond Evers
Department of Drug Metabolism, Merck Research Laboratories,
Rahway, New Jersey, 07065, U.S.A.

Introduction - Clinically important pharmacokinetic drug interactions can occur when one drug alters the metabolism of a co-medication. Such interactions can be due to enzyme inhibition or induction (1). This review focuses on enzyme induction, which is an undesirable drug interaction, since this can result in reduced efficacy of the administered or co-administered drug and can have associated safety implications (2, 3, 4). While reference is made to enzyme induction in animal species, the main focus of this review is on enzyme induction in humans.

Up until the mid-1990's, studies to investigate enzyme induction were largely restricted to the cytochrome P450 (CYP) family of enzymes, underlining their important role in the Phase 1 metabolic clearance of pharmaceutical drugs. In particular, these studies have tended to focus on the up-regulation of CYP3A4, an enzyme responsible for the metabolism of ≥50% of marketed drugs (5, 6), and which comprises approximately 30% of the total CYP450 in human liver (7). The study of protein induction has evolved since to represent one of the fastest growing scientific disciplines. Indeed, that we can now refer to rifampicin as a "pleiotropic inducer of drug metabolism genes" (4), is testimony to the relatively recent advances made in understanding the biomolecular premise of gene induction, and in bioanalytical technology as it pertains to gene regulation. This has largely been achieved through high throughput screens to identify nuclear receptor ligands, RT-PCR (reverse transcriptase-polymerase chain reaction) to quantify mRNA induction, and cDNA microarrays to obtain gene "signature" responses for drugs.

When the induced CYP enzyme is primarily responsible for the metabolic clearance of the administered drug, values of systemic exposure are likely to fall upon repeat dose administration. This phenomenon is referred to as auto-induction. Interestingly, in a nonclinical FDA database, 11 of 35 compounds had a relative systemic exposure (RSE) of ≤ 1 (8); the RSE being the ratio of the rat plasma area under the plasma concentration-time curve (AUC) at the maximum tolerated dose to the human plasma AUC at the maximum recommended daily dose. Drugs are therefore clearly developable despite poor RSE margins, in one species at least. In developing such drugs, however, the complexity and time taken to undertake the toxicology and clinical programs can often serve as strong disincentives to continue with development. For example, for an auto-inducer undergoing non-clinical evaluation, having to demonstrate plateau in systemic exposure to both parent drug and its circulating metabolites in toxicology studies may well represent a required study to support drug registration. Implicit to this process is that the circulating metabolites are well characterized and available as synthetic standards for which toxicokinetic assays are validated. From a clinical viewpoint, (auto)-induction will only become apparent following repeat, dose-ranging, pharmacokinetic studies. These studies occur at a time when considerable financial investment has already been made in the drug and may, in some instances, result in reduced pharmacological efficacy.

These comments on the disadvantages of developing an enzyme inducer need to be balanced with the observation that the attrition of compounds as a consequence of

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enzyme induction in the clinic is small. In one organization, for example, induction as a reason for failure was <2% of drugs entering development during the approximate period, 1990 – 2000 (9). This may reflect a general trend in the pharmaceutical industry to develop compounds which are extremely potent, and which are able to exert their pharmacological effect(s) from low systemic concentrations of drug; the magnitude of the enzyme induction response generally being considered to increase with increased dose. Despite these observations, for a "first in class" drug there is always a great degree of initial uncertainty about what drug levels are required for clinical efficacy, and hence anticipation about whether, for a drug which is an enzyme inducer in vitro, enzyme induction in vivo will be encountered in patients. The calibration of second entry compounds tends to be more straightforward since the dose-to-exposure-to-efficacy relationship, driven by improvements in pharmacological potency and pharmacokinetic properties, is often better defined.

The overall conclusion from these deliberations is that it is reasonable to effort assays which aid in the selection of non-inducers for clinical development. These assays need to be undertaken early in order to impact decision making during structure-activity-relationship (SAR) optimization. In the event that medicinal chemists cannot abrogate the property of enzyme induction from their lead pharmacophores, then this property should not preclude further evaluation of the drug in clinical studies; this is especially true for a "first in class" drug. Indeed, the relevance, if any, of induction data in vitro, to the clinical environment, will be used to calibrate the back-up medicinal chemistry program.

This review presents information on the mechanism of nuclear receptor mediated gene regulation, the pharmacophore modelling of these receptors, and assays used to assess enzyme induction. Selected prototypic examples of induction from a "victim" perspective are also presented, since these provide perspective on the role of induction with respect to the loss of clinical efficacy and hence re-emergence of the disease state for which therapy was initially provided.

MECHANISM OF NUCLEAR RECEPTOR MEDIATED GENE REGULATION

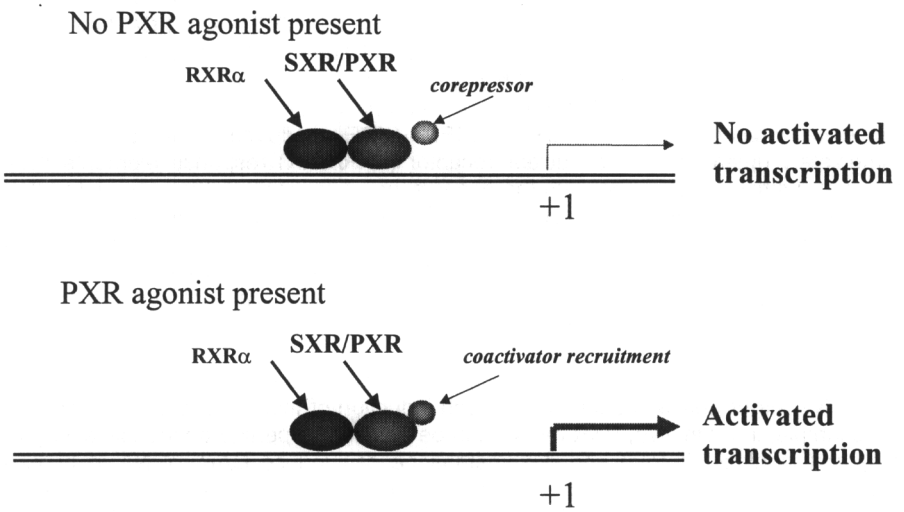
The role of the human orphan nuclear receptors, pregnane X receptor (hPXR, NR1I2), and constitutive androstane receptor (hCAR, NR1I3), in the regulation of target gene transcription has been reviewed extensively (6, 10-14) The aryl hydrocarbon receptor (AhR) has been similarly reviewed (15). While the AhR belongs to the basic helix-loop-helix/Per-Arnt-Sim family, not the nuclear hormone receptor family, it is described here for the sake of completeness since the AhR regulates members of both the CYP1A and glucuronyl transferase family of enzymes.

Both hPXR and hCAR belong to the same NR1I receptor subfamily and show high sequence homology to each other. They each contain DNA-binding domains (binding to DNA response elements, REs) and a ligand-binding domain (LBD). Upon ligand activation, both hPXR and hCAR form a heterodimer with the 9-cis retinoic acid receptor α (RXRα), wherein this complex binds to consensus DNA binding elements, ER-6 (everted repeat separated by six nucleotides) and DR-4 (direct repeat separated by four nucleotides), respectively, which are localized in specific gene promoters of nuclear hormone responsive genes, like CYP3A4 (10, 16, 17).

The vitamin D receptor (VDR) also belongs to the NR1I subfamily and has the second highest homology to hCAR in the ligand binding domain (18). The physiological ligand for VDR, from which this receptor derives its name, is 1α,25-dihydroxyvitamin D_3. hPXR has a wide variety of ligands, but bona fide ligands of hCAR are essentially limited to androstenol (5α-androst-16-en-3α-ol), and androstanol (5α-androstane-3α-ol), and these are considered inverse agonists (14). Although

members of the same nuclear receptor super family, hPXR and hCAR are believed to act through very different and complex mechanisms, both of which are not entirely understood. The hPXR is thought to reside in the nucleus, and in the absence of ligand is complexed with co-repressor proteins, which dissociate when PXR is bound by ligand (Figure 1). Although CAR has been coined a constitutively active receptor, this description was derived from the high ligand-independent "basal" activity of this receptor in various cell-based reporter assay, which is suppressed by the inverse agonists androstanol and androstenol (14). However, evidence suggests that, in vivo and in non-transfected cells, CAR is complexed in an inactive state in the cytoplasm, and is only translocated to the nucleus in the presence of a ligand or a non-CAR binding CAR-activator (*e.g.* phenobarbital) (19).

Figure 1. Schematic representation of PXR mediated transcription



Human PXR - Human PXR responds to a wide variety of drugs, xenobiotics and endogenous compounds, and plays a critical role in gene regulation and, thereafter, in potentially mediating drug-drug interactions in humans. It is expressed predominantly in the liver and small intestine – organs important in the processes of drug and bile acid metabolism (20). In addition to CYP3A4, other genes up-regulated by hPXR ligands include CYP2C8, CYP2C9, CYP2C19, CYP2B6, members of the glutathione S-transferase family, carboxylesterases, UDP-glucuronyl transferases, MDR1 (efflux transporter, P-glycoprotein), and the multidrug resistance protein, MRP2 (efflux transporter in the canalicular membrane of hepatocytes, and in the apical membranes of kidney proximal tubules and enterocytes), as well as genes critical to bile acid metabolism (6). hPXR functions as a PXR-RXRα heterodimer and can bind to specific sequences on the xenobiotic DNA response elements (PXRE) in the regulatory regions of many genes, notably CYP3A4. The PXR-RXRα heterodimer can bind members of the p160 / steroid receptor co-activator (SRC) family of transcriptional co-activators and, through direct interaction with other transcriptional co-activators, activate basal transcriptional machinery to up-regulate transcription (20) (Figure 1).

From the perspective of CYP3A4, the PXR response element is localized to a sequence in the CYP3A4 promotor (~ -153 to –170 bp), containing two copies of the nuclear receptor half site sequence AG(G/T)TCA organized as an everted repeat (ER) and separated by 6 base pairs, collectively termed the ER-6 motif. The distal CYP3A4 enhancer (~ -7800 bp upstream of the transcription start site) contains three elements

(termed dNR1, dNR2 and dNR3) capable of binding hPXR-RXRα heterodimers, in addition to two additional *cis*-acting elements, FP1 and FP2, immediately upstream of the latter PXR-binding regions which are essential for full responsiveness to transactivation by rifampicin (10, 21). Of note, the MDR1 gene has a regulatory cluster at approximately -8 kbp in its 5'-upstream regulatory region which contains a DR4 motif that serves as a locus for the binding of hPXR (22), thus providing a potential mechanism for how chemical structures can induce both CYP3A4 and P-glycoprotein. Known hPXR ligands, most of which are also known to induce CYP3A, include rifampicin, dexamethasone, St. John's Wort (hyperforin), lithocholic acid, SR12813 (16), clotrimazole, troglitazone, lovastatin, phenobarbital, coumestrol, RU-486, dexamethasone-t-butylacetate, paclitaxel (taxol), 5-β-pregnane-3,20-dione, 9-cis-retinoic acid, corticosterone (20), ecteinascidin, LGD1069 (targretin), nifedipine, carbamazepine, phenytoin, and sulfinpyrazone.

It has recently been reported that the orphan nuclear receptor, hepatocyte nuclear factor-4α (HNF-4α), is an important determinant of the hPXR- and hCAR-mediated transcriptional activation of CYP3A4 in a human cell line; for PXR and CAR also, in a mouse cell line (12). HNF-4α is most highly expressed in the liver and intestine, and is capable of binding to a putative response element containing a direct repeat (DR1) sequence located within the distal XREM (XRE modulator) site of CYP3A4, immediately upstream of two nuclear receptor (PXR/CAR) response elements (23). This observation is consistent with the observation of Jover et al. (24) who noted that in human hepatocytes transfected with HNF-4 antisense RNA, down regulation of CYP3A4 and CYP3A5 was observed. These workers have also recently published on the *down regulation* of CYP3A4 by the proinflammatory cytokine, interleukin 6 (25). The down-regulation of genes represents a relatively new area of research for which clinical relevance has yet to be defined.

An alternative mechanism whereby PXR- or CAR could elicit induction of their respective target genes could occur through induction of these receptors themselves. For example, in rat in vivo, PXR mRNA can be induced by perfluorodecanoic acid (10-fold) and isoniazid (8-fold) and dexamethasone can induce CAR mRNA in human hepatocytes providing a potential alternative mechanism, beyond nuclear receptor activation, by which gene products of PXR and CAR can be up-regulated (26, 27).

Human CAR (hCAR) - A distinguishing feature of hCAR is that it is liver enriched. Mouse CAR is considered to be a phenobarbital (PB) responsive transcription factor, but interestingly does not bind PB. Acting as a RXR heterodimer, it binds to the PB-responsive enhancer module to activate gene transcription (13). A question remaining is, therefore, "How is the constitutive activity of CAR suppressed in vivo so that CAR can be activated in response to inducers?" One potential clue comes from the limited SAR of the hCAR ligands (ibid), which is specific for 5α-reduced compounds with a 3α-hydroxy group. As a consequence of this observation, it has been speculated that production of a CAR inhibitor in vivo (in mouse at least) may require the activity of steroid 5α-reductase (14).

From a mechanism perspective, it has been shown that in mouse liver, CAR is localized in the cytoplasm. Following treatment with an inducer (*e.g.* phenobarbital), the protein translocates to the nucleus. The signals that have been proposed to be responsible for the cytoplasmic localization of CAR are a C-terminal LXXLXXL motif and a okadaic acid sensitive dephosphorylation step (13). Currently, it is not clear whether dephosphorylation of CAR itself or another factor induces translocation to the nucleus. Human and mouse CAR, when expressed in mouse liver, can translocate into the nucleus in response to PB or TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene) (13). In addition, upon PB treatment of cultured human hepatocytes, CAR translocation to the nucleus was reported (19).

The identification of CAR target genes in humans has been hampered by the lack of a specific hCAR agonist. Very recently, however, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime (CITCO) has been identified as a hCAR agonist (28). In three batches of human primary hepatocytes treated with CITCO or rifampicin, CYP2B6 was induced more efficiently by CITCO than by rifampicin, suggesting that this gene is more responsive to hCAR than hPXR.

It recently has also been shown that UGT1A1 is a target gene for both PXR and CAR. Moreover, several DR-3- and DR-4-like hCAR and hPXR responsive promoter elements approximately 3 kb upstream of the UGT1A1 transcription start site have been identified (29). Interestingly, it was shown that in mice transgenic for a constitutive form of hPXR, or wild-type hCAR, that both receptors are able to induce mouse UGT1A1 and increase the rate of clearance of bilirubin (29, 30). In neonatal mice and humans CAR expression is very low. It is therefore possible that a functional deficit of CAR expression may be a contributing factor in neonatal jaundice, but that residual CAR expression is sufficient to confer a response to phenobarbital treatment (30, 31).

Human glucocorticoid receptor (hGR) - At least two groups have proposed that GR controls the expression of hPXR, hCAR, and hRXRα, thus contributing indirectly to the inducible expression of many genes (32-34). The evidence to support this is that dexamethasone, a GR agonist, activates CYP3A4 via a GR-mediated increase in both hPXR and hRXRα. Similarly, the GR agonists dexamethasone, prednisolone and hydrocortisone have been observed to induce hCAR mRNA in human hepatocytes (33). The observation that the GR antagonists, RU486 and PCN, can induce CYP3A4 (35) can perhaps be rationalized by the observation that RU486 can activate the translocation of the GR from the cytoplasm to the nucleus in a mouse cell line (36).

Aryl Hydrocarbon Receptor (AhR) - A model of AhR action has been described wherein an inducing chemical enters the cell and binds with high affinity to the cytosolic AhR (15). The AhR is believed to exist as a multiprotein complex, containing two molecules of the chaperone protein hsp90 (a heat-shock protein of 90 kDa), the immunophilin-like X-associated protein-2 (XAP2), and a 23 kDa co-chaperone protein referred to as p23. Following ligand binding, the AhR is believed to undergo a conformation change that exposes a nuclear localization sequence(s) that results in translocation of the complex into the nucleus. Release of the ligand:AhR from this complex and its subsequent dimerization with a Ah nuclear translocator (Arnt) protein converts the AhR into its high affinity DNA binding form. Binding of the heteromeric ligand:AhR:Arnt complex to its specific DNA recognition site, the DRE, upstream of the CYP1A1 and other AhR-responsive genes, stimulate transcription of these genes.

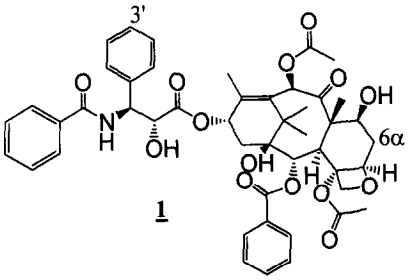
A physiological role of the AhR remains in question since, to date, no high affinity endogenous ligand has been identified. However, the ability of hydrodynamic shear stress conditions to induce CYP1A1 in vitro (37), and for hypoxia to induce CYP1A1 in rat lungs and liver in vivo (38, 39), are consistent with the formation of an endogenous AhR ligand. Interestingly, some compounds have been shown to induce AhR target genes but do not appear to competitively bind to the AhR. The underlying reasons for this are not clear, but include well known compounds such as omeprazole (40), and caffeine (41).

NUCLEAR RECEPTOR PHARMACOPHORE MODELLING

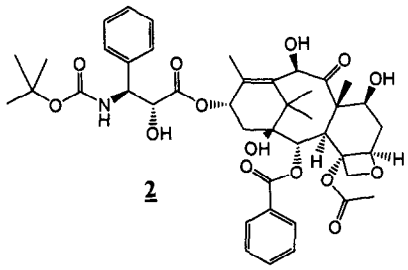
Human PXR (hPXR) - The structure of the hPXR-LBD has been resolved (16, 20). The hPXR-ligand binding domain is closely related in structure to the vitamin D receptor (VDR), sharing 45% sequence identity; less similarity is shared with RXR,

PPARγ, and the estrogen and progesterone receptors. It has been reported that the hPXR-LBD has evolved several structural features that permits it to function as a broad chemical "sensor". These features include a large elliptical ligand binding cavity (1150 $Å^3$) that offers few surface features to impact the steric selection of its ligands. This represents a potential problem since there may well not be a defined SAR for hPXR within any one chemical template. This highlights the importance of having structural template diversity for all basic research programs. It should be noted, however, that while paclitaxel (1) is a reasonably potent activator of hPXR, with commensurate induction CYP3A4, CYP2C8 and MDR1 in LS180 cells, its close structural analog, docetaxel (2), is not (42); highlighting some potential at least for hPXR SAR to emerge in the future.

Other features of hPXR include a flexible binding cavity which can accommodate ligands in more than one orientation (three were observed for SR12813), and can bind ligands which are both large (taxol, 854 Da; rifampicin, 823 Da) and small (phenobarbital, 232 Da). Mutation of Asp^{205} resulted in a marked decrease in basal



1



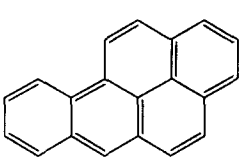
2

transcriptional activity of hPXR, highlighting this to be an important determinant of activity. Of the three energetically favorable conformations in which SR12813 binds, only one of twenty eight amino acid residues involved in ligand contact, Phe^{288}, interacted with SR12813 in all three conformations highlighting, in addition to Asp^{205}, the importance of this residue. The most potent ligands defined to date include a constituent in the St. John's Wort herbal antidepressant, hyperforin (Kd = 27 nM, 514 Da), and the hypocholesterolemic compound, SR12813 (Kd = 41 nM, 505 Da) (20, 43).

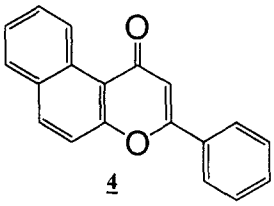
Using literature data for EC_{50} values for 12 hPXR ligands a pharmacophore model for hPXR has been developed (44). This pharmacophore was also used to predict the binding affinity for 28 molecules not in the model but known to be hPXR ligands of differing potencies. The pharmacophore distinguished the most potent activators of hPXR (that display >5-fold activation/deactivation), like ecteinascidin, troglitazone, nifedipine, and dexamethasone-t-butylacetate, from poor activators, such as scopoletin and kaempferol. Such a model may offer insights into the SAR of hPXR ligands as they strive to synthesize compounds, or alternative chemical templates, which are devoid of this property.

Human CAR (hCAR) - Several groups have reported on structural comparisons between hCAR and hPXR (18, 43, 45). A 3D model of the ligand binding domain of the hCAR was constructed based on the available X-ray structures of hPXR and VDR (18). The model shows that the size of the ligand binding cavities of hCAR and hPXR are similar, but larger than that of VDR. However, in contrast to hPXR which can bind extremely large ligands such as rifampicin through the flexibility of a surface loop, hCAR would only be expected to bind the smaller hPXR ligands. In support of this observation, it has been reported that helices 6 and 7 provide the walls of the ligand binding pocket in hCAR, but its Helix1-Helix3 insert is too short to reach into the volume occupied by the analogous region in hPXR (43).

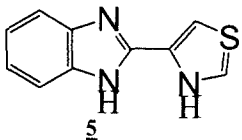
Aryl Hydrocarbon Receptor (AhR) - The AhR ligand binding pocket can bind planar ligands with maximal dimensions of 14 x 12 x 5 Å which have certain thermodynamic and electronic properties characterized by molecules such as benzo(a)pyrene (**3**) and β-naphthoflavone (**4**) (46-48). By using an AhR-reporter gene assay based on green fluorescent protein (49), a more diverse range of ligands has now been identified, characterized by thiabendazole (**5**) and 5-methyl-2-phenylindole; other substrates are summarized in detail elsewhere (15).



3



4



5

ASSAYS TO MONITOR FOR ENZYME INDUCTION IN VITRO

Our current understanding of the mechanisms of gene regulation offers opportunities to develop assays to evaluate each step of the gene regulation cascade. These include nuclear receptor, mRNA, protein, or enzyme activity assays, and examples of each are either referenced or described below. An enzyme induction assay based on human hepatocyte preparations using enzyme activity as the readout is still considered by many to be the assay of "best practice". An assay based on apoprotein detection is semi-quantitative, and those based on mRNA are open to interpretation because of the potential for post-transcriptional modifications which may result in a lack of correlation with either apoprotein or enzyme activity.

Assays based on nuclear receptors offer the advantage of high throughput in order to develop SAR. The other assays described provide information on specific gene products, such as CYP3A4. Increasingly, however, scientists want to derive a characteristic "signature" gene response which is prototypic of, for instance, a hPXR activator. This is important since, historically, scientists have been "CYP3A4 centric". Nowadays, the totality of gene responses can be considered in the context of potential implications for interactions between co-administered medications. These higher throughput assays also allow the use of human hepatocytes for induction studies to be limited to those compounds of real interest.

The technology of cDNA microarrays allows the investigator to monitor for changes of gene expression on a relatively massive scale; typically 25 - 50 K oligonucleotide probes or cDNA fragments at one time (50, 51). Such experiments are likely to be undertaken in the context of attempting to understand a drug-drug interaction or a specific toxicology. In the context of a drug-drug interaction, once potential associations between selected gene sets and proteins involved in the disposition of the co-administered drugs have been identified, then assays to quantify induction of specific genes is undertaken and will typically be performed using a technique such as quantitative RT-PCR (*e.g.* TaqMan, Applied Biosystems).

To perform microarray and Taqman studies requires the availability of mRNA from living cells which have been challenged with a drug or test compound (52, 53). The gut and liver can be considered important organs in which to monitor for protein induction since these organs modulate exposure to parent compound via the action of

drug transporters and drug metabolizing proteins. In vitro induction studies are therefore undertaken in fresh and cryopreserved human hepatocytes, and in human liver slices (54, 55). Hepatocytes are often in limited supply, and the additional issue of inter-preparation/individual variability have, in addition, made these assays less attractive as a primary screen.

Preparing primary enterocytes is relatively routine, but reliable protocols for plating them for subsequent drug challenge during the course of undertaking an induction study is not trivial (56). The availability of gut cell lines represent one alternative, but it should be noted that Caco-2 cells, for instance, express the VDR, but not the PXR (57). Induction of the CYP3A4 gene in these cells is therefore controlled by the binding of the VDR-RXR heterodimer to the CYP3A PXR response elements (58). An alternative to using Caco-2 cells is the immortalized human colon carcinoma LS180 cell line which contains both the VDR and PXR (42, 58). Induction of both CYP3A4 and Pgp activity by the hPXR ligand, paclitaxel, in this cell line, has been reported (42).

While there are several in vitro systems in which to evaluate enzyme induction, the main problem lies with interpretation of data in the context of clinical relevance. Factors such as, potency at the nuclear receptor, dose level (exposure), duration of dosing, free versus bound concentrations of drug, the effect of drug transporters on cell concentrations, all potentially confound interpretation of data. Clearly the role of in vitro induction assays is to provide choice; compounds with low potential to induce enzymes being favored over those which do not, all other considerations being equal.

Induction Studies in Human Hepatocytes - Hepatocytes are cultured on an extracellular matrix (*e.g.* Matrigel® or collagen) for a period of 48 - 72 hr and then challenged with drug. It is common, although not essential, to overlay hepatocytes in culture with extracellular matrix in order to recapitulate a cytoarchitecture synonymous with that encountered physiologically. Drug challenge is typically for at least 48 hr if protein levels and enzyme activities are to be assayed. The appropriate controls to run include a positive control (*e.g.* 10 μM rifampicin), and a vehicle control (*e.g.* methanol, acetonitrile or dimethylsulfoxide; all < 1%). Induction of enzyme activity is typically performed with midazolam or testosterone for CYP3A4, caffeine, theophylline, or phenacetin for CYP1A, and S-warfarin, diclofenac, or tolbutamide for CYP2C9. For compounds which are inhibitors of enzyme activity, at least in vitro, then assessment of alternative measures of enzyme induction (*e.g.* mRNA or apoprotein) are recommended.

Nuclear receptor reporter gene assay - A hPXR reporter gene assay based on the transient transfection of HepG2 cells with a full length PXR construct and a reporter plasmid consisting of the CYP3A-5′ flanking region linked to the luciferase gene has been developed (59). These workers then evaluated 14 drugs for PXR activation and for their ability to induce CYP3A4 in cultured primary human hepatocytes (using testosterone 6β-hydroxylase (T6βH), CYP3A4 mRNA, and protein assays). The overall correlation (not considering the CYP3A4 inhibitors, ritonavir and troleandomycin) between hPXR activation and CYP3A4 activity (T6βH) was 0.864 ($P < 0.001$). An important component of this analysis was variability of response. For the positive control, rifampicin, the magnitude of the induction over solvent control, based on enzyme activity, was 2- to 10-fold. In contrast, the hPXR reporter gene assay showed less variation, 2 μM rifampicin providing a 21- to 25-fold increase over control in three separate experiments. In recognizing that hPXR activation was not the only means by which CYP3A4 induction occurs (vide infra), these workers suggested that enzyme induction should be evaluated in more than one experimental system.

hPXR Scintillation proximity assay (60) - In this assay hPXR was immobilized on a scintillant-containing bead and then incubated with the radioligand, [^{3}H]SR12813, a

potent activator of hPXR (Kd 41 nM) which has been shown to efficiently induce CYP3A gene expression in human hepatocytes. The binding of non-radioactive ligands was measured as a function of their ability to compete with the radioligand for binding to hPXR. Since this assay does not require separation of bound from free ligand, this assay is amenable to high throughput screening.

CYP3A4 reporter gene assay - One group has investigated the effect of 17 xenobiotics on an approximately 1 kilobase pair fragment of the CYP3A4 proximal promoter, cloned upstream of the secretory placental alkaline phosphatase gene, which was then transfected into the HepG2 cell line (61). They also evaluated a smaller region of the proximal promotor covering the first 301 bp and determined it to be sufficient to act as a basal transcription unit, and to be able to mediate induction of the CYP3A4 reporter gene by compounds previously shown to activate the 1105 bp proximal promotor reporter (35). These workers used a four point concentration response curve (1, 10, 30 and 50 μM) to calculate maximal induction (I_{max}) and EC_{50} values, where the ratio I_{max}/EC_{50} (the "overall inductive ability" (IA)) was representative of intrinsic transcriptional activation (61). It was concluded that this in vitro model is capable of identifying CYP3A4 transcriptional inducers and yields an IA value allowing the ranking of compounds for their overall ability to induce CYP3A4 transcription. It would be of interest also to determine the effect of free drug concentration on the IA in an expansion of the equation which describes the activation of a reporter gene by a compound that follows the law of mass action for binding of a ligand to its receptor (61):

$$\text{Effect} = \frac{I_{max} * \text{Free Drug Concentration}}{EC_{50} + \text{Free Drug Concentration}}$$

CLINICAL EXAMPLES OF ENZYME INDUCTION - MEASUREMENT, TIME OF ONSET, AND TYPES OF PROTEIN INDUCED

This section provides some clinical perspective on enzyme induction. Assessing enzyme induction at any defined dose in the clinic is relatively straightforward. The challenge of undertaking these studies is to perform them at the correct dose; namely the pharmacologically relevant dose to be used in chronic therapy.

There is little data on the rate of onset of induction in humans. Data are provided below for CYP3A4, but few, if any, additional examples exist. To our knowledge, only one report has appeared showing the rate of recovery of mRNA levels for gut CYP3A to baseline following oral dosing of rifampicin (62). This group reported that CYP3A mRNA levels in gut biopsies returned to normal 3 days after the last rifampicin dose. Lastly, examples of proteins induced other than CYP3A are presented. This is to underline the scope, beyond CYP3A, to which enzymes can be induced in humans.

Clinical Assays for CYP3A Induction - Measuring the urinary 6β-hydroxycortisol to cortisol ratio (6β-OHC/C) has been reported to be an effective and efficient method for evaluating the potential of investigational agents to induce CYP3A4 (63, 64). Disadvantages of using this ratio as a marker of endogenous CYP3A activity are reported as including variability of the response due to stress or circadian rhythm, and daily interindividual variability (65). Despite these limitations, this assay has been used to characterize rifampicin, antipyrine, phenobarbital, troglitazone, phenytoin, and carbamazepine as CYP3A inducers in clinical studies (Table 1). Midazolam has likewise been used to probe for CYP3A induction in the clinic, where the magnitude of the response is typically 10-fold higher than that observed for the 6β-OHC/C ratio. Regardless of the specific probe substrate used, there are benefits to standardizing on a single probe substrate to calibrate enzyme inducers in the clinic as being either a

"strong", "medium" or "weak" inducer. This will allow potential prospective liabilities related to the property of enzyme induction to be perceived more readily.

Rate of Onset of CYP3A Induction - In human subjects receiving 200 mg phenytoin every 8 hr for 11 doses, and 100 mg every 8 hr for 8 doses, a rapid increase in 6β-hydroxycortisol excretion in urine was observed (66). Urine samples were collected each morning on days –1 through +7, and urinary concentrations of 6β-hydroxycortisol and cortisol were determined. The baseline 6β-OHC/C ratio on day –1 was 5.05. Ratios on days 4 (10.4), 5 (10.8), and 7 (12.0) were significantly higher than baseline, and the value on day 3 (7.56) almost reached significance. These data indicate that phenytoin mediated elevations in CYP3A activity in humans were apparent by day 3.

Table 1. Urinary Excretion of 6β-Hydroxycortisol as a Marker of CYP3A Induction in Humans

Compound	Oral Dose (mg)	% Increase in 6β-OHC Excretion/day	% Increase in Urinary 6β-OHC/C[1]	Reference
Rifampicin	600 mg, qd, 14 days	327	N.R.	97
	600 mg, qd, 14 days	268	271	98
	1200 mg, qd, 14 days	416	490	
Antipyrine	1000 mg, qd, 14 days	66	90	98
	1200 mg, qd, 14 days	93	107	
Phenobarbital	100 mg, qd, 14 days	105	135	98
Troglitazone	400 mg, qd, 11 days	103	118	99
Phenytoin	200 mg every 8 hr for 11 doses, then 100 mg every 8 hr for 8 doses	N.R.	237	66
Carbamazepine	200 mg, bid, 5 weeks	250	N. R.	100
Eletriptan	40 mg tid or 80 mg bid for 7 days	N.R.	N. S. I.	73
Pioglitazone	45 mg, qd, 14 days	N.S.I.	N.S.I.	65

N.S.I. - no significant increase
N.R. - not reported
[1] Ratio of 6β-hydroxycortisol (6β-OHC)/17-hydroxycorticosteroids or 6β-OHC/free cortisol

Induction of MDR1, MRP2 and UDP-Glucuronyl Transferase in Humans - Rifampicin (600 mg, P.O. once daily, typically for 14 days) has been shown to induce P-glycoprotein (MDR1) (67), MRP2 (68), and UDP-glucuronyl transferase (UGT) (69, 70) in humans. Enzyme induction has been assessed in gut tissue in human volunteers undergoing esophagogastroduodenoscopy pre-dose and after dose cessation (67). The level of P-glycoprotein induction in gut biopsies, as defined by Western-blot analysis, was 3.5-fold over control (67). The extent of this induction was strongly correlated with digoxin clearance (administered as a 1 mg intravenous infusion over 30 minutes). Duodenal MRP2 mRNA was elevated in 14/16 patients; elevations in MRP2 protein levels were detected in 10/16 patients (68). It was concluded that the increased elimination of MRP2 substrates (*e.g.* drug conjugates) into the lumen of the gastrointestinal tract could represent a new mechanism of drug interactions (68). Both rifampicin and phenytoin (300 mg P.O., once daily for at least 2 weeks) were also shown to increase the paracetamol glucuronide to paracetamol ratio from 18 ± 5 in healthy volunteers who were all non-smokers without medication, to 41 ± 11 and 35 ± 7, respectively, in medicated patients. It was concluded that monitoring the paracetamol glucuronide to paracetamol ratio in urine may be a useful tool for evaluating UDP glucuronyl transferase induction in humans (69).

DECISION MAKING IN ENZYME INDUCTION STUDIES

As has been alluded to earlier, the main problem associated with all studies conducted in vitro is defining their in vivo relevance. Rifampicin (71) and troglitazone (72) induce CYP3A4, both in the clinic and in vitro in human hepatocytes, whereas both eletriptan (73) and omeprazole (74) can induce CYP3A and CYP1A, respectively, in vitro, but this had no clinical relevance at the doses used therapeutically (Table 2). Interestingly, when the clinical dose of omeprazole was increased from a standard 40 mg dose to a dose of 120 mg (75), CYP1A induction was demonstrated, as defined by the caffeine plasma index (the ratio of concentrations of paraxanthine to caffeine). This highlights the importance of dose level on mediating enzyme induction in vivo.

Troglitazone can reduce the plasma concentrations of drugs that are substrates of CYP3A4 and/or active transport such as terfenadine, cyclosporin, atorvastatin and fexofenadine (76). The high values of plasma C_{max} (median value ~5 μM) and AUC (median value ~50 μM.hr) determined for troglitazone, values which are not corrected for plasma protein binding, appear to distinguish it from the relatively low values of C_{max} and AUC obtained for its close structural analog, rosiglitazone (C_{max} ~0.35 μM, AUC 2.2 μM.hr), and also from eletriptan (C_{max} ~0.6 μM, AUC 3.9 μM.hr) (Table 2), neither of which are regarded as enzyme inducers in vivo at therapeutic dosages. Correcting plasma concentrations of drug for plasma protein binding removed this distinction; values of C_{max} and AUC for troglitazone after accounting for plasma protein binding being relatively low and indistinguishable from those values observed for rosiglitazone and eletriptan (Table 2). While it is difficult to conceive mechanistically of a situation where free drug is not the governing force which drives an induction response, in the absence of being able to define what concentration of drug is responsible for initiating the induction event in tissues, we conclude that it is better to use concentrations of drug in plasma, uncorrected for plasma protein binding, as a conservative approach to evaluating the potential for enzyme induction in vivo.

Table 2. A Comparison of Enzyme Induction In Vitro Versus In Vivo – Effect of C_{max} and AUC at Doses Used Clinically

Compound (Molecular weight)	Putative Enzyme Induced	Enzyme Inducer In Vivo?	CYP3A Inducer In Vitro?	Dose (mg)	fu	Total C_{max} (μM)	fu C_{max} (μM)	Total AUC (μM.hr)	fu AUC (μM.hr)	Reference
Rifampicin (823 Da)	CYP3A	Yes	Yes	600	0.30	8 - 12	2 - 4	N.R.	N.R.	101
Troglitazone (442 Da)	CYP3A	Yes	Yes	200 - 600	0.01	0.8 -10	0.01 - 0.1	7.7 - 92	0.08 - 0.9	72
Dexamethasone (392 Da)	CYP3A	Yes	Yes	12	0.3	0.2 - 0.5	0.06 - 0.15	N.R.	N.R.	101
Phenytoin (252 Da)	CYP3A	Yes	Yes	100 -200	0.09	40 - 79	3.6 - 7.1	>100	>9	66, 101
Carbamazepine (236 Da)	CYP3A	Yes	Yes	200	0.50	20 - 40	10 - 20	N.R.	N.R.	101
Eletriptan (382Da)	CYP3A	No	Yes	80	0.24	0.6	0.14	3.9	0.93	73, 103-105
Rosiglitazone (357 Da)	CYP3A	No	N.R.	2 - 8	n.d.	0.3 - 0.4	N.R.	2.2	N.R.	101, 102
Omeprazole (345 Da)	CYP1A	No	Yes	40	0.05	3.9	0.2	8.7	0.44	74

fu, fraction unbound in plasma; C_{max}, maximum observed concentration of drug in plasma; fu C_{max}, C_{max} corrected for plasma protein binding to reflect fraction unbound; AUC, area under the plasma concentration-time curve; fu AUC, AUC corrected for plasma protein binding to reflect fraction unbound; N.R. – data are not reported

In having an understanding of the expected exposures of drug required for clinical efficacy, in addition to an understanding of "Inductive Ability" in vitro (ibid), it is tempting to conclude that certain approximations as to the risk of encountering enzyme

induction in the clinic can potentially be made. For example, the hPXR EC_{50} values (P, Potency) for rifampicin and troglitazone are approximately 0.7 and 0.5 μM, respectively (44). When these values are considered in the context of clinical C_{max} and AUC (E, Exposure) (Table 2), then values of E/P for these compounds are ≥ 10 for C_{max} and ≥ 100 for AUC. This is certainly an oversimplified analysis since the contribution of drug metabolites to induction, and induction of proteins through mechanisms not associated with hPXR, need to be considered. Implicit in this simple analysis, however, is that targeting E/P ratios <10 for C_{max}, or <100 for AUC, could represent a reasonable goal when considering the potential induction liability of a lead compound. Pragmatically, however, such imprecise analyses are unlikely to impact decision making on advancing a compound for more thorough evaluation in the clinic.

ENZYME INDUCTION IN DIFFERENT TISSUES

The mechanism(s) underlying why enzymes are induced in certain tissues and not in others has not been extensively studied. In studies performed in mice, PXR regulated a number of genes in small intestine that were not regulated by CAR, and CAR stimulated the expression of several genes in liver that were not regulated by PXR (77). PXR and CAR have overlapping but distinct biological functions, but each may play more dominant roles in xenobiotic metabolism in small intestine and liver, respectively. PXR and CYP3A have been observed to be co-localized in the liver, small intestine and colon of human, rabbit, rat, and mouse (6). In rodents, lower levels of PXR mRNA have also been detected in kidney, stomach, lung, uterus, ovary and placenta. In human, PXR mRNA has also been detected in both normal and neoplastic breast tissue (6). From our current understanding of the gene induction process, host cell environment (*e.g.* transcription factors) and physiological factors (*e.g.* pregnancy) are likely to play important roles. On this latter point, PXR expression in the mouse liver and ovary was increased ~50-fold during pregnancy (78). The expression of CYP enzymes in extrahepatic tissue was reviewed recently (79).

SPECIES DIFFERENCES IN ENZYME INDUCTION

It is well known that species differences exist in respect of gene response(s) to pharmaceutical agents. For instance, rat does not readily respond to rifampicin and rabbit does not respond to pregnenolone 16α carbonitrile (PCN) (80), yet rifampicin can induce CYP3A in both rabbit (81, 82) and human (83, 84). By far the main determinant of species differences in response to PXR activators can be attributed to sequence differences in PXR ligand-binding domains. The PXR receptors from human, mouse, rat, and rabbit have been cloned and characterized (80). While they share approximately 95% identity in their DNA binding domains, they share only 75-80% identity in their amino acid sequences in the ligand-binding domain, hence showing that the LBD sets have diverged through evolution. This finding provides a molecular basis for the species differences in CYP3A induction observed in vivo. Interestingly, although the amino acid sequence identity between the human and rhesus LBD is 96%, differences are observed in activation potential for some compounds. Whereas rhesus PXR is activated by progesterone, PCN and dihydroepiandrosterone (DHEA), hPXR is activated by reserpine and the bile acids cholic acid and lithocholic acid (85).

There are also species differences in the downstream consequences of enzyme induction. Numerous phenobarbital-type inducers and peroxisomal proliferators are tumor promoting agents in rats, but not in humans. Moreover, prolonged treatment of humans with the anticonvulsants, phenobarbital and phenytoin (human CYP3A4 inducers), does not lead to liver or thyroid tumor formation in humans. Elevation of thyroid stimulating hormone (TSH) in humans does not lead to tumor formation but causes goiter, a reversible enlargement of the thyroid gland treatable with drugs that

block thyroid hormone synthesis (86, 87). In contrast, in phenobarbital treated rats, sustained stimulation of the thyroid gland by TSH leads to the development of thyroid follicular tumors. Similarly, chemicals that cause peroxisomal proliferation in rodents do not do so in humans and other primates, possibly because of low levels of PPAR in primate liver (88).

PROTOTYPIC VICTIMS OF ENZYME INDUCTION

The consequences of administering strong enzyme inducers to human subjects (*e.g.* rifampicin and phenytoin) have been well documented, and several are described here to highlight the clinical issues associated with potential reductions in clinical efficacy. These include oral contraceptives (risk of conception), calcium channel blockers (loss of blood pressure control), anti-HIV drugs (lack of suppression of HIV replication), and several anti-cancer agents (reduced anti-tumor activity?).

Oral contraceptive - In humans, the metabolism of ethinylestradiol (**6**), the usual estrogenic component of the oral contraceptive pill, involves sulfation (sulfotransferases) to form the EE-3-O-sulfate, and glucuronidation (UGT1A1) to form EE-3-O-glucuronide. Alternatively, ethinylestradiol is hydroxylated at C2, C4, C6, or C16 to the corresponding hydroxy-derivatives, which subsequently are methylated to form methoxy derivatives and conjugated to form the corresponding methoxy sulfate or methoxy glucuronide. Following oral administration of ethinylestradiol to humans, AUC values for parent compound vary ~10-fold among different individuals and the oral bioavailability ranges from ~20-65%. The low bioavailability of ethinylestradiol is not due to poor absorption, but to first pass metabolism. The contribution of the gut and liver to [^{3}H]ethinylestradiol first pass metabolism has been studied in humans. The extraction of ethinylestradiol by gut (E_g) and liver (E_h) has been estimated to be ~44% and 25% and the mean bioavailability is ~45% (89, 90).

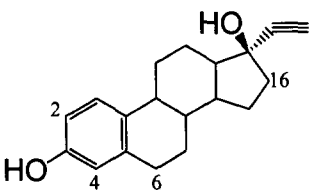
In another study, premenopausal women were treated with 35 μg ethinylestradiol/1 mg norethindrone (91). Subjects received 14 days of rifampicin (600 mg per day) from days 7-21 of their menstrual cycle. Rifampicin significantly decreased the mean area of the concentration of ethinylestradiol (66%) and norethindrone (51%). The mean C_{max} decreased by 43% from base line for ethinylestradiol and the $t_{1/2}$ decreased by 48%. The mechanism of this interaction was attributed to CYP3A4 induction. Interestingly, troglitazone administration (600 mg daily, 22 days) reduced AUC values for ethinylestradiol on day 21 by ~30%, and it was reported that troglitazone may enhance the conjugation pathways of ethinylestradiol metabolism (72). This is one of the few reports citing the up-regulation of Phase 2 pathways as a mechanism by which ethinylestradiol interacts with other drugs.

Calcium-channel blockers - The interaction between rifampicin and the dihydropyridine calcium-channel blockers resulted in loss of efficacy with potential adverse consequences of controlling blood pressure in the elderly. Rifampicin was given to treat tuberculosis in four elderly hypertensive patients whose blood pressure was well-controlled by one or more dihydropyridine calcium-channel blockers (nisoldipine, nifedipine, or barnidipine and manidipine). Shortly after the start of antituberculosis therapy, their blood pressures rose. Either much greater doses of dihydropyridines or additional antihypertensive agents had to be given to keep blood pressure under control. After withdrawal of rifampicin, blood pressure fell in all patients and the doses of the antihypertensive agents had to be reduced. These findings indicated that rifampicin could lessen the antihypertensive effects of dihydropyridine calcium-channel blockers (92).

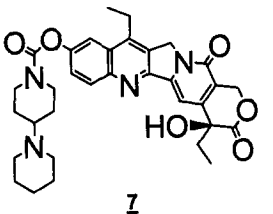
Anti-HIV drugs - Co-administration of rifampicin (600 mg) with nelfinavir (750 mg, t.i.d.), a CYP3A substrate, resulted in a 76% reduction in C_{max} and an 82% reduction in

AUC. Since the degree and duration of suppression of HIV replication is significantly correlated with plasma concentrations, this co-medication has been contraindicated because of the potential for drug interactions which could result in clinically significant adverse events (93).

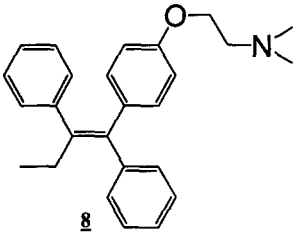
Irinotecan (CPT-11, 7) - is a water-soluble topoisomerase I inhibitor used for the treatment of patients with metastatic carcinoma of the colon or rectum whose disease has recurred or progressed following 5-fluorouracil-based therapy (94). Irinotecan is metabolized by carboxylesterase enzymes to form an active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), which is 100 to 1000-fold more potent than parent compound. Irinotecan can also undergo CYP3A4-mediated metabolism to form a



6



7



8

pharmacologically inactive metabolite, 7-ethyl-10-[4-N-[(5-aminopentanoic acid)-1-piperidino]-carbonyloxy-camptothecin (APC), formed following α-N-oxidation of the outer piperidine, and NPC, a primary amine metabolite formed by oxidative cleavage of both α-carbons of the outer piperidine. Co-administration of irinotecan with strong inducers of the CYP3A4 pathway have the potential to reduce its efficacy by increasing the proportion of drug undergoing oxidative metabolism to APC and NPC. The pharmacokinetic profile of irinotecan and its major metabolites with and without concomitant phenytoin administration in an individual patient has been reported (95). These studies revealed that phenytoin administration resulted in a marked decrease in the systemic exposure to irinotecan and SN-38 and an increase in the exposure to APC. The area under the curve of irinotecan and SN-38 decreased by 63% and 60%, respectively; the area under the curve of APC increased by approximately 16%, indicating that phenytoin administration had induced CYP3A4. These authors concluded that further detailed pharmacokinetic studies of irinotecan in patients receiving concomitant therapy with enzyme-inducing anticonvulsants (*e.g.* phenytoin, dexamethasone) are required so that rational dosing recommendations can be provided for this patient population.

Tamoxifen (8) and toremifene - Rifampin (600mg once daily for 5 day) reduced the area under the plasma concentration-time curve (AUC) of tamoxifen (80 mg, given on day 6) by 86%, peak plasma concentration (C_{max}) by 55%, and elimination half-life ($t_{1/2}$) by 44%. The AUC of toremifene (120 mg given on day 6) was reduced by 87%, C_{max} by 55%, and $t_{1/2}$ by 44% with rifampin. Rifampin therefore markedly reduced the plasma concentrations of tamoxifen and toremifene by inducing their CYP3A4-mediated metabolism. It was concluded that concomitant use of rifampin or other strong inducers of CYP3A4 with tamoxifen and toremifene may reduce the efficacy of these antiestrogens (96).

CONCLUSIONS

The drug interaction literature for rifampicin provides good reason why it is better to avoid developing a strong enzyme inducer. As with all potential liabilities, however, the challenge is really one of defining a therapeutic window between the desired pharmacological effect and the unwanted effect of enzyme induction. Since neither of

these variables are very well understood for a first entry compound, early assessment of the potential to induce enzymes at therapeutically relevant dosages in human subjects is clearly warranted. Compounds optimized to exert their pharmacological effects from low doses will, in all likelihood, have minimal potential to induce enzymes in human subjects, and for many, this represents a common basic research goal. The role of in vitro assays is to attenuate the property of enzyme induction during the drug discovery stage, when there is typically greater choice of chemical templates available for potential development.

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Chapter 32. The use of bioisosteric groups in lead optimization

Xiaoqi Chen[1] and Weibo Wang[2]
[1]Tularik Inc. 1200 Veteran Drive, South San Francisco, CA 94080
[2]Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608

Introduction – With the rapid advance of the human genome program, high throughput screening (HTS) and combinatorial chemistry, an unprecedented large number of hits are generated and funneled down to the hands of medicinal chemists at an ever-increasing speed. Additional extensive work is often required to increase the potency or *in vitro* profile in order to move these hits forward to *in vivo* testing. Unfortunately, the technological advances of combinatorial chemistry and HTS have not yet fulfilled all their promises to industrialize the drug discovery effort. This is partially evidenced by the historically high investments in R&D in the drug discovery industry, only accompanied by a declining number of NDAs approved by the FDA in recent years. In reality, careful handcrafting of medicinal chemists is often needed to turn a hit into a promising candidate for *in vivo* validation of clinically unproven genomic targets or into a promising clinical candidate to test in human. These elaborate SAR studies that focused on potency, specificity and ADME properties often require medicinal chemists to make compromises to accommodate all the *in vitro* and *in vivo* parameters set for the target profile of the candidate molecule (1). To add more difficulties to the problem, there are very few fixed routes to guide the individual SAR effort to transform the hit into a clinical candidate. The tortuous road of SAR studies, more often than not, encompasses an individual solution to an individual problem for an individual lead. To finish this challenging task requires medicinal chemists' insight, creativity and experience. Bioisosteric replacement is one of the tools available to help medicinal chemists solve some of the problems in their SAR studies.

The concept of bioisosteres refers to compounds or a substructure of compounds that share similar shapes, volumes, electronic distributions and physiochemical properties which together produce similar biological activities (2). It is difficult to clearly define how much similarity is deemed necessary for consideration as a bioisostere. However, despite its ambiguity, medicinal chemists have widely adopted this concept for drug discovery efforts. Bioisosteric replacement is often explored for the lead compound to optimize the potency and selectivity or to improve the overall ADME profile. Sometime, bioisosteres are used simply to circumvent previous patent coverage of leads in the literature. There are many excellent review articles that cover this topic extensively (3). The current review will cover some of the recent successful bioisosteric replacement work in lead optimization in the literature. Some typical, yet unsuccessful, examples of bioisosteric replacements will also be included in this review to demonstrate the principles and limitations of this technique.

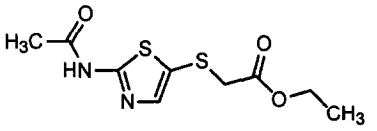
Bioisosteric replacements can be divided into several common types as illustrated in the next section.

FUNCTIONAL GROUP REPLACEMENT

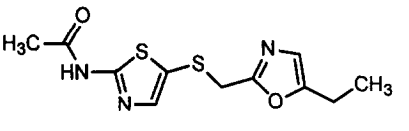
Ester - Carboxylic ester functionality is metabolically labile *in vivo* due to the ubiquitous presence of esterases throughout the body. This liability commonly prevents ester-containing compounds from being used as oral drugs unless the compounds were designed as "soft drugs" to achieve a desirable short duration of action. Oxazoles and oxadiazoles commonly serve as bioisosteric replacements of ester moieties with increased stability to hydrolytic degradation. (4-6),

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In the development of the cyclin-dependent kinase 2 (CDK2) inhibitors as anticancer agents, 2-amino-5-thio-substituted thiazole **1** was identified during high throughput screening with good selectivity against protein kinases A and C (7). Despite its potent CDK2 inhibitory activity, compound **1** lacks cellular activity in cell proliferation assays due to hydrolysis of the ethyl ester to the corresponding carboxylic acid that is completely devoid of CDK2 inhibitory activity. Attempts to increase the cellular stability of **1** by replacing the ethyl ester with a bioisostere oxazole moiety led to compound **2** which is metabolically stable against esterases, maintains the selectivity and shows increased CDK2 inhibitory potency, as well as potent antiproliferative activity in cancer cell lines.

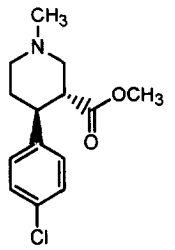


1

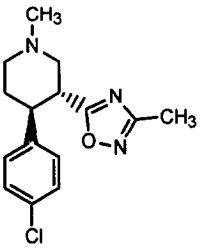


2

Another successful example of a bioisosteric replacement of an ester functionality with an oxadiazole moiety, for improving the metabolic stability, was illustrated in the studies of piperidine-based analogs of cocaine. In an effort to increase the duration of action of the dopamine transporter **3**, a 3,4-substituted piperidine-based cocaine analog **4** was synthesized in which the methyl ester group in **3** was replaced with an oxadiazole moiety (8). The affinities of **4** to the dopamine and norepinephrine transporters are very similar to **3**, however, **4** showed at least a 2-fold longer duration of action when compared to ester **3**.

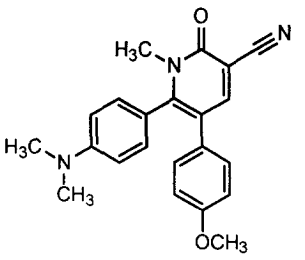


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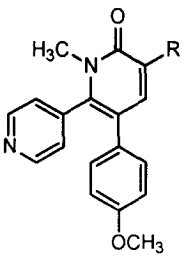


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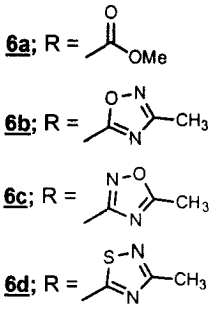
Thiazoles and thiadiazoles have also been used as bioisosteres for ester groups (9). In the continuing search for benzodiazepine site ligands with functional selectivity for α2/α3-subtypes of human $GABA_A$ receptor-ion channels, 1-methyl-3-cyano-2-pyridone **5** emerged from screening as a low-affinity ligand with weak binding selectivity for α2 and α3 $GABA_A$ subtypes over α1. Modification of the 3-cyano group in **5** to a methyl ester and introduction of a basic pyridine to the C-6 position gave **6a** which possessed higher affinities than the lead at α1, α2, and α3 subtypes. However, the pharmacokinetic behavior of **6a** in rat was poor, with rapid metabolism observed. Various heteroaryls were incorporated at the 3-position of the pyridone core of **6a** as replacements for the metabolically labile methyl ester. While **6b** showed negligible affinity for $GABA_A$ subtypes, isomer **6c** retained some affinity, and the corresponding thiadiazole **6d** showed excellent binding affinity and was a full agonist at all subtypes.



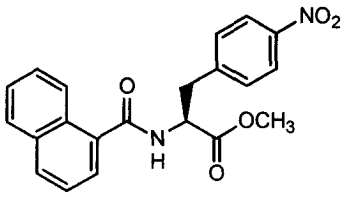
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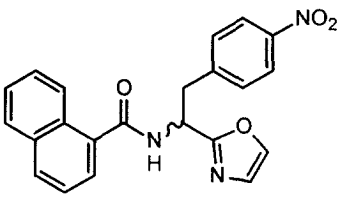
6a-d



Bioisosteric replacement of the ester with oxazole and other heteroaryls may not be universally effective, however, as demonstrated in the design and synthesis of a series of highly selective and potent phenylalanine derived CCR3 antagonists as anti-inflammatory agents. While compound **7** was highly effective in blocking both the binding and the functional activity of a number of CCR3 agonists, compound **8** lacks all CCR affinity which suggests a more subtle role for the ester moiety that the heterocycles were unable to mimic (10).



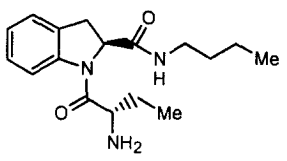
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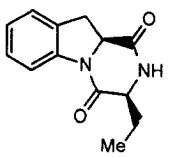
8

Amide - Bioisosteric replacements for an amide bond have been extensively studied because of their importance in peptide chemistry and the development of peptide mimetics. The most successful replacements were demonstrated in the design and development of HIV-1 protease inhibitors. Several review articles on the replacement of peptide bonds have been published (11,12).

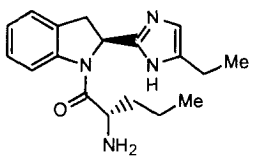
Imidazole- or benzimidazole-derived amide bond replacements were successfully incorporated in the design of HIV-1 and MMP inhibitors (13,14). Recently, this replacement has also been applied to the synthesis of tripeptidyl peptidase II (TPPII) inhibitors. Under physiological conditions, butabindide **9**, a potent inhibitor of the serine protease enzyme TPPII, underwent cyclization to form the diketopiperazine **10**, which is void of any TPPII enzymatic activity. Bioisostere replacement of the amide moiety in **9** with imidazole generated compound **11** which maintained TPPII inhibitory activity, and avoids of the inherent chemical instability of **9** (15).



9



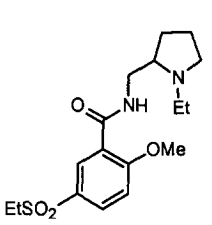
10



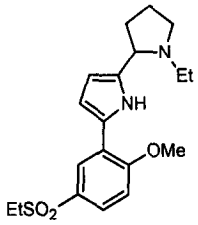
11

In the course of searching for high affinity dopamine D3 receptor antagonists, conformationally restricted benzamide isosteres, such as pyrroles, oxazoles, and

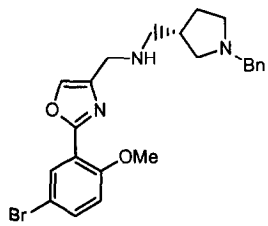
thiazoles were investigated. Replacement of the amide of **12** (sultopride) by a pyrrole ring led to compound **13** which maintained affinity for the dopamine D_3 receptor and introduced modest selectivity over the dopamine D_2 receptor (16). Replacement of the benzamide with oxazole resulted in compound **14** which exhibits dopamine D3 and dopamine D4 binding affinities comparable to those of the atypical neuroleptics sultopride and clozapine, respectively (17).



12

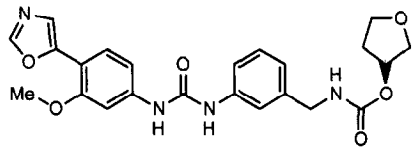


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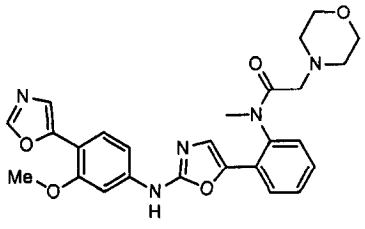


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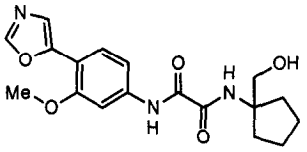
Urea - Inosine monophosphate dehydrogenese (IMPDH), a key enzyme that is involved in the *de novo* synthesis of purine nucleotides, is an attractive target for immunosuppressive, anticancer, and antiviral therapies (18). Compound **15** (VX-497) is an orally active inhibitor of IMPDH (19) recently discovered using a structure-based design approach. Using a bioisosteric replacement of the amide portion of the urea moiety with an oxazole, a novel series of potent inhibitors such as **16** with low nanomolar potency against the enzyme were developed. Compound **16** also showed excellent *in vivo* activity in the inhibition of antibody production in mice and in the adjuvant induced arthritis model in rats (20). Replacing the urea linkage in **15** with an oxalamic diamide linkage also led to a series of potent IMPDH inhibitors as exemplified by **17** (21).



15

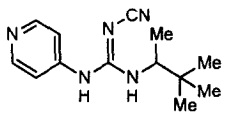


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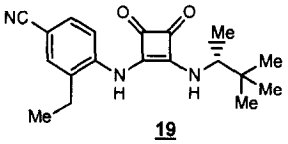


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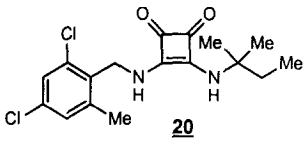
Guanidine - A novel bioisosteric replacement of the *N*-cyanoguanidine moiety in **18** (pinacidil) with a 1,2-diaminocyclobutene-3,4-dione template has been described which led to a series of bladder-selective KATP openers as exemplified by compound **19** (22). Subsequent optimization of the metabolic properties of **19** resulted in the discovery of **20**, which is 166-fold selective for bladder effects versus hemodynamic effects, and is currently under clinical evaluation for the treatment of urge urinary incontinence (23).



18

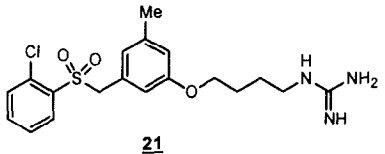


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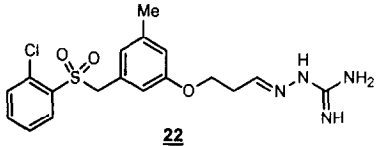


20

Due to the highly basic nature of the guanidine group, guanidine- containing compounds usually display poor oral bioavailabilities *in vivo*. Attaching electron-withdrawing groups such as cyano, nitro, acyl or sulfonyl to the nitrogen atom to attenuate the basicity of guanidine is one of the most classical techniques used in medicinal chemistry. Recently, another novel bioisosteric replacement of the guanidine group with an amidinohydrazone motif was reported in the search for direct antithrombins with good pharmacokinetic properties (24). Although compound **21** and its related series of analogs exhibited potent and selective inhibition of thrombin, *in vivo* evaluation was limited by its poor solubility. Amidinohydrazone replacement of the guanidine moiety resulted in compound **22**, which is slightly more potent than compound **21** *in vitro*. More importantly, compound **22** showed good bioavailability and long half-life in rabbits and dogs, respectively.

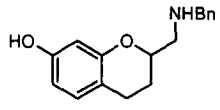


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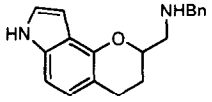


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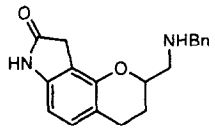
Phenol - In the search for more potent and selective dopamine (DA) D2 agonists for their implication in several psychiatric and neurological illnesses such as schizophrenia, Parkinson's disease and drug addiction, Mewshaw and coworkers published a series of modifications centered around the bioisosteric replacement of the metabolically labile 3-OH-phenol moiety in **23** with the aim of improving the oral bioavailability of this class of compounds (25). The bioisosteric analogs, such as indole **24**, indolone **25**, 2-trifluoromethyl-benzimidazole **26**, and benzimidazol-2-one **27**, were observed to have excellent affinity for the D2 receptor. Compounds **24** and **27** also demonstrated *in vivo* efficacy when administered both orally and subcutaneously.



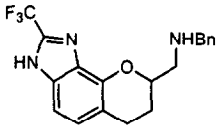
23



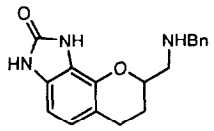
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25

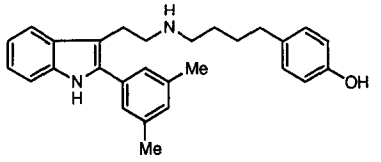


26

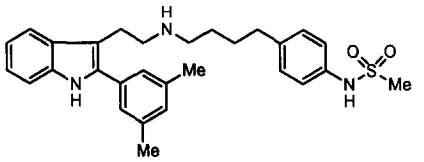


27

The alkylsulfonamide group is commonly used as a bioisosteric replacement for the phenol group as it has similar pKa values to that of the phenolic hydroxy group. As demonstrated in a recent publication on the lead optimization of a gonadotropin releasing hormone (GnRH) antagonist, methylsulfonamide replacement of the phenol in **28** resulted in compound **29**, with a 4-fold increase in binding affinity compared to **28** (26).

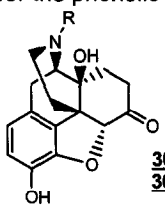


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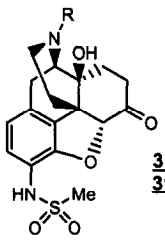


29

When the same replacement was applied to the opioid antagonist, naltrexone **30a** and its receptor agonist, oxymorphone **30b**, the opiate sulfonamides (**31a**, **31b**) were found to be devoid of agonist or antagonist activity (27). The lack of significant binding affinity of **31a** and **31b** may be attributed to the steric bulk of the sulfone moiety, which makes the sulfonamide group an unsuitable bioisosteric replacement for the phenolic hydroxy in this particular series.



30a; R = $CH_2CH(CH_3)_2$
30b; R = CH_3

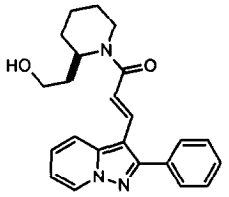


31a; R = $CH_2CH(CH_3)_2$
31b; R = CH_3

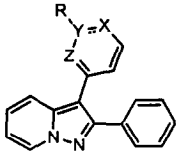
CONFORMATIONAL RESTRICTION

Restriction of conformation is the most commonly used technique in SAR development (28). The conformational restrictions of a lead structure are often associated with decreased entropy and increased binding affinity and specificity with targets if the vectors of the binding elements are orientated toward the correct binding pockets of the targets. Additionally, by systematically reducing the rotatable bonds in the lead compounds, the resulting compound may have a better chance to have oral bioavailability. After analyzing 1100 compounds, it has been reported that the candidate compounds with fewer than 10 rotatable bonds had a higher instance of good oral bioavailability in rat (29).

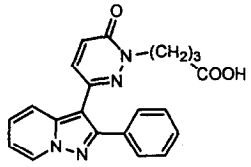
Adenosine receptor agonists or antagonists play important roles in a variety of physiological functions in human. Recently, **32** was discovered as a novel non-xanthine adenosine A_1 receptor antagonist. The oral bioavailability of **32** was relatively low due to a rapid first-pass effect in the liver and poor water solubility. Additionally, **32** was readily converted to the less active cis isomer in solution due to a facile photochemical trans-cis isomerization process. Compound **33** was designed using heteroaryl groups to mimic the double bond and carbonyl group of the acryloyl moiety (30). Compared with the original lead, compound **34** is more hydrophilic and soluble in water and has improved oral bioavailability. As a result, compound **34** was found to be 10 times more potent than **32** for *in vivo* diuretic activity despite a relatively low affinity for the adenosine A1 receptor.



32

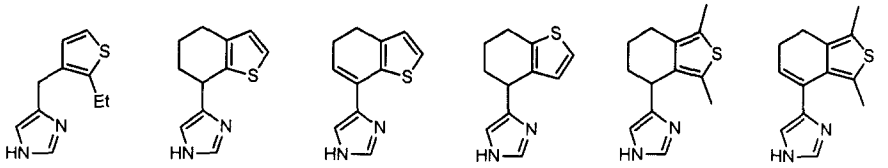


33



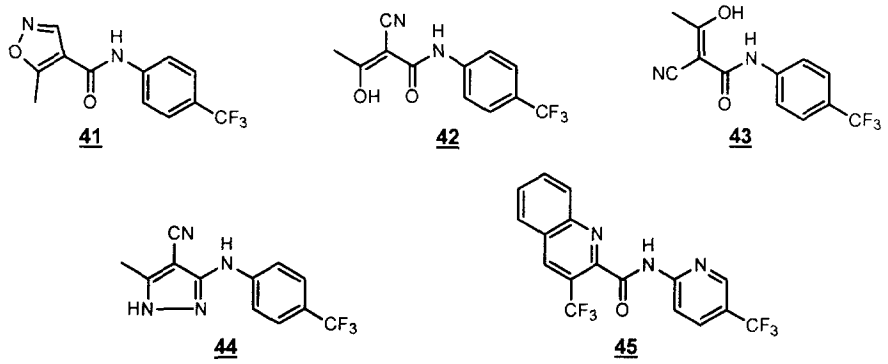
34

α_{2D} Adrenergic agonists are used in clinic for treatment of severe pain. Following early identification of potent lead compound (imidazolemethyl)thiophene **35**, a series of compounds **36**-**40** were designed to restrict the rotation of the two single bonds between the imidazole and thiophene (31,32). These compounds are all very potent α_{2D} adrenergic receptor ligands. Among them, **38** showed *in vivo* efficacy upon oral dosing. Compound **39** is the most potent α_{2D} ligand with Ki of 0.009 nM, and more than 10,000-fold separation against α_1. These analogs are useful pharmacological tools for studies involving the α_{2D} adrenergic receptor.



35 **36** **37** **38** **39** **40**

Transplant rejection is substantially dependent on the immuno-response of antibody production mediated by B-cells independent of T-cell signals. Leflunimide **41** is a pro-drug which converts to hydroxypropenamide *in vivo* and *in vitro* quantitatively in the cellular system. The resulting hydroxypropenamide inhibits dihydrooroate dehydrogenase (DHODH) at low concentrations, but can operate by additional protein tyrosine kinase related mechanisms at its therapeutically relevant concentrations. Hydroxypropenamide has two conformers **42** and **43** and no information is available on the DHODH-bound conformation of **42**. Prazole **44** was initially designed to mimic **42** and the inhibitory activity of DHODH is similar to leflunimide (33). Compound **45** was then designed to mimic the conformer **43** (34). Compound **45** has negligible enzyme activity, however, it was found to be a highly potent and selective B-cell immunosuppressant over T-cells. Additionally, compound **45** effectively suppressed rejection in the antibody- mediated mouse xenograft model using 0.3mg/kg oral once-daily dosing.

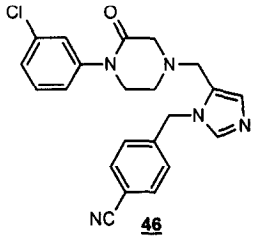


41 **42** **43**

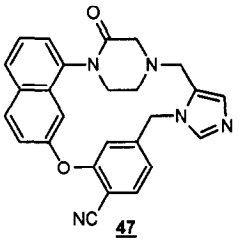
44 **45**

Macrocyclic constrained inhibitor design has become of great interest recently. A number of potent, orally bioavailable macrocyclic inhibitors targeting farnesyltransferase, thrombin, metalloproteases, HCV protease, and HIV protease have been discovered (35-42). Since the lead structures all have extended and highly flexible backbones, macrocyclization is one way to reduce the number of potential conformers of the leads while still preserving all the necessary binding elements. The increased the rigidity may also allow the inhibitors to adopt the optimal binding conformation that may have higher energy barrier for a free rotate solution structure to overcome.

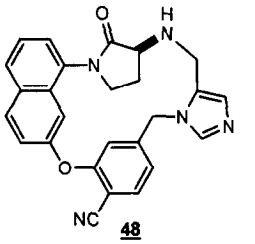
Detailed SAR studies of a series of macrocyclic farnesyltransferase inhibitors were reported following the early discovered macrocyclic lead (43,44). Compared with previous linear leads such as **46**, macrocyclic inhibitors in this series showed significant increases in potency. Compound **48** also had improved pharmacokinetic properties and lowered inhibition of hERG channel. After X-ray crystallography and NMR spectroscopy studies of these inhibitors, the report concluded that the improvements in *iv* half-lives due to lower plasma clearance appeared to correlate with the rigidity of the molecules.



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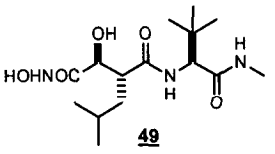


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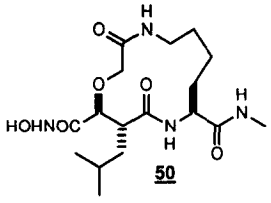


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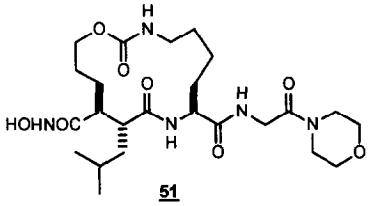
Tumor necrosis factor-α converting enzyme (TACE) is responsible for the conversion of the pro-TNF-α to soluble TNF-α that plays a central role in symptoms of a variety of infectious, autoimmune and inflammatory disorders. Analogous to the development of the macrocyclic MMP inhibitors **50** as a macrocyclic mimic of marimast **49**, the design, synthesis and SAR of a new class of macrocyclic hydroxamic acid TACE inhibitors has been reported (45). Additional SAR studies following the early macrocyclic lead **51**, the undesired cross activities toward MMP1, MMP2 and MMP9 were suppressed without compromising the TACE activity (46). Compound **52** showed an IC_{50} of 70 nM in human whole blood and more than 100-fold selectivity over a panel of 11 MMPs.



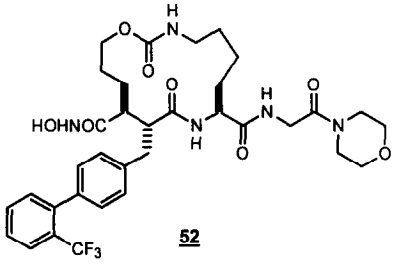
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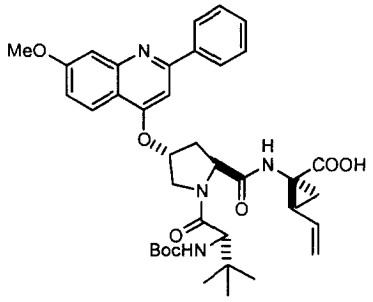
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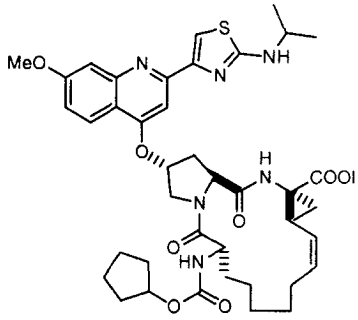
52

Hepatitis C virus is a major anti-infective research focus for many pharmaceutical companies, due to the presence of a large infected population in western society (47). HCV encoded NS3 serine protease has been under close scrutiny to develop orally bioavailable agents for many years (48-50). Due to its large and shallow binding pocket, even with the aids of X-ray crystallography and NMR studies of enzyme complex with peptide inhibitors, very few orally bioavailable small molecule inhibitors have emerged from extensive research (51,52). Recently, a macrocyclic inhibitor structurally related to **54** was reported (53,54). This highly potent NS3 inhibitors showed sub nM Ki which is more potent than the early acyclic lead **53** that has an IC_{50} of 15nM (55). More interestingly, a clinical trial with b.i.d. dosing at

200mg/kg for two days in 10 patients with chronic HCV infection showed at least 2 log_{10} copies/ml reduction of HCV RNA levels with no drug related toxicity (56).



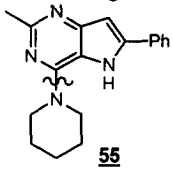
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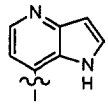
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ATOM REPLACEMENT

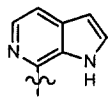
A detailed SAR study of a pyrrolo[3,2-d]-pyrimidine lead for NYP5 receptor antagonist has been reported (57). The N atom of the core ring of **55** was systematically replaced with an O, S, or CH. A working hypothetical model of the human NPY5 receptor binding site was thus proposed and could be used for future novel antagonist design.



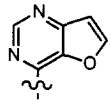
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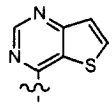
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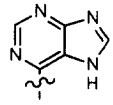
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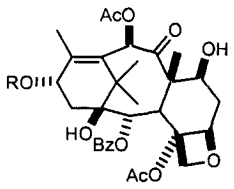


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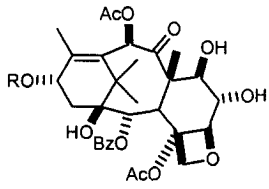


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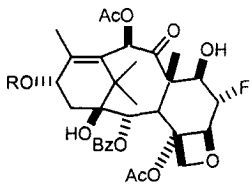
Fluorine replacement of a hydrogen atom to block the metabolic site of the drug is widely used in drug design. With the availability of *in vitro* testing with human liver microsome, S9 fraction and human liver hepatocyctes, it becomes much easier to accurately locate the site of metabolic liability and to install the fluorine atom.



61



62



63

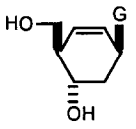
A significant portion of paclitaxel **61** was found to be excreted through the bile as 6-α-hydroxypaclitaxel **62** in human (58). The hydroxylated metabolite is 30-fold less active than the parent drug. The fluorine analog was designed to overcome this problem. The 6-α-F, Cl, Br paclitaxels were found to have similar *in vitro* and *in vivo* activities. The human liver S9 incubation of the newer inhibitors did not produce the hydroxylated paclitaxels.

Due to the high electronic negativity of the fluorine atom, it is often used to modulate the physicochemical properties of a molecule. A fluorine atom was often employed to affect the basicity of the amine, thus influencing the bioavailability and metabolic stability. In a recent report, the basicity of the fluorinated molecule was measured to quantify the β and γ induction effects of the fluorine atom (59). It was

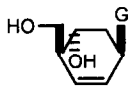
found that the basicity has a dramatic, beneficial influence on oral absorption, but the effect on oral bioavailability could not always be accurately predicted.

HETEROCYCLIC RING REPLACEMENTS

Herdewijin and De Clercq reported using a cyclohexene ring to replace a saturated furanose ring for antiviral nucleoside chemistry (60). A cyclohexene mainly exists in the half-chair form, which interconverts via the symmetrical boat form. The conformational equilibrium of cyclohexenyl nucleosides which is very similar to the equilibrium observed in furanose nucleosides. In comparison with acyclovir, the D-and L-cyclohexenyl guanines were slightly more potent against HSV-1 and slightly less potent against HSV-2. D- and L-Cyclohexenyl G were equipotent with acyclovir in inhibiting VZV and equipotent with ganciclovir in inhibiting HCMV replication. Additionally, (±)-cyclohexenyl G is about 10 fold more potent against HBV than penciclovir.

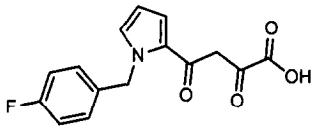


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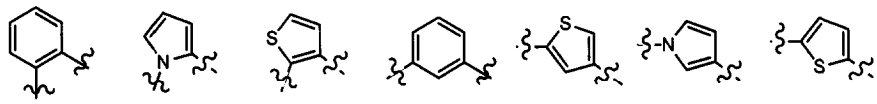


65

Heterocycle replacement in the HIV intergrase inhibitors also showed some interesting results (61). Systematically replacing the central ring of **66** with a series of aromatic systems having various substitution patterns provided a quick survey of biological activity in relation to the bisected angle of benzyl and diketo acid side chains. The intrinsic potency of these inhibitors increases as the angle of bisection increases from 60° to 118°. The angle of bisection was based on X-ray coordinates of similarly substituted heterocyclic/aromatic compounds.



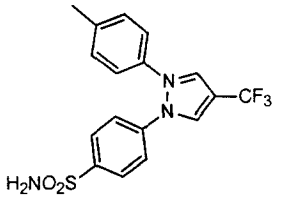
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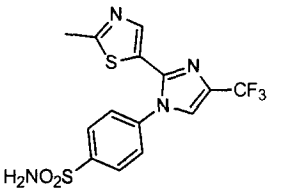
60.1°	69.3°	74.6°	118.2°	138.5°	138.6°	148.9°
67	**68**	**69**	**70**	**71**	**72**	**73**

Recently, celecoxib and refecoxib became the first cyclooxygenase-2 selective inhibitors to enter the market. A series of heteroaryl modified 1,2-diarylimidazoles were evaluated and shown to be potent and highly selective inhibitors of the human COX-2 (62). Bioisosteric replacement of the pyrazole of celecoxib with an imidazole and subsequent replacement of the phenyl group with various hetero aryl rings yielded inhibitors that exhibited desirable pharmacokinetic profiles. Additionally, excellent efficacy compounds in both acute and chronic models of inflammation was observed for these compounds with no GI toxicity in the rat up to 100mg/kg. Another series of COX-2 inhibitors with a central oxazole ring was also reported. The best compound in the series has an IC_{50} of 85nM against COX-2 and has a >1100-fold separation against COX-1. The leading compound also demonstrated good oral pharmacological activities in the rat carrageenan paw edema model and

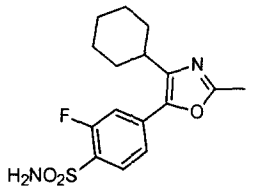
rat adjuvant arthritis model, without acute ulcerogenic toxicity in rats at doses up to 300mg/kg.



74

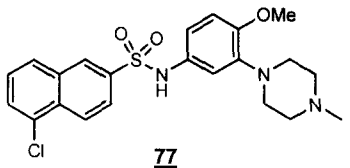


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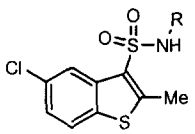


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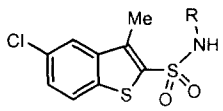
Replacing a phenyl ring with a thiophene system has been, and still is, one of the most classic examples of bioisosteric replacement. There are many reports of lead optimization using this technique. A series of 3-substituted-3,4-dihydroisoquinolinamines was replaced by 4,5-tetrahydrothieno[2,3-c]pyridines or 6,7-dihydrothieno[3,2-c]pyridines as nitric oxide synthase inhibitors (63). Not surprisingly, these analogs showed rather different activity and selectivity profiles. Similar heterocyclic replacements were used for the antagonists of the adenosine A1 receptor (64). The isoquinoline core was replaced with a thiadiazole or a thiazole. The resulting compounds showed quite different activities and selectivities.



77



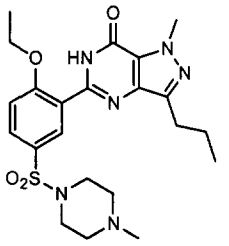
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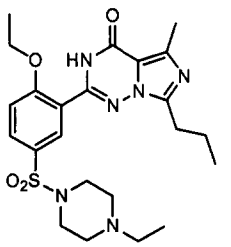
79

Bioisosteric replacement of 4-quinolones with 2-pyridones for the bacterial topoisomerase inhibitors resulted in several highly potent agents (65). The comparison of biological activities of these two series of compounds was recently reviewed (66). Although no marketed drugs emerged from this highly promising series, the concept of introducing a bridgehead nitrogen to replace a carbon atom in a heterocyclic ring is a well validated bioisosteric replacement. The recently discovered vardenafil **81**, awaiting FDA approval, is a creative and successful way to apply this strategy (67,68). While vardenafil and sildenafil are structurally similar, the two compounds show rather different PDE subtype selectivities and PK profiles. Vardenafil is slightly more potent than sildenafil and has a faster on-set of action. The IC_{50} ratios of PDE6/PDE5 are 7-fold and 160-fold for sildenafil and vardenafil, respectively (69). A larger activity separation of PDE6/PDE5 could potentially reduce the side effect of blue vision in some patients.

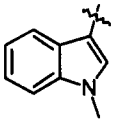
Another example is shown in the attempted replacement of the indole ring with pyrrolo[2,3-b]pyridine **83**, pyrazolo[1,5-a]pyridine **84**, tetrahydropyrazolo[1,5-a]pyridine **85**, and imidazo[1,2-a]pyridine **86** in the search for dopamine D4 receptor ligands (70). Replacement with these heterocycles did not yield compounds of similar potency compared with the indole lead. An electrostatic potential map was calculated to further rationalize the molecular property and activity differences against respective receptor subtypes. This helps to gain some understanding of the electrostatic differences and binding requirements for these apparently similar heterocyclic rings.



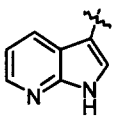
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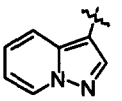
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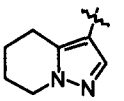
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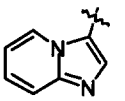
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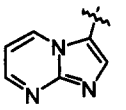
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CONCLUSION

Bioisosteric replacement is an excellent tool for lead optimization to produce the desired potency and selectivity and the requisite ADME profile for a marketable drug. Like any tool used in modern drug discovery, it has limitations that requires medicinal chemists with insight, creativity and experience to use it intelligently in the solution of the daily problems encountered in drug discovery.

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SECTION VII. TRENDS AND PERSPECTIVES

Editor : Annette M. Doherty
Pfizer Global Research & Development

Chapter 33 . To Market, To Market - 2002

Cécile Boyer-Joubert, Edwige Lorthiois and François Moreau
Pfizer Global Research & Development
Fresnes, France

A significant increase in the number of new therapeutic chemical and biological entities was observed in 2002 compared to 2001 (33 *vs* 25) (1-6). From the 31 NCEs and 2 NBEs launched on the market last year, the US market saw 17 new entities introduced, the Japanese market 8 and the European only 6. These drugs originated for the most part from Europe (13) mostly from UK (8), followed by the US (11) and Japan (9). Astra Zeneca discovered the highest number of products in 2002 with a total of 4 new entities, followed by Pharmacia and Glaxo-Smithkline with 3 compounds discovered, and Merck & Co and Ono Pharmaceuticals (2 compounds). Finally, Novartis and Pfizer were at the origin of 1 new molecular entity and marketed or co-marketed 2 substances.

With 8 new launches, antiinfectives were the most active therapeutic area. Three quinolones, Q-Roxin® (balofloxacin), Pasil® (pazufloxacin) and Sword® (prulifloxacin) were launched for the treatment of urinary tract or bacterial infections.Two new 1-β-methyl carbapenems with broad spectrum antimicrobial activity reached the market, Omegacin® (biapenem) and Invanz® (ertapenem sodium). Funguard® (micafungin), the second member of the echinocandin class, and Vfend® (voriconazole) are two new agents introduced for the treatment of fungal infections caused by *Aspergillus* and *Candida* spp. Hepsera® (adefovir dipivoxil) is the first nucleotide analog to be launched against hepatitis B virus infections.

The anticancer field was also well represented with 5 new drugs launched. Calsed® (amrubicin hydrochloride) is a completely synthetic anthracycline derivative launched for the treatment of small-cell and non-small-cell lung cancer (NSCLC). Iressa® (gefitinib) was introduced for the treatment of NSCLC. The first "pure" steroidal estrogen antagonist, Faslodex® (fulvestrant), was marketed for the treatment of breast cancer in postmenopausal women with disease progression following estrogen therapy. Foscan® (temoporphin), a second generation photosensitizer, was launched for the photodynamic therapy of advanced head and neck cancers. Finally, Zevalin™, the first radiolabeled antibody, was launched for the treatment of non-Hodgin lymphomas.

In the cardiovascular area, 4 drugs were introduced: Arixtra® (fondaparinux sodium), a synthetic copy of the heparin pentasaccharide sequence, for the prophylaxis of deep vein thrombosis following major orthopaedic surgery, Onoact® (landiolol), an ultra-short acting β1-adrenergic blocker, for the treatment of tachyarrhythmia during surgery, Benicar® (olmesartan medoxomil), for the treatment of hypertension and Remodulin® (treprostinil sodium), a prostacyclin mimetic for the treatment of pulmonary hypertension.

In the field of allergic and respiratory diseases, 3 NCEs were marketed. Elidel® (pimecrolimus) was introduced for the treatment of atopic dermatis in patients for whom conventionals therapies are inadvisable. Elaspol® (sivelestat), a neutrophil elastase inhibitor was launched for the treatment of acute lung injury associated with systemic

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inflammatory response syndrome. Finally, Spiriva® (tiotropium bromide), an antimuscarinic agent was developed for the treatment of chronic obstructive pulmonary disorder.

In the CNS area, Abilify® (aripiprazole), a partial dopamine D2 agonist which has a new mechanism of action compared to typical and atypical antipsychotic, was launched for the treatment of psychoses including schizophrenia. Focalin® (dexmethylphenidate hydrochloride), the eutomer of Ritalin® was marketed for the treatment of attention deficit hyperactivity disorder in children. Cipralex® (escitalopram oxalate), the *S*-enantiomer of citalopram, was introduced for the treatment of depression and panic disorders.

The antiinflammatory field was represented by 3 COX-2 inhibitors: Arcoxia® (etoricoxib), launched for the treatment of osteoarthritis, rheumatoid arthritis, dysmenorrhoea, gout, ankylosing spondylitis and pain; Bextra® (valdecoxib) for the treatment of osteoarthritis, rheumatoid arthritis and menstrual pain and Dynastat® (parecoxib), an amide prodrug of valdecoxib, marketed as an antiinflammatory agent for the management of acute pain.

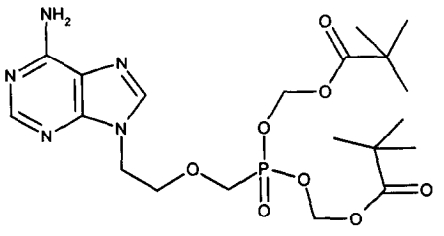
In the area of metabolism regulators, 2 NCEs appeared on the market. Ezetrol® (ezetimibe), a new lipid-altering drug with a novel mechanism of action, was launched as a hypolipaemic agent. Nerixia® (neridronic acid), a second generation bisphosphonate, was introduced for the treatment of the "orphan disease"; osteogenesis imperfecta.

Frova® (frovatriptan), a long-acting antimigraine drug, is the eighth member of the triptan class. Ortho Evra® (norelgestromin) is the first transdermal patch to be launched as a female contraceptive. Orfadin® (nitisinone), an inhibitor of 4-hydroxyphenylpyruvate dioxygenase, was marketed for the treatment of hereditary tyrosinaemia type I. Avodart® (dutasteride), a dual type 1 and type 2 5α-reductase inhibitor, was introduced for the symptomatic treatment of benign prostatic hyperalgesia.

Finally, 3 additional biological entities were launched in 2002 but they are not considered as NBEs:

- new formulation of already existing products such as pegfilgrastim from Amgen (Neulasta™, a pegylated form of recombinant human GCSF with prolonged half-life for the treatment of chemotherapy-induced neutropenia in patients with thoracic tumors and breast cancers);
- existing products launched for new indications such as teriparatide from Eli Lilly (Forsteo®, a synthetic peptide of parathyroid hormone launched for the treatment of postmenopausal osteoporosis) and porcine secretin from Repligen (SecreFlo™, a synthetic peptide of porcine secretin hormone launched as diagnostic agent for the prevention of ERCP-induced pancreatitis).

Adefovir dipivoxil (Antiviral) (7-10)

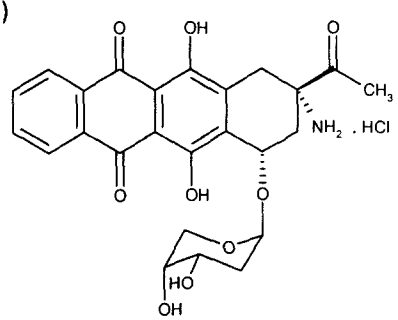


Country of Origin : Czech Republic, Belgium
Originator : Institute of Organic Chemistry and Biochemistry of the Academy of Sciences in the Czech Republic and the REGA Stichting Research Institute

First Introduction : US
Introduced by : Gilead
Trade Name : Hepsera
CAS Registry No.:142340-99-6
Molecular Weight : 501.48

Adefovir dipivoxil is the first nucleotide analog to be launched in the US as an oral treatment for hepatitis B virus (HBV) infections. It can be easily prepared in 4 steps from adenine. Adefovir dipivoxil acts as a bioavailable ester prodrug which is rapidly hydrolyzed to free adefovir and further anabolized to its active form, adefovir diphosphate, by two intracellular phosphorylation steps. The diphosphate competitively inhibits reverse transcriptase and/or causes chain termination when incorporated into growing DNA. Adefovir dipivoxil has a broad antiviral spectrum against retro-, herpes- and hepadnaviruses. The drug inhibits HBV replication, decreases HBV DNA levels and improves liver histology of patients infected with HBV wild type and resistant to other antivirals such as lamivudine. It also demonstrated activity in hepatitis B"e" antigen-negative, or precore mutant, patients and in patients co-infected with HIV. To date, no adefovir dipivoxil-associated resistance mutations have been identified in patients up to 136 weeks with the drug. The oral bioavailability of adefovir after oral administration of its dipivoxil prodrug is approximately 30%. It is mainly excreted unchanged in the urine and its plasma elimination half-life is 4.2 h. However, a long intracellular half-life (17 h) of the active bisphosphorylated metabolite enables once-daily dosing. The most prominent adverse effect of adefovir dipivoxil is nephrotoxicity (which has prevented the drug from being marketed for HIV infections where the drug required administration at higher doses).

Amrubicin hydrochloride (antineoplastic) (11-14)



Country of Origin : Japan
Originator : Sumitomo
First Introduction : Japan
Introduced by : Sumitomo
Trade Name : Calsed
CAS Registry No. : 92395-36-5
Molecular Weight : 519.13

Amrubicin was launched in Japan as an injectable preparation for the treatment of non-small and small-cell lung cancers. Amrubicin, a completely synthetic anthracycline derivative, mediates its growth inhibitory action via topoisomerase II inhibition. It is activated *in vivo* by the formation of its 13-OH metabolite, amrubicinol, via reaction with carbonyl reductase. In contrast to doxorubicin and daunorubicin whose metabolites are inactive in the blood, amrubicinol is 10-100 fold more cytotoxic than amrubicin. In phase II clinical trials, amrubicin showed antitumor activity against non-small-cell lung cancers (response rate exceeding 20%) and against untreated extensive stage small-cell-lung cancers (response rate 78.8%). Amrubicin demonstrated a smaller distribution-volume, a shorter half-life in mice and also less chronic cardiotoxicity in preclinical studies compared to doxorubicin. Amrubicin was generally well tolerated with major adverse events being anaemia, leucopenia, thrombocytopenia and neutropenia.

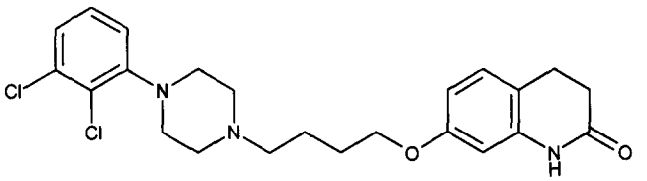
Anti-digoxin polyclonal antibody (antidote) (15-17)

Country of Origin : UK
Originator : Protherics
First Introduction : US
Introduced by : Savage Laboratories
Trade Name : DigiFab
CAS Registry No : 339086-83-8

Class : Polyclonal antibody
Type : Anti digoxin immune Fab
Molecular Weight : 46 kDa
Expression system : Immunized sheep
Manufacturer : Protherics

Digoxin is widely prescribed for the treatment of cardiac conditions such as atrial arrhythmias and congestive heart failure. Because of its narrow therapeutic range, digoxin-related toxicity resulting from acute or chronic overdose is common. Digoxin toxicity can be rapidly and safely reversed by intravenous administration of anti-digoxin immune fragments (Fab) such as DigiFab™ which act by binding digoxin with high affinity (10^{9}–10^{10} L/mol), favoring movement of digoxin out of tissue and thus promoting elimination. DigiFab™ is obtained from the blood of healthy sheep immunized with a digoxin derivative, digoxin-dicarboxymethoxylamine. The final product is prepared by isolating the immunoglobulin fraction of the serum, digesting it with papain and isolating the digoxin-specific Fab fragments by affinity chromatography. Digibind® from Glaxo-SmithKline has been available in the US since 1986 but produced by immunization with digoxin. Comparison of the pharmacokinetics and in vivo bioaffinity of DigiFab™ versus Digibind® showed that both drugs are equally effective in binding and neutralizing serum free digoxin (40mg of Fab binds 0.5 mg of digoxin approximately). In this clinical study involving 15 DigiFab™-treated patients, 93% had complete resolution of the induced digoxin toxicity within 20 hours. No patients developed a measurable immune response (human anti-ovine antibodies) to DigiFab™. The elimination half life of DigiFab™ is 15 hours.

Aripiprazole (neuroleptic) (18-22)



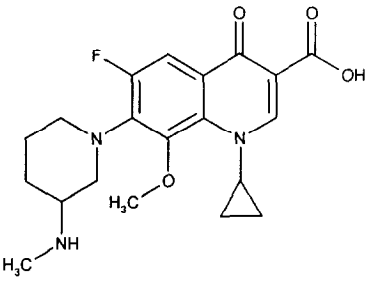
Country of Origin : Japan
Originator : Otsuka
First Introduction : USA
Introduced by : Bristol-Myers Squibb

Trade Name : Abilify
CAS Registry No : 129722-12-9
Molecular Weight : 448.40

Aripiprazole was launched for the treatment of psychoses including schizophrenia and offers a novel mechanism of action as a partial D2 receptor agonist. Aripiprazole can be synthesized in three steps beginning by the condensation of 7-hydroxy-1,2,3,4-tetrahydroquinolin-2-one with 1,4-dibromobutane followed by reaction with 1-(2,3-dichlorophenyl)piperazine. Aripiprazole is a significant D2 agonist/antagonist, 5-HT_2 antagonist and 5-HT_{1a} agonist combined with minimal affinity for α_1-adrenergic, H1 and M1 receptors. It has a low D4:D2 selectivity ratio and a D2:5-HT_2 affinity ratio that exceeds 15; resulting in different pharmacological characteristics compared to other atypical antipsychotics agents such as clozapine. In animal models, aripiprazole inhibits apomorphine-induced stereotypy without causing catalepsy and ptosis. Moreover, in contrast to classical antipsychotics that produce disabling movement disorders,

aripiprazole does not cause an upregulation of D2 receptors or an increase in immediate early gene expression of *e.g.* the c-fos mRNA in the striatum. In patients with acute relapse of schizophrenia, treatment with aripiprazole provided significant improvement in both positive and negative syndrome scale (PANSS) total score in both short- and long-term evaluations. These results were comparable to those observed with haloperidol or risperidone; however, the early response rate was greater with aripiprazole. Aripiprazole was well tolerated with mild to moderate adverse events such as nausea, dizziness, somnolence and weight gain. The rates of extrapyramidal symptoms were lower than with haloperidol, prolactin levels increase has been uncommon and no significant Q-Tc interval prolongation was observed compared with placebo. Finally, studies suggested a minimal impact of aripiprazole administration on total cholesterol levels and on fasting blood sugar in contrast to other antipsychotics. Aripiprazole has a bioavailability of 87%, a t_{max} of 3-5 h and a half-life time of 48-68 h. Aripiprazole has been found to have linear kinetics and is mainly metabolized *via* the cytochrome systems CYP2D6 and CYP3A4. It has little effect on the blood levels of other medications; interaction with both lithium and divalproex sodium found minimal impact. Aripiprazole has also been studied in other psychiatric disorders, including bipolar disorders and has shown great efficacy.

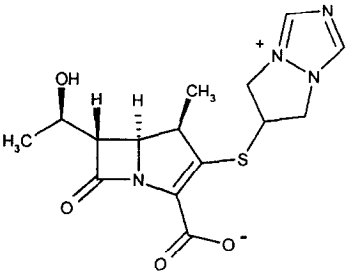
Balofloxacin (antibacterial) (23-28)



Country of Origin : Japan
Originator : Chugai Pharmaceutical
First Introduction : South Korea
Introduced by : Choongwae Pharma Corporation
Trade Name : Q-Roxin
CAS Registry No : 127294-70-6
Molecular Weight : 389.43

Balofloxacin, a novel orally-active fluoroquinolone antibiotic, was introduced in South Korea for the treatment of urinary tract infections (UTI). It can be synthetized by reaction of 3-(methylamino)piperidine with the classical 4-quinolone-3-carboxylic acid template. *In vitro* antibacterial activity of balofloxacin against gram-positive bacteria (*Staphylococcus aureus* including methicillin-resistant *S. aureus, Staphylococcus epidermis, Streptococcus pneumonia, Streptococcus pyrogenes*) was almost equal to that of sparfloxacin or tosufloxacin, in contrast its activity against gram-negative bacteria was 2 times or more lower. In clinical trials, balofloxacin was well tolerated and showed comparable efficacy to ofloxacin in patients with UTIs. After oral administration, balofloxacin was well absorbed, and was primarily eliminated unchanged in the urine with an elimination half-life of approximately 8 h. In animal studies, balofloxacin did not exhibit any phototoxicity.

Biapenem (antibacterial) (29-31)

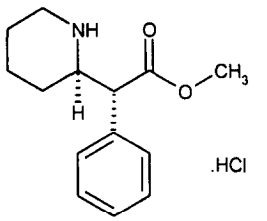


Country of Origin : US
Originator : Wyeth
First Introduction : Japan
Introduced by : Meiji Seika
Trade Name : Omegacin
CAS Registry No. : 120410-24-4
Molecular Weight : 350.42

Biapenem was introduced last year in Japan as a parenteral treatment for bacterial infections. This new 1-β-methylcarbapenem can be prepared by reaction of commercially available 4-nitrobenzyl protected β-methylcarbapenem enolphosphate with mercapto bicyclotriazolium chloride, obtained in 11 steps starting from hydrazine, followed by deprotection of the carboxylic acid function. Biapenem is a bacterial cell wall synthesis inhibitor with a broad spectrum *in vitro* antibacterial activity encompassing many Gram-negative and Gram-positive aerobic and anaerobic bacteria, including species producing β-lactamases. Like imipenem, biapenem is moderately active against *Enterococcus faecalis* and *E. faecuim* and is inactive against methicillin-resistant *Staphylococcus aureus*. Biapenem is stable to hydrolysis by human renal dihydropeptidase I (DHP-I) and therefore does not require the coadministration of a DHP-I inhibitor. In clinical trials, biapenem showed good clinical and microbiological efficacy in the treatment of patients with intra-abdominal, lower respiratory tract and complicated urinary tract infections. After intravenous administration, the drug is widely distributed, has linear pharmacokinetics and is mainly excreted in the urine with an elimination half-life of approximately 1 h. Biapenem is generally well tolerated, the most common adverse events being skin eruptions/rashes, nausea and diarrhea.

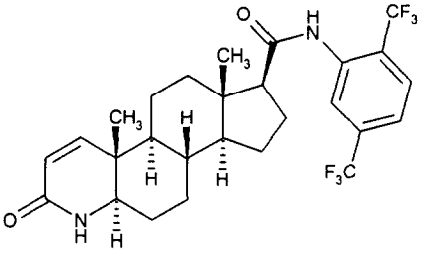
Dexmethylphenidate hydrochloride (psychostimulant) (32-35)

Country of Origin : USA
Originator : Celgene
First Introduction : USA
Introduced by : Novartis
Trade Name : Focalin
CAS Registry No : 19262-68-1
Molecular Weight : 269.77



Dexmethylphenidate, the pharmacologically effective enantiomer of *d,l*-methyl phenidate (Ritalin®) was developed as an improved treatment for attention deficit hyperactivity disorder (ADHD) in children. Dexmethylphenidate acts *via* the inhibition of reuptake of dopamine (by binding to dopamine transporter) and noradrenaline. It is thought to block dopamine and noradrenaline reuptake into the presynaptic neuron and increase neurotransmitter release into the extraneuronal space. Dexmethylphenidate, at half the usual dose of racemic methylphenidate, improved the symptoms of attention deficit hyperactivity disorder to a similar extent to methylphenidate in both home and school settings (SNAP-ADHD scores) at 3 h post dosing. Moreover, some studies showed that dexmethylphenidate has a statistically significant longer duration of action than the racemic form as measured by a behavioral scale at 6 h post dosing compared to placebo. In patients with ADHD, plasma dexmethylphenidate concentrations increased rapidly, reaching a maximum in the fasted state at approximately 1-1.5 h post-dose. The mean plasma half-life for dexmethylphenidate is approximately 2.2 h. Dexmethylphenidate is metabolized to *d*-α-phenyl-piperidine acetic acid, its main urinary metabolite which has negligible pharmacological activity. *In vitro* studies showed that dexmethylphenidate did not inhibit cytochrome P450 isozymes. Dexmethylphenidate was well tolerated; the most commonly reported adverse events (abdominal pain, headache, anorexia, insomnia) were mild in severity and consistent with those known to be associated with agents containing methylphenidate. Current labeling states that dexmethylphenidate should be administered twice daily with an interval of at least 4 hours between doses. Stimulant medications have been used for over sixty years and remain, until now, the first line pharmacological therapy for children with ADHD, demonstrating effectiveness in roughly 70% of patients.

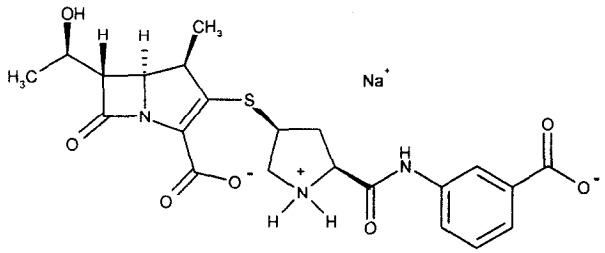
Dutasteride (5α-reductase inhibitor) (36-41)



Country of Origin : UK
Originator : GlaxoSmithKline
First Introduction : USA
Introduced by : GlaxoSmithKline
Trade Name : Avodart
CAS Registry No : 164656-23-9
Molecular Weight : 528.54

Dutasteride was launched for the symptomatic treatment of benign prostatic hyperplasia. Dutasteride can be prepared from 3-oxo-4-androstene-17β-carboxylic acid by several ways in 6 or 8 steps. Dutasteride is a dual inhibitor of type 1 and 2 isoforms of 5α-reductase unlike finasteride, the first marketed 5α-reductase inhibitor, which only acts on type 2 isozyme. Dutasteride is a 3-fold greater inhibitor of type-2 5α-reductase than finasteride in men and has greater effect on the type-1 than on type-2 isozyme. In animal models, dutasteride exhibited superior efficacy and pharmacokinetics compared to finasteride. In patients with benign prostate hyperplasia, administration of dutasteride was shown to dose-dependently decrease serum dihydrotestosterone levels with greater efficacy as compared to finasteride (95% *vs* 67%). Serum testosterone levels increased with both active drugs, in conjunction with dihydrotestosterone suppression but remained within normal ranges. In long term studies, in men with moderate to severe benign prostate hyperplasia, once daily dutasteride significantly reduced prostate volume, reduced the risk of acute urinary retention and surgery by 57% and improved lower urinary tract symptoms and urinary flow measurements. After oral administration, dutasteride is rapidly absorbed, has a short distribution phase and a mean bioavailability of 60%. The high volume of distribution, combined with its low linear clearance results in a prolonged dose dependent half-life (from 3 days at low concentrations to 5 weeks at high concentrations) whereas finasteride's half-life time is approximately 10 h. Dutasteride is well tolerated and the most occurring adverse events are impotence, decrease in libido, ejaculation disorders and gynaecomastia. Unlike α-blockers which primarily act acutely on benign prostatic hyperplasia symptoms, 5α-reductase inhibitors can alter disease progression. Concomitant administration of dutasteride did not affect the pharmacokinetics of either tamsulosin or terazosin. In addition, the tolerability of both terazosin and tamsulosin were improved during combination therapy.

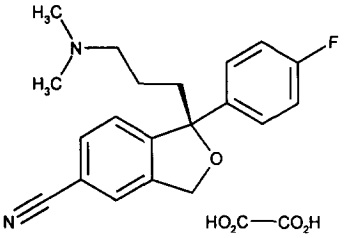
Ertapenem sodium (antibacterial) (42-44)



Country of Origin : UK
Originator : Astra Zeneca
First Introduction : US
Introduced by : Merck & Co.
Trade Name : Invanz
CAS Registry No. : 153773-38-3
Molecular Weight : 497.5

Ertapenem sodium was introduced in the US as a once daily intravenous or intramuscular injection for the treatment of adult patients with moderate to severe bacterial infections. This new 1-β-methyl carbapenem can be assembled from commercially available 4-nitrobenzyl protected β-methyl carbapenem enolphosphate and the appropriate thiol derivative. The last intermediate can be synthesized from a suitably N-protected 4-hydroxy proline derivative in a one pot operation involving bis activation of the carboxy and hydroxy groups, reaction with sodium sulfide yielding the corresponding thiolactone, aminolysis with 2-aminobenzoic acid and *N*-deprotection. This bacterial cell wall synthesis inhibitor has broad spectrum antimicrobial activity including common Gram-positive and Gram-negative aerobic pathogens and restricted activity against nosocomial pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter* species, methicillin-resistant staphylococci and enterococci. Ertapenem is resistant to a broad and extended spectrum of β-lactamases (excluding metallo-beta-lactamase) and is also more resistant than imipenem to human renal dehydroxypeptidase-I-inactivation (DHP-I). In drug-resistant strains of *P. aeruginosa*, resistance to ertapenem and imipenem was common but almost all strains remain susceptible to at least one antipseudomal agent. In phase III studies, ertapenem showed efficacy in the treatment of obstetric and gyneacological infections, skin and soft tissues infections, community-acquired pneumonia, urinary tract infections and in intra abdominal infections. Ertapenem has improved pharmacokinetics over currently available carbapenems and cephalosporins with an extended serum half-life of 4 h. The overall safety and tolerability profile of ertapenem was comparable to that seen with comparator antibacterials.

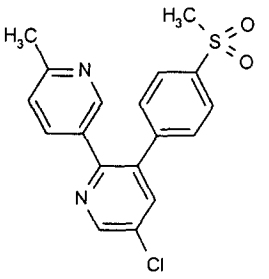
Escitalopram oxalate (antidepressant) (45-48)



Country of Origin : Denmark
Originator : Lundbeck
First Introduction : Switzerland, Sweden, UK
Introduced by : Lundbeck
Trade Name : Cipralex
CAS Registry No. : 219861-08-2
Molecular Weight : 414.44

Escitalopram was launched last year as Cipralex® in Switzerland, Sweden and UK for the treatment of depression and panic disorder. It is the *S*-enantiomer version of the selective serotonin reuptake inhibitor (SSRI) citalopram approved in 1989. It can be obtained from 5-cyanophthalide by successive reactions with 4-fluorophenyl magnesium bromide and 3-(dimethylamino)propyl magnesium chloride. The resulting racemic diol can be resolved by several routes such as crystallization with a chiral acid. Finally, a two step cyclisation procedure affords escitalopram. Escitalopram is twice as effective as the racemate and over 100 fold more potent than the *R*-enantiomer in inhibiting the 5-HT reuptake *in vivo* in rat brain synaptosomes. Moreover, it exhibits higher selectivity for the human serotonin transporter relative to the noradrenaline or dopamine transporters than any other currently available SSRI's. In the mouse forced swim test, the duration of immobility (which reflects antidepressant activity) for escitalopram was comparable to citalopram and greater than (*R*)-citalopram. Clinical trials in patients with panic disorders or depression have shown that escitalopram has a clinically relevant and significant effect. Additionally, it has a faster onset of antidepressant action than citalopram. Escitalopram has linear pharmacokinetics, with a long half-life (27-32 h). It is extensively metabolized in the liver via cytochromes P450 to *S*(+)-desmethyl and *S*(+)-didesmethyl citalopram. However, it has been shown to be a weak or negligible inhibitor of CYP450 drug-metabolizing enzymes *in vitro*. Escitalopram is well tolerated with nausea being the most common side effect.

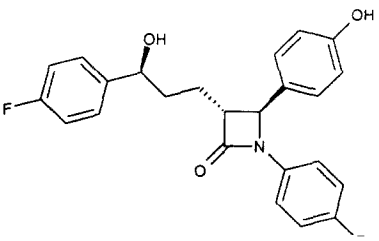
Etoricoxib (antiarthritic, analgesic) (49-52)



Country of Origin : USA
Originator : Merck & Co
First Introduction : Mexico
Introduced by : Merck & Co
Trade Name : Arcoxia
CAS Registry No : 202409-33-4
Molecular Weight : 358.85

Etoricoxib is a COX-2 inhibitor developed as a follow-up of rofecoxib for the treatment of osteoarthritis, rheumatoid arthritis, dysmenorrhoea, gout, ankylosing spondylitis and pain. Several processes describe the preparation of etoricoxib in 4 or 5 steps from 6-methylnicotinate. The key step is the novel pyridine construction using annulation of a ketosulfone with a vinamidinium synthon. In human whole blood, *in vitro*, the IC_{50} value obtained for inhibition of COX-2 is 1.1 μM as compared to 116 μM obtained for inhibition of COX-1. Thus, etoricoxib is the most selective COX-2 inhibitor to date, with a COX-1/COX-2 ratio of IC_{50} values of 106 for etoricoxib as compared to 35, 30, 7.6 for rofecoxib, valdecoxib and celecoxib, respectively. Its *in vivo* potency is generally comparable to that of rofecoxib in animal models against inflammation (carrageenan-induced paw edema), pyrexia (LPS-induced pyresis), pain (carrageenan-induced hyperalgesia) and arthritis (adjuvant-induced arthritis). Etoricoxib is well tolerated with dose-proportional pharmacokinetics. It has no effect on bleeding time or platelet aggregation. The gastrointestinal tolerability of etoricoxib is excellent as demonstrated by [^{51}Cr] models of excretion in rats and squirrel monkeys. Moreover, etoricoxib, unlike naproxen is not associated with significant inhibition of gastric mucosal PGE_2 synthesis compared to placebo. Etoricoxib is highly absorbed, has a t_{max} of 1.5 h and a half-life time of approximately 15 - 22 h. Five metabolites, weak inhibitors of COX-1 and COX-2 have been identified after renal excretion. Finally, although multiple CYP enzymes are involved in the metabolism of etoricoxib (CYP3A4 being the major contributor), etoricoxib is not a potent CYP3A4 inhibitor or inducer. In patients undergoing molar extraction, etoricoxib showed similar efficacy to naproxen sodium with a longer duration of analgesia than acetaminophen/codeine (approximately >24 h, 22 h and 5.2 h, respectively) and a better total pain relief score over 8 h. Similar efficacy of etoricoxib and naproxen was also seen in patients suffering of osteoarthritis. In the treatment of rheumatoid arthritis and ankylosing spondylitis, etoricoxib demonstrated significantly superior efficacy compared to naproxen and placebo. Etoricoxib did not affect the pharmacokinetics of prednisolone (*i.v.* or *p.o.*) and its co-administration with antacids showed insignificant effects on the maximal concentration and its absorption.

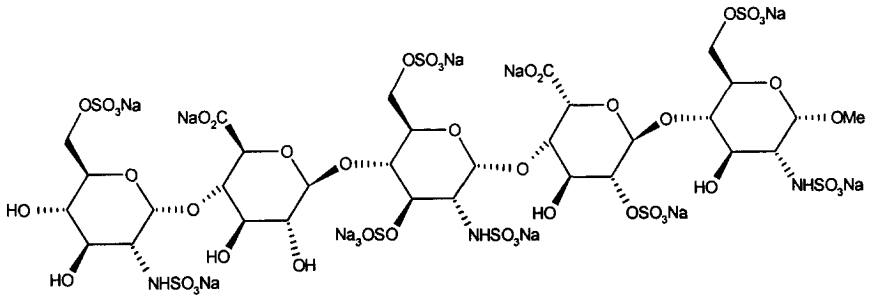
Ezetimibe (hypolipidemic) (53-59)



Country of Origin : USA
Originator : Schering-Plough
First Introduction : Germany
Introduced by : Merck
Trade Name : Ezetrol
CAS Registry No : 163222-33-1
Molecular Weight : 409.44

Ezetimibe is a once-daily orally active cholesterol absorption inhibitor, launched as a hypolipidemic agent. The one-step diastereo- and enantioselective formation of β-lactams starting from commercially available (3*S*)-hydroxy-γ-lactone is the key point of the asymmetric synthesis of ezetimibe. The 2-azetidinone class was initially designed as acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitors but experimental data suggest that this compound acts in the intestinal wall to inhibit cholesterol through a novel mechanism with an as yet undiscovered target. Orally administered ezetimibe inhibited increases in plasma cholesterol in four cholesterol-fed animals species (hamster, rats, dogs and rhesus monkeys). In rats cannulated in the intestine and bile duct, [^{3}H]-ezetimibe inhibited cholesterol absorption by more than 95%. In cholesterol-fed LDL receptor+apoE knockout mice, treatment with ezetimibe reduced atherosclerotic lesion cross sectional area by 48% in the aorta and 20% in the carotid artery. Moreover, the plasma cholesterol levels were reduced and the progression of lesions was inhibited. Ezetimibe is highly protein bound and is metabolized by the liver to its glucuronide metabolite, which represents 80-90% of circulating ezetimibe. About 90% of ezetimibe and/or the glucuronide metabolite are excreted in the feces and 10% in the urine. The parent compound and its glucuronide metabolite undergo enterohepatic recirculation; in consequence, the drug is slowly eliminated. In hypercholesterolemic patients, ezetimibe (10 mg/day, 12 weeks) reduced LDL cholesterol by 18% and total cholesterol by 12%, with a similar safety profile to placebo. Co-administration of ezetimibe with statins or fenofibrate lowered LDL cholesterol levels more than either monotherapy. Ezetimibe was well tolerated and interaction studies provided evidence that ezetimibe had no significant effect on the activity of major CYP450 drug-metabolizing enzymes. Moreover, no pharmacokinetic/pharmacodynamic interactions were seen between ezetimibe and statins and others frequently administered drugs.

Fondaparinux sodium (antithrombotic) (60-63)



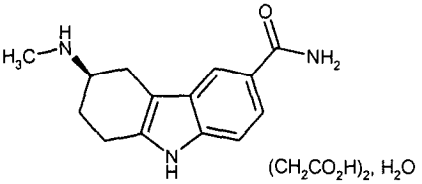
Country of Origin :	France	Trade Name :	Arixtra
Originator :	Sanofi-Synthelabo	CAS Registry No. :	114870-03-0
First Introduction :	US	Molecular Weight :	1728.08
Introduced by :	Akzo Nobel (Organon) and Sanofi-Synthelabo		

Fondaparinux sodium was first introduced in the US for prophylaxis of deep vein thrombosis which may lead to pulmonary embolism following major orthopaedic surgery. Fondaparinux is the first of a new class of antithrombic agents distinct from low molecular weight heparin (LMWH) and heparin. This entirely synthetic molecule is a copy of the heparin pentasaccharide sequence, the shortest fragment able to catalyze antithrombin III-mediated inhibition of factor Xa thereby inhibiting thrombin generation without antithrombin action. Fondaparinux does not display significant effects on coagulation tests (such as

activated partial thromboplastin time and prothrombin time), does not bind to platelet factor 4 or promote heparin-induced thrombocytopenia. In phase III studies, fondaparinux significantly reduced the incidence of thromboembolism following orthopedic surgery, with an overall risk reduction of 50% in comparison to the LMWH, enoxaparin. Following subcutaneous administration, fondaparinux has a nearly complete bioavailability, a rapid onset of action, a prolonged half-life (17.2 h) enabling once daily dosing and is not metabolized preceeding renal excretion. The drug appears to be generally safe, with haemoragic complications either comparable to or higher than those for LMWH.

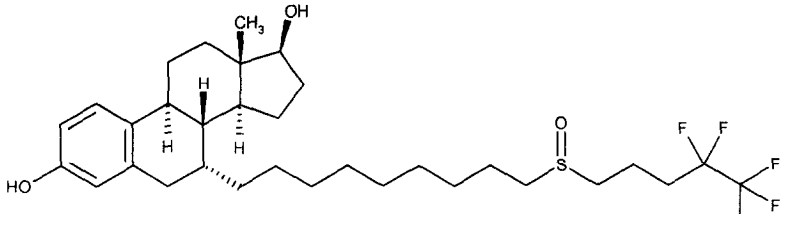
Frovatriptan (antimigraine) (64-69)

Country of Origin : UK
Originator : GlaxoSmithKline/Vernalis
First Introduction : USA
Introduced by : Elan
Trade Name : Frova
CAS Registry No : 158930-09-7
Molecular Weight : 379.42



Frovatriptan succinate was launched as an oral treatment for acute migraine attacks with or without aura in adults. It is the eighth member of the "triptan" class. Frovatriptan is a conformationally-restricted analog of the 5-HT_1-receptor agonist 5-carboxytryptamine which can be prepared in six steps. The key intermediate (*R*)-6-cyano-3-N-methylamino-1,2,3,4-tetrahydrocarbazole is obtained by Fischer reaction of 4-cyanophenylhydrazine with the appropriate ketone followed by resolution using L-pyroglutamic acid. This drug acts as a dual 5-HT_{1D}/$_{1B}$ receptor partial agonist and has high and selective affinity for 5-HT_{1B} and 5-HT_{1D} receptors in cranial vessels. It has no significant activity at 5-HT_2, 5-HT_3, 5-HT_4, α-adrenergic, histaminergic or $GABA_A$ receptors. Frovatriptan is also a moderately potent full agonist at 5-HT_7 receptors, which have a dilatory action and are expressed in the human coronary artery. *In vitro* studies appear to indicate frovatriptan's functional selectivity for cerebral circulation as shown by the concentrations needed to induce threshold contractile activity and maximum response in basilar arteries as compared with coronary arteries. Frovatriptan is mainly metabolized by CYP1A2 and most of its metabolites are excreted renally. Co-administration of frovatriptan with the monoamine oxidase-A inhibitor moclobemide or with the potent CYP1A2 inhibitor fluvoxamine did not affect its pharmacokinetics parameters. Although frovatriptan has a poor bioavailability (24-30%), it has a very long half-life compared to other triptans (25 h) and has an onset of action and efficacy similar to those of naratriptan. The most striking features of this drug are the low headache recurrence rate, which is one of the lowest among the triptans and which may be attributed to its long half-life, and excellent tolerance profile. No significant effect on the cardiovascular system was seen after administration of a single oral dose of 14 fold the therapeutic dose of frovatriptan.

Fulvestrant (anticancer) (70-73)

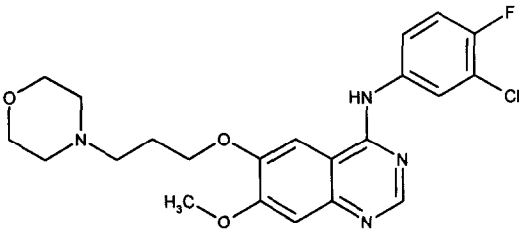


Country of Origin :	UK	Trade Name :	Faslodex
Originator :	Astra Zeneca	CAS Registry No. :	129453-61-8
First Introduction :	US	Molecular Weight :	606.79
Introduced by :	Astra Zeneca		

Fulvestrant was launched in the US as a novel once monthly injectable steroidal estrogen antagonist for the treatment of hormone receptor positive metastatic breast cancer in postmenopausal women with disease progression following estrogen therapy. This 7α-alkylsulphinyl derivative of estradiol can be prepared in 10 steps from 6,7-didehydro-19-nortestosterone by successive conjugate addition of the organocuprate derived from O-protected 9-bromononan-1-ol followed by aromatization of the resulting enone, then activation of the protected primary alcohol, substitution with 4,4,5,5,5-pentafluoropentanthiol and oxidation to the sulfoxide. Fulvestrant is the first "pure" estrogen antagonist from a novel class known as selective estrogen receptor down regulators (SERDs). It binds to the estrogen receptor (ER), with affinity close to that of estradiol and 100 fold greater than that of tamoxifen (a partial estrogen antagonist), preventing estrogen-stimulated gene activation, thereby interfering with the estrogen-related processes essential for cell-cycle completion. Fulvestrant also appears to downregulate the ER by 80-90% often to non detectable level both *in vitro* and *in vivo*. In comparison to tamoxifen, fulvestrant is devoid of systemic estrogenic activity, it displays no uterotrophic activity and is able to block the uterine stimulation of estradiol or tamoxifen. Furthermore, fulvestrant completely blocks the cell growth in tamoxifen-resistant breast cancer cell-lines and prevents growth of tamoxifen resistant tumor in mice. In clinical trials, it was also shown that fulvestrant is comparable to anastrozole (a third generation aromatase inhibitor) both in efficacy and tolerability in postmenopausal women with tamoxifen-resistant advanced breast cancers.

Gefitinib (antineoplastic) (74-77)

Country of Origin :	UK
Originator :	Astra Zeneca
First Introduction :	Japan
Introduced by :	Astra Zeneca
Trade Name :	Iressa
CAS Registry No. :	184475-35-2
Molecular Weight :	446.91



Gefitinib was introduced in Japan as a daily oral monotherapy for the treatment of inoperable or recurrent non-small cell lung cancers (NSCLC). This anilinoquinazoline derivative can be synthesized in 6 steps starting from 6,7-dimethoxyquinazolin-4(3H)-one by successive monodemethylation/acetylation of the 6-hydroxy-group followed by chlorination and reaction with 3-chloro-4-fluoroaniline, finally deacetylation and alkylation with 3-(4-morpholinyl)propylbromide complete the synthesis. Gefitinib reversibly inhibits the activity of the epidermal growth factor receptor tyrosine kinase (EGRF TK). This inhibits autophosphorylation of EGRF and blocks the cascade of intracellular events which have been implicated in the proliferation, survival and metastasis of cancer cells. Gefitinib diplays good selectivity for the EGRF TK relative to other growth factors in human umbilical endothelial cells. It is similarly selective relative to other kinases, for example c-erB2. Data from two large phase II studies in patients with pretreated NSCLC have shown that gefitinib induces a response rate approaching 20% in patients receiving the agent as a

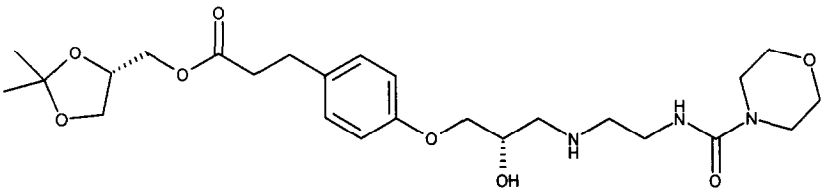
second line therapy and approximately 10% in those pretreated with more lines of chemotherapy. Gefitinib has good bioavailability and is metabolized in the liver via the cytochrome P450 3A4 enzyme system with a mean elimination half life of 28 h. Gefitinib has been generally well tolerated in cancer patients with predominant side effects being acne-like skin-rash, diarrhea, nausea, vomiting and mild to moderate myelosuppression.

Ibritumomab tiuxetan (anticancer) (78- 81)

Country of Origin :	US	Class :	Monoclonal antibody
Originator :	IDEC	Type :	murine anti (human CD20) radioimmunoconjugate
First Introduction :	US		
Introduced by :	Syncor	Molecular Weight :	148 kDa
Trade Name :	Zevalin	Expression system :	CHO cells
CAS Registry No :	206181-63-7	Manufacturer :	Syncor

Radioimmunotherapy (RIT) is a new treatment modality for B-cell non-Hodgkin's lymphoma (NHL). The goal of RIT is to deliver ionizing radiation selectively to tumors while minimizing radiation absorbed in normal tissues. ^{90}Y-Ibritumomab tiuxetan is the first commercially available radiolabeled antibody for cancer therapy and more specifically for the treatment of relapsed or refractory low-grade, follicular, or transformed B-cell NHL including patients with rituximab-refractory follicular NHL. Ibritumomab is a murine immunoglobulin G1 kappa isotype monoclonal antibody produced in Chinese hamster ovary cells. It targets CD20, a B-lymphocyte antigen. The purified antibody is subsequently reacted with the isothiocyanatobenzyl derivative of DTPA to form ibritumomab tiuxetan. Tiuxetan forms a stable covalent urea type bond with the antibody and can chelate a radionuclide via its five carboxyl groups: either indium-111 for imaging (medium energy gamma emitter) or yttrium-90 for radiotherapy (pure high-energy beta-emitter, mean=0.94 MeV). Rituximab (Rituxan®, MabThera) is an unlabeled chimeric antibody also directed against CD20. The Zevalin™ therapeutic regimen starts with the imaging protocol: infusion of 250 mg/m^2 rituximab to clear peripheral B-cells and improve targeting of radioisotope to tumor cells, followed by 5 mCi ^{111}In-Zevalin™ for whole body imaging to enable determination of favorable biodistribution of radiolabeled antibody. The therapeutic dose (0.3-0.4 mCi/kg) of ^{90}Y-Zevalin™ is delivered on days 7-9 following another predosing of 250 mg/m^2 rituximab. The pure beta-emitting ^{90}Y can be given with few radiation precautions. It has a long path length (χ_{90}=5 mm) allowing the delivery of a cytotoxic radiation dose to tumor cells more distant to the antibody-bound cell. Its short half-life (64 h) approximates the biological half-life of the radiolabeled antibody (47 h) which may minimize radiotoxicity to nontarget organs. The non tumor distribution is primarily to the bone. In a phase III clinical trial of 143 patients with relapsed or refractory low-grade, follicular, or CD20-positive transformed B-cell NHL, Zevalin™ combined with Rituxan® showed an overall response rate (ORR) of 80%, compared to Rituxan® alone which gave an ORR of 56%. Also, 30% of Zevalin™-treated patients achieved complete responses compared to 16% of Rituxan® patients.

Landiolol (antiarrhythmic) (82-85)

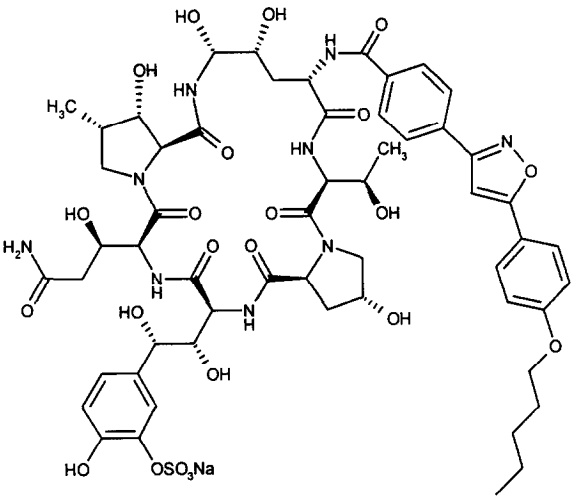


Country of Origin : Japan
Originator : Ono Pharmaceutical
First Introduction : Japan
Introduced by : Ono Pharmaceutical

Trade Name : Onoact
CAS Registry No. : 133242-30-3
Molecular Weight : 509.60

Landiolol was launched last year as *iv* infusion for the treatment of tachyarrhythmia during surgery. This structurally related derivative of esmolol can be synthesized in 3 linear steps from 3-(4-hydroxyphenyl)propionic acid by successive esterification followed by alkylation of the phenol function with (*2S*)-glycidyltosylate and opening of the resulting epoxide by the appropriate amine. Landiolol is an ultra short acting β1-adrenergic blocker more cardioselective (β1/ β2 = 255) than esmolol (β1/ β2 = 32). It showed 6-8 times greater efficiency compared to esmolol in reducing isoproterenol-induced increase in heart rate and ventricular contraction in anesthetized dogs. In clinical trials, landiolol was effective against a variety of arrhythmias with efficacy seen in patients with atrial fibrillation, proxysmal supraventricular tachycardia, ventricular tachycardia and premature complexes. Landiolol produced a dose-related pharmacokinetic behavior, has a rapid onset of action (10 min.) and is rapidly hydrolyzed to inactive acidic metabolites by esterases after *iv* administration. This results in an ultra-short half-life (approx. 3 min.) and β-blocade, allowing rapid termination of the drug effect by termination of infusion if side effects occur. Hypertension was the most frequent adverse event and resolved in less than 30 min. after drug withdrawal.

Micafungin (antifungal) (86-88)



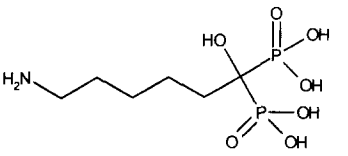
Country of Origin : Japan
Originator : Fujisawa
First Introduction : Japan
Introduced by : Fujisawa
Trade Name : Funguard
CAS Registry No. : 235114-32-6
Molecular Weight : 1292.30

Micafungin, the second member of the echinocandin class of antifungal agents was launched last year in Japan for the parenteral treatment of various fungal infections caused by *Aspergillus* and *Candida* spp. such as fungaemia and respiratory and gastrointestinal mycoses. This water-soluble semisynthetic cyclic lipopeptide is synthesized by acylation with (5-(4-pentyloxyphenyl)isoxazol-3-yl)benzoate of the cyclic peptide nucleus (FR-179642),

obtained by enzymatic cleavage of the naturally occurring echinocandin FR-901379, derived from the fungus *Coleophoma empedri*. Micafungin acts by inhibiting the synthesis of 1,3-beta-glucan, an essential polysaccharide of the cell wall of many pathogenic fungi. Micafungin has a marked fungicidal effect on almost all species of *Candida*, including fluconazole-resistant spp. *C. albicans*, *C. glabrata*, *C. Krusei*, *C. parapsilosis* and *C. tropicalis* and a fungistatic effect on a range of *Aspergillus* species including *A. flavus*, *A. fumigates* and *A. terreus*. Like caspofungin, micafungin is inactive against *Cryptococcus neoformans*, and the emerging pathogen *Trichosporon cutaneum* and *Fusarium solani*. Micafungin has proved highly effective in mouse models of *Candidiasis* and *Aspergillus* infections (including those using an amphotericin B- and itraconazole-resistant isolate of *A. fumigatus*). In phase I studies, micafungin had linear pharmacokinetics with an elimination half-life ranging from 11.7 to 15.2 h after injection and was well tolerated.

Neridronic acid (Hypocalcaemic) (89-94)

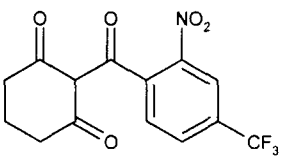
Country of Origin : Italy
Originator : Instituto Gentili
First Introduction : Italy
Introduced by : Abiogen (Merck and Co)
Trade Name : Nerixia
CAS Registry No. : 79778-41-9
Molecular Weight : 277.15



Neridronic acid was launched in Italy as intravenous or intramuscular injection for the treatment of osteogenesis imperfecta. It is the first drug launched worldwide for this "orphan disease" characterized by a fragility of the bone. Sufferers risk frequent fractures commencing during infancy, eventually leading to bone deformation and a severe deterioration of the quality of life and life expectancy. Neridronic acid is structurally related to the second generation of bisphosphonates characterized by an amino terminal group and can be synthesized by treating ε-aminocaproic acid with phosphoric acid and phosphorous trichloride. Bisphosphonates, synthetic analogs of the endogenous mineral deposition inhibitor pyrophosphate, are extremely potent inhibitors of bone resorption and increase bone mineral density, possibly by inhibiting the activity of osteoclasts and promoting the death of these bone-eroding cells. *In vitro*, neridronic acid was a poor inhibitor of phagocytosis, but significantly and dose dependently inhibited β-glucuronidase release and superoxide anion production in the rat peritoneal macrophage, two parameters that may have a part in bone resorption. In a clinical study on patients with osteogenesis imperfecta, neridronic acid increased remarkably bone mass in young growing individuals. In adults the changes were less important but still significant.

Nitisinone (antityrosinaemia) (95-101)

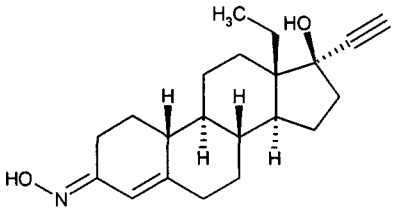
Country of Origin : UK
Originator : AstraZeneca
First Introduction : USA
Introduced by : Rare Disease Therapeutics
Trade Name : Orfadin
CAS Registry No : 104206-65-7
Molecular Weight : 329.23



Nitisinone was originally developed as a pesticide and then launched as an adjunct to dietary restriction of tyrosine and phenylalanine for the treatment of hereditary tyrosinaemia type I. In this inborn error of metabolism, fatal liver disease results either from liver failure during infancy or early childhood or from development of hepatocellular carcinoma during childhood or adolescence. This is caused by accumulation of toxic metabolites due to deficiency of fumarylacetoacetase, the last enzyme of the tyrosine catabolic pathway. Nitisinone, which acts as an inhibitor of the 4-hydroxyphenylpyruvate dioxygenase, prevents the formation of toxic metabolites such as succinylacetoacetate in the liver. Administration of a single dose of nitisinone in mice showed a rapid, significant and persistent inhibition of 4-hydroxyphenylpyruvate dioxygenase. In a murine model of tyrosinaemia type I, administration of nitisinone abolished acute liver failure. Additional dietary tyrosine restriction in the same model on long term follow-up (> 2 years) showed complete correction of liver function tests and succinylacetone levels, and cancer-free survival improvements when compared to historical controls. In healthy volunteers, nitisinone was well tolerated, peak plasma concentrations were rapidly attained following a single dose of 1 mg/kg and the half-life time was approximately 54 h. Following the administration of nitisinone (1 mg/kg), the concentrations of tyrosine in plasma increased, were still 8 times those of background at 14 days after dosing, but had returned to background levels within 2 months of the second dose. Elevated tyrosine levels are a potential risk of corneal opacities. No treatment related corneal lesions were seen after administration of high dose of nitisinone in mice. In children diagnosed when they were less than 2 months old, when nitisinone treatment was combined with a restricted diet, the four-year survival rate was 88%, compared to 29% from historical data of children treated with restricted diet alone. So, there is some clear evidence that nitisinone treatment associated with restricted diet can reduce the risk of early hepatocellular carcinoma when started before two years of age. On the contrary, in patients with late start of nitisinone treatment there is a considerable risk of liver malignancy. Even if 10% of patients have not clinically responded to nitisinone, studies have shown that oral nitisinone treatment plus dietary restriction has greatly improved the survival of patients and reduced the need of liver transplantation during early childhood.

Norelgestromin (contraceptive) (102-104)

Country of Origin : USA
Originator : Johnson & Johnson
First Introduction : USA
Introduced by : Ortho-McNeil
Trade Name : Ortho Evra
CAS Registry No : 53016-31-2
Molecular Weight : 327.47

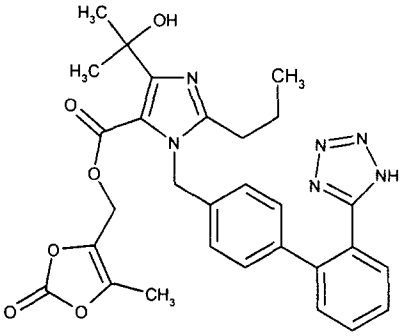


Ortho Evra® is the first birth control transdermal patch and contains a combination of norelgestromin and ethinylestradiol. The patch can be applied to the buttocks, lower abdomen, upper torso or arms; changed weekly for 3 weeks, followed by a patch-free week. Norelgestromin is the active metabolite produced following oral administration of norgestimate, the progestin component of the contraceptive, Ortho-Cyclen®. The half-life value of norelgestromin was approximately 28 h. Following application, norelgestromin rapidly appeared in the serum, reached a plateau by approximately 48h and was maintained at an approximate steady state throughout the wear period. Hepatic metabolites of norelgestromin included norgestrel and various hydroxylated and

conjugated metabolites that were eliminated by renal and fecal pathways. Ortho Evra produced ovarian suppression and cycle control. It was significantly more effective in suppressing follicular development than the leading oral contraceptive Triphasil® (ethinyl estradiol/levornorgestrel); was as effective in preventing pregnancies and had similar tolerability profile. The patch offers 99% efficacy when used appropriately, as shown by three clinical trials. However, a statistically significantly greater proportion of pregnancies occurred among women weighing 90 kg or more. Ortho Evra® was well tolerated, with 2.6% of women discontinuing treatment due to mild to moderate reactions at the site of application. Thus, the patch combined the efficacy of oral contraceptives with the convenience of just once-weekly dosing.

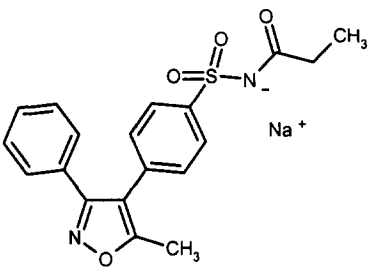
Olmesartan Medoxomil (antihypertensive) (105-107)

Country of Origin : Japan
Originator : Sankyo
First Introduction : US
Introduced by : Sankyo and Forest
Trade Name : Benicar
CAS Registry No : 144689-63-4
Molecular Weight : 558.22



Olmesartan medoxomil was launched last year in the US as benicar®, an orally administered treatment for hypertension. This ester prodrug of olmesartan can be synthesized in 8 steps from diaminomaleonitrile by successive reactions with trialkylorthopropanoate to access 2-propyl-imidazole-4,5-dicarbonitrile, conversion of the two nitrile functions to the corresponding ethyl esters, followed by methylmagnesium bromide addition to give the corresponding 4-(1-hydroxyalkyl)imidazole derivative. The latter is alkylated with the trityl-protected biphenyl tetrazole derivative. Finally detritylation and alkaline hydrolysis leads to olmesartan, that is esterified to its corresponding prodrug. Olmesartan, is a new selective and competitive nonpeptide angiotensin II type 1 receptor antagonist and potently inhibits the Ang.II-induced pressor responses. The drug competitively inhibited binding of [^{125}I]-AII to AT_1 receptors in bovine adrenal cortical membranes, but had no effect on binding to AT_2 receptors in bovine cerebellar membranes. In comparative clinical studies in patients with essential hypertension, olmesartan reduced sitting cuff diastolic blood pressure significantly more than losartan, valdesartan and ibesartan, while reductions in systolic blood pressure were similar for all treatments. Olmesartan medoxomil was also shown to reduce blood pressure significantly more effectively than losartan and the ACE inhibitor captopril and as effectively as the β-bloker atenolol. Olmesartan medoxomil is rapidly and completely bioactivated by ester hydrolysis to its active metabolite, olmesartan, during absorption from the gastrointestinal tract. Olmesartan has an absolute bioavailability of approximatively 26%, a mean elimination half-life of 14 h in patients with hypertension and is not further metabolized. Olmesartan medoxomil is well tolerated, has a side-effect profile similar to that of placebo and unlike ACE inhibitors, the incidence of dry cough is rare.

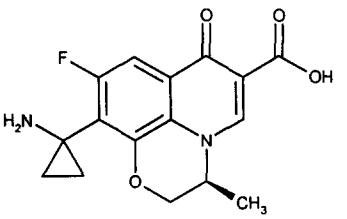
Parecoxib sodium (analgesic) (108-113)



Country of Origin : USA
Originator : Pharmacia (Searle)
First Introduction : UK
Introduced by : Pharmacia
Trade Name : Dynastat
CAS Registry No : 197502-82-2
Molecular Weight : 392.41

Parecoxib sodium is an injectable COX-2 inhibitor, launched as an anti-inflammatory agent and for the management of acute pain. Parecoxib is an amide prodrug of Pharmacia's valdecoxib also launched last year. It can be administered by either *i.m.* or *i.v.* routes, in contrast to other currently available COX-2 inhibitors which are poorly water-soluble. Parecoxib sodium can be prepared from valdecoxib by acylation of the sulfonamide with propionic anhydride. Parecoxib sodium exhibited potent acute and chronic antiinflammatory activity in rats, as demonstrated in the carrageenan air pouch model, where 98% of inhibition was achieved with the dose of 0.3mg/kg, and in the adjuvant arthritis model (ED_{50} = 0.08mg/kg). Moreover, in the carrageenan footpad edema model, parecoxib sodium showed excellent efficacy (ED_{50} = 5mg/kg) and a rapid onset of action comparable with the most potent analgesic ketorolac. In this model, it produced a complete blockade of the carrageenan-induced hyperalgesia within 1 h after *i.v.* administration. Pharmacokinetic studies indicated that parecoxib sodium completely and rapidly converted in valdecoxib with bioequivalence found between parenterally administered parecoxib sodium and orally administered valdecoxib. The maximum plasma concentrations of valdecoxib were 25-30% greater after *i.v.* than *i.m.* administration of parecoxib and t_{max} was respectively 0.5 h after *i.v.* and 1.5 h after *i.m.* administration. Parecoxib sodium had greater gastrointestinal tolerance than ketorolac and had no significant effect on platelet aggregation. Parecoxib sodium was as effective as ketorolac or even superior to morphine and placebo in the treatment of severe pain (such as that observed following gynaecologic or orthopaedic surgery). Mean times to rescue medication were longer with parecoxib compared to morphine. Parecoxib sodium works 12-14 min after *i.v.* administration and a dose of 40 mg provides good or excellent relief of pain in approximately 90% of patients. Thus, parecoxib sodium is a non-narcotic, specific COX-2 inhibitor, which provides potent analgesic ability and fulfils some important requirements for the pain management in the post-operative period such as *i.v.* formulation, rapidity of action, and short half-life. Furthermore, although valdecoxib is a substrate for CYP3A4 as other drugs used in the perioperative period, it does not interfere either with the metabolism of the sedative/tranquilizer, midazolam, or with the intravenous anesthetic propofol.

Pazufloxacin (antibacterial) (114-121)

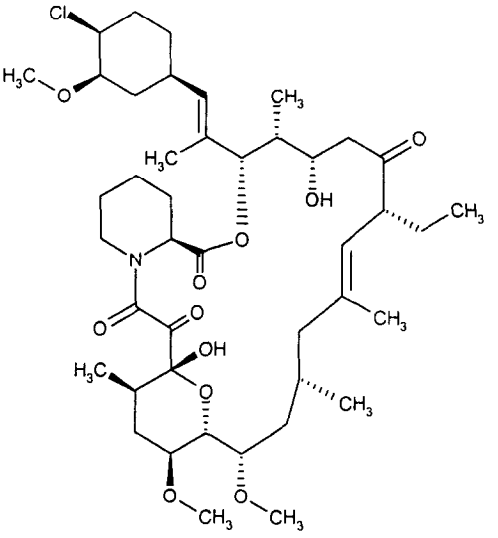


Country of Origin : Japan
Originator : Toyama
First Introduction : Japan
Introduced by : Toyama and Mitsubishi (formely Welfide)
Trade Name : Pasil, Pazucross
CAS Registry No : 127045-41-4
Molecular Weight : 318.30

Pazufloxacin is a novel quinolone marketed for the treatment of bacterial infections in Japan. This tricyclic fluoro-quinolone can be synthesized in 11 steps from commercially available 2,3,4,5-tetrafluorobenzoic acid. The cyclopropyl substituent is first introduced in 6 steps including 4-F-substitution with *tert*-butylcyanoacetate, decarboxylation, αα alkylation with 1,2-dibromoethane, partial nitrile hydrolysis and Hoffmann-rearrangement. The pyridoxazine ring is then introduced in 5 steps including β-ketoester formation and pryridoxazine annulation. Pazufloxacin displays a broad spectrum activity against Gram-positive and Gram-negative bacteria, although it is less active that ciprofloxacin against pneumococci and is not active against ciprofloxacin-resistant isolates. In patients with gonococcal urethritis a high prevalence of fluoroquinolone-resistant *N. gonorrhoeae* isolates with the Ser-91-to-Phe mutation in GyrA was observed. However, good clinical responses have been seen in clinical trials of patients with urinary tract infections and to a lesser extent with respiratory tract infections. Pazufloxacin is mainly excreted in urine with a short half-life (2-2.5 h). It has a phototoxicity equal to that of ciprofloxacin and its adverse effect profile resembles that of other quinolones.

Pimecrolimus (immunosuppressant) (122-129)

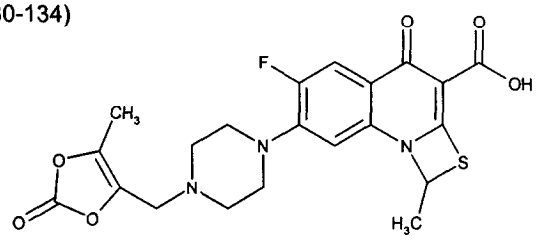
Country of Origin : USA
Originator : Novartis
First Introduction : USA
Introduced by : Novartis
Trade Name : Elidel
CAS Registry No : 137071-32-0
Molecular Weight : 810.46



Pimecrolimus is an ascomycin macrolactam derivative, developed as a topical formulation (1% cream) for the treatment of mild to moderate atopic dermatitis for patients aged two years and over in whom the use of conventional therapies is inadvisable. Pimecrolimus is an inflammatory cytokine inhibitor that works by selectively targeting T-cells in the skin. It inhibits *in vitro* the production and release of pro-inflammatory cytokines after antigen-specific or non-specific stimulation in T cells and mast cells. Pimecrolimus binds specifically to cytosolic receptor macrophilin-12 at nanomolar concentrations leading to inhibition of the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin. Pimecrolimus is a chlorine derivative of the known FK-520, from which it can be synthesized. In a pig model of dinitrofluorobenzene-induced allergic contact dermatitis, pimecrolimus was shown to inhibit erythema and induration, had equivalent efficacy compared to clobetasol-17-propionate, but did not cause atrophogenic effects. In a mouse model of allergic contact dermatitis, pimecrolimus given orally was as potent as tacrolimus and more effective than cyclosporin. The agent also decreased the intensity of cutaneous manifestations in an atopic dermatitis model involving hypomagnesemic hairless mice. Both in adults and in pediatric patients with atopic dermatitis, treatment with pimecrolimus has demonstrated greater efficiency than conventional treatment in reducing the incidence of disease flares, as well as the use of second-line corticosteroids. Moreover, in a 26 week study, in pediatric patients (2-17 years), from 65% of subjects showing improvement, 85% were cleared of

the disease. Oral pimecrolimus administration resulted in an elimination half-life of about 30 to 40 h. It is not metabolized or degraded during skin permeation after topical administration. However, following oral administration, it is metabolized *via* the liver CYP3A4 pathway and excreted mainly in the feces. Pimecrolimus is well tolerated; no systemic accumulation is seen and the most commonly reported side effect is reaction at the site of application. Moreover, pimecrolimus did not cause skin atrophy in contrast to corticosteroids. At this time, as a replacement therapy for topical corticosteroids, the only competing agent for pimecrolimus is topical tacrolimus (ointment). Preliminary studies demonstrated comparable efficacy and safety between these two agents.

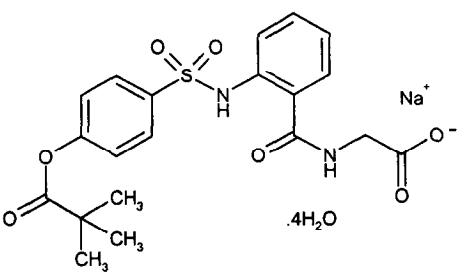
Prulifloxacin (antibacterial) (130-134)



Country of Origin : Japan
Originator : Nippon Shinyaku
First Introduction : Japan
Introduced by : Nippon Shinyaku and Meiji Seika
Trade Name : Sword
CAS Registry No. : 123447-62-1
Molecular Weight : 461.50

Prulifloxacin was the third fluoroquinone to be launched last year. It was introduced in Japan as an oral treatment for urinary tract infections (UTIs), respiratory tract infections (RTIs) and bacterial pneumoniae. It can be synthesized in 10 steps from commercially available 3,4-difluoroaniline. Key steps involve the cyclization of 6,7-difluoro-4-hydroxy-2-thioquinoline-3-carboxylic acid ethyl ester with 1,1-dibromomethane to give the corresponding thiazeto-[3,2a]quinoline. Aromatic nucleophilic substitution of the 7-fluoro atom with piperazine followed by hydrolysis of the ethyl ester and finally alkylation of the piperazinyl moiety with 4-(bromomethyl)-5-methyl-1,3-dioxol-2-one complete the synthesis. Prulifloxacin is a lipophilic prodrug, which is rapidly hydrolyzed to the corresponding N-dealkylated piperazine, NM 394, by paraoxonase type enzymes in blood and liver following intestinal absorption. The DNA gyrase inhibitor NM 394 accounts for all antimicrobial activity: it shows a similar or greater activity against gram-positive bacteria compared to ciprofloxacin, and a greater activity in the case of gram-negative bacteria. In clinical studies, prulifloxacin has shown good efficacy against UTIs and RTIs. The drug is mainly excreted in the urine and in the feces as unchanged NM 394, which has a plasma half-life of approximately 8 h. Phototoxicity in animal models is less severe than with other quinolones. Prulifloxacin is well tolerated with an adverse effect profile similar to that of other fluoroquinolones.

Sivelestat (antiinflammatory) (135-141)

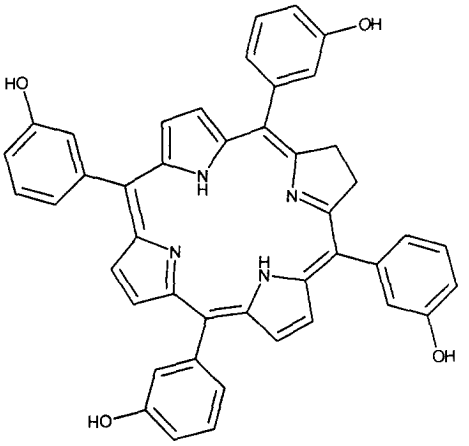


Country of Origin : Japan
Originator : Ono Pharmaceutical
First Introduction : Japan
Introduced by : Ono Pharmaceutical
Trade Name : Elaspol
CAS Registry No : 201677-61-4
Molecular Weight : 528.53

Sivelestat is an acyl enzyme inhibitor of neutrophil elastase, developed as an injectable formulation for the treatment of acute lung injury associated with systemic inflammatory response syndrome. A neutrophil predominant inflammation associated with excessive release of human neutrophil elastase (HNE) from azurophilic granules is capable of damaging both the lung parenchymal cells and the extracellular matrix allowing an alveolar capillary barrier disruption. Sivelestat is a sulfonanilide-containing pivaloyloxy benzene derivative prepared in a three step synthesis. This agent acts as a reversible and selective inhibitor of HNE with an IC_{50} value of 0.044 μM. Sivelestat has exhibited potent protective effects against various causes of lung injuries in animal models. In an acid-induced acute lung injury model in conscious hamster, administration of sivelestat for 48 h following HCl instillation, dose-dependently reduced mortality and significantly improved the protein levels in bronchoalveolar lavage fluids and pulmonary artery pressure. In a similar study, the agent inhibited the endotoxin-induced acute lung dysfunctions (marked elevation of pulmonary vascular permeability, leukocyte migration, hemorrhage and parenchymal injury) in different animal species. Moreover, in a cardiopulmonary bypass model in dog, sivelestat ameliorated the respiratory index and interstitial-intra-alveolar edema. Sivelestat has a relatively poor bioavailability, due to an extensive first-pass metabolism and is easily hydrolyzed *in vitro* to an inactive metabolite. In two animal species, the half-life time was approximately 5-7 min. Clinical trials have shown that treatment with the agent improves respiratory function and facilitates early removal of patients from mechanical ventilation. However, in the last clinical study, conducted by Eli Lilly in patients with acute lung injury, no difference in mortality and safety was seen between sivelestat and placebo.

Temoporphin (antineoplastic/photosensitizing) (142-145)

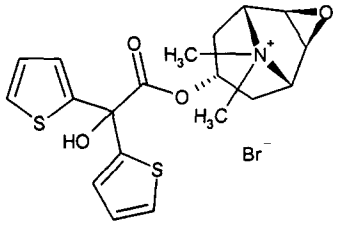
Country of Origin : UK
Originator : Quanta Nova
First Introduction : UK
Introduced by : Quanta Nova
Trade Name : Foscan
CAS Registry No. : 122341-38-2
Molecular Weight : 680.75



Temoporphin, a second generation photosensitizer, was launched last year in UK for the photodynamic therapy (PDT) of advanced head and neck cancers. This porphyrin derivative can be synthesized from pyrrole and 3-hydroxybenzaldehyde. The pharmacological activity is initiated, 4 days after intravenous injection, by laser-light photoactivation of temoporphin, that has selectively accumulated in cancer tissues. The resulting generation of highly reactive oxygen species leads to malignant cells death thereby inducing tumor necrosis up to a depth of 15 mm. An advantage of temoporphin over other photosensitizing agents is its extreme sensitivity to wavelengths of light that penetrate tissues, resulting in lower light/dose and irradiation time. PDT with intravenous temoporphin has produced relatively high complete and partial response rates in head and neck cancers, with higher response rates generally observed with higher light dose. Temoporphin is well-tolerated and does not preclude surgery/radiotherapy as a later option. Adverse effects were photosensitivity and pain at the injection site.

Tiotropium bromide (bronchodilator) (146-150)

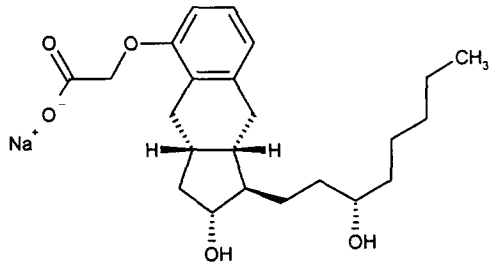
Country of Origin : Germany
Originator : Boehringer Ingelheim
First Introduction : Netherlands / Philippines
Introduced by : Boehringer Ingelheim / Pfizer
Trade Name : Spiriva
CAS Registry No : 136310-93-5
Molecular Weight : 472.42



Tiotropium bromide is a long-acting inhaled muscarinic antagonist, developed for the once-daily treatment of chronic obstructive pulmonary disease. Tiotropium bromide can be prepared in three steps. The Grignard condensation of 2-thienyl magnesium bromide with oxalic acid dimethyl ester, followed by a transesterification with scopine provided the ester which was quaternized with methyl bromide. Tiotropium bromide binds to human recombinant muscarinic receptors M_1-, M_2- and M_3-subtypes with high and similar affinity, comparable to those obtained with ipratropium. Tiotropium bromide is characterized by its novel property of kinetic selectivity : while ipratropium rapidly dissociated from each of the receptor subtypes, tiotropium dissociated rapidly from M_2 receptors ($t_{1/2}$=3.6 h) but slowly from M_1 ($t_{1/2}$=14.6 h) and M_3 ($t_{1/2}$=34.7 h) receptors. Inhibition of cholinergic bronchospasm by tiotropium bromide was demonstrated in anesthetized guinea pigs, rabbits and dogs. In healthy volunteers, inhalation of tiotropium bromide resulted in an absolute bioavailability of 19.5%, a t_{max} value of 5 min. and the terminal half-life value of 5-6 days. There was no evidence of drug accumulation after repeated administration. The extent of biotransformation was small with a urinary excretion of 74% of unchanged substance after *i.v.* administration. Long term studies in patients with stable COPD have demonstrated that tiotropium bromide gave an effective bronchodilation that was maintained over 24h, significantly improved lung function as measured by FEV1 (+11-12%) and showed progressive reduction in dyspnea. It also reduced exacerbations of COPD patients and improved quality of life. Tiotropium bromide produced greater and more sustained bronchodilation than ipratropium bromide. Tiotropium has been shown to cause superior bronchodilatation and symptomatic improvements when compared to twice daily salmeterol in COPD. Tiotropium bromide was well tolerated and caused few adverse effects. The most common side effect reported was the mechanism-related effect of dry mouth.

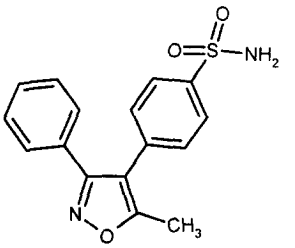
Treprostinil sodium (antihypertensive) (151-154)

Country of Origin : USA
Originator : Pharmacia/ GSK
First Introduction : USA
Introduced by : United Therapeutics
Trade Name : Remodulin
CAS Registry No : 289840-64-4
Molecular Weight : 412.51



Treprostinil sodium has been launched for the treatment of pulmonary hypertension. This synthetically designed tricyclic benzindene analog of the natural potent vasodilator prostacyclin has been developed for subcutaneous infusion. Treprostinil sodium can be prepared in a 15 step stereoselective process. It has been suggested that treprostinil sodium acts via the prostacyclin receptor since the agent could only increase cAMP in human embryonic kidney cells transiently transfected with the human prostacyclin receptor. In a conscious spontaneously hypertensive rat model, treprostinil sodium reduced hypoxia-induced increases in pulmonary arterial pressure and pulmonary vascular resistance in a dose-related manner. At higher doses, the test compound reduced systemic arterial pressure and systemic vascular resistance. In patients with primary pulmonary arterial hypertension, a one year therapy with treprostinil improved hemodynamics parameters: a 22% improvement in cardiac output, a 24% significant decrease in peripheral vascular resistance, a decrease in mean pulmonary arterial pressure (55 *vs.* 58 mmHg at baseline) and an improvement in NYHA functional class were observed. In addition, 6-min walk distance was significantly improved (463 vs. 399 m at baseline). A larger study conducted in 470 patients with pulmonary arterial hypertension has demonstrated the same type of result. In patients with severe intermittent claudication, infusion of treprostinil was well tolerated and increased the blood flow in most of the peripheral arteries of the lower limb. Treprostinil is eliminated in a biphasic distribution with a terminal half-life of 2-4 h. Approximately 79% of the administrated dose is excreted in the urine. Thus, treprostinil, in contrast to the currently available epoprostenol remains biologically active for a longer time and is not sensitive to light and temperature. Moreover, the subcutaneous administration avoids the epoprostenol systemic side effects due to continuous surgically implanted intravenous infusion. Treprostinil is well tolerated by patients and the main adverse effects currently observed are injection site reaction and injection site pain.

Valdecoxib (antiarthritic) (155-158)

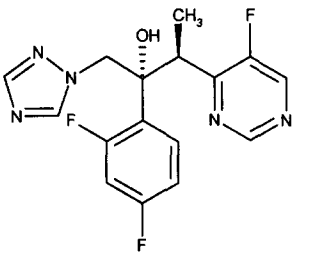


Country of Origin : USA
Originator : Pharmacia (Searle)
First Introduction : USA
Introduced by : Pharmacia / Pfizer
Trade Name : Bextra
CAS Registry No : 181695-72-7
Molecular Weight : 314.37

Valdecoxib is a second-generation COX-2 inhibitor, developed as a follow-up to celecoxib for the oral once-daily treatment of osteoarthritis, adult rheumatoid arthritis and menstrual pain. The first step for the preparation of valdecoxib is the conversion of deoxybenzoin to its corresponding oxime. Deprotonation of this oxime with butyl lithium followed by condensation with ethyl acetate gave the corresponding isoxazoline, which, after treatment with chlorosulfonic acid and reaction with aqueous ammonia afforded valdecoxib. Valdecoxib is approximately 28,000-fold more selective against human recombinant COX-2 than human recombinant COX-1. In an *ex vivo* human whole blood assay, the IC_{50} values against COX-2 and COX-1 were respectively 0.89 μM and 25.4 μM. In animal models, valdecoxib possesses excellent oral activity as an antiinflammatory. In rats, valdecoxib potently inhibited carrageenan footpad edema and adjuvant-induced arthritis. In the rat carrageenan air pouch model of inflammation, valdecoxib showed an efficient inhibition of prostaglandin production. In pharmacokinetic studies, valdecoxib has a rapid onset of action (t_{max} = 2.25 h) and a prolonged duration of action ($t_{1/2}$ = 8 h).

Valdecoxib is a substrate of CYP3A4 but no metabolism interference was seen with commonly used synthetic narcotics, alfentanil and fentanyl. Clinical studies have shown that valdecoxib is as effective as naproxen in treating osteoarthritis, rheumatoid arthritis and dysmenorrhoea. The efficacy of valdecoxib was also demonstrated in managing post-operative pain (oral and orthopedic surgery) with effective analgesia and time to rescue medication superior to those obtained with rofecoxib. Several clinical trials showed that valdecoxib has a better upper gastrointestinal safety profile compared to naproxen, ibuprofen or diclofenac and does not affect platelet function. Less abdominal pain, dyspepsia and constipation were observed with valdecoxib than with naproxen. Valdecoxib is contraindicated in patients with a history of allergic reactions to sulfonamides due to reported anaphylactic and skin reactions.

Voriconazole (Antifungal) (159-163)



Country of Origin : UK
Originator : Pfizer
First Introduction : US
Introduced by : Pfizer
Trade Name : Vfend
CAS Registry No. : 137234-62-9
Molecular Weight : 349.32

Voriconazole was introduced in the US for the treatment of acute invasive aspergillosis, candidosis and other emerging fungal infections seen in immuno compromised patients. It can be synthesized in 3 steps by reaction of readily available 6-(1-bromoethyl)-4-chloro-5-fluoropyrimidine with 1-(2,4-difluorophenyl)-2-(1,2,4-triazol-1-yl) ethanone in the presence of zinc metal. The resulting racemic mixture was submitted to a reductive dechlorination step followed by resolution with (*R*)-camphorsulfonic acid. Voriconazole is structurally related to fluconazole (Pfizer, diflucan®) and acts by inhibiting the cytochrome P450-dependant enzyme 14α-sterol demethylase of ergosterol synthesis (thereby resulting in the formation of a cell membrane with abnormal characteristics and accumulation of toxic sterol intermediates). Voriconazole was more active than itraconazole and fluconazole against *Cryptococcus neoformans* and a variety of *Candidas* species such as *C. albicans*, *C. glabrata C. krusei*. It also exhibits similar or superior activity compared to amphotericin B and itraconazole against filamentous fungi such as *Aspergillus*, an important pathogen which is not susceptible to fluconazole. In clinical trials, voriconazole was effective in the treatment of neutropenic patients with acute invasive aspergillosis, non-neutropenic patients with chronic invasive aspergillosis and HIV patients with oropharyngeal candidiasis. Voriconazole is available as oral or intravenous formulations. Following oral administration, absorption is rapid and the bioavailability is greater than 80%. Voriconazole exhibits non linear pharmacokinetics, a large volume of distribution (2 L/Kg) and a relatively short half-life (6 h). It was extensively metabolized via hepatic cytochrome P450 and has a drug interactions potential similar to itraconazole. Voriconazole was generally well tolerated, the most common treatment-related adverse events were transient visual disturbances.

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Chapter 34. Health and Climate Change: Implications for the Pharmaceutical Sector

Julian E. Salt
Climate Solutions Consultancy
Canterbury, UK

Introduction - With the advent of climate change becoming an accepted reality by the mainstream of science it is worth extrapolating the various scenarios for climate change in the immediate future to explore potential implications for the health of mass populations.

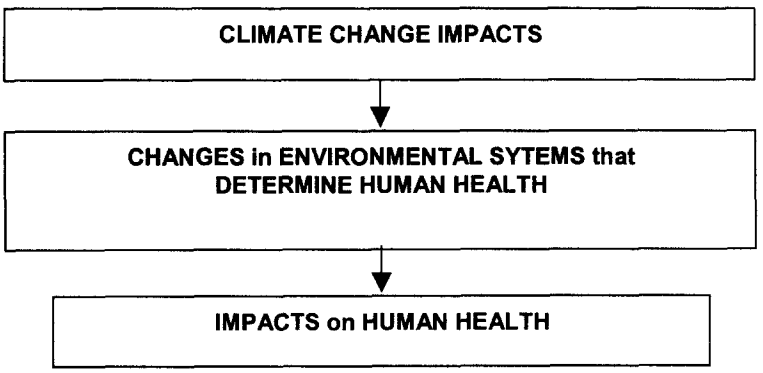
In general, the scenarios predict that temperatures globally will rise by between 1.4-5.8°C by 2100 and that weather events will become more extreme, leading to increased flooding and droughts.

This sort of behaviour superimposed on a background of increased temperatures will inevitably lead to an increase in a variety of diseases, primarily of the water borne and insect–vector types. This will place a huge burden on Third World countries that are already struggling against increasing disease patterns and malnourished populations. However, these developing world diseases, through the changing weather patterns brought on by climate change, may begin to visit the developed world in the next century.

These impacts will have important implications for the United Nations and in addition, its member governments. The United Nations through its various agencies such as the World Health Organisation (WHO), World Food Programme (WFP), Food and Agricultural Organisation (FAO), United Nations Commissioner for Refugees (UNHCR), United Nations Children Fund (UNICEF) Human and the Red Cross will need to deal with this human catastrophe. The pharmaceutical industry will also have an important role to play.

CLIMATE CHANGE

Human-induced climate change will impact societies and their health in many ways. The chain that creates the effect is composed of three distinctive steps:-



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There are many uncertainties attached to each of the three categories. Each uncertainty will make the prediction of future events more difficult.

To give an idea of the potential uncertainties that may exist with each of the levels of the "climate chain", the following is a summary of potential variations that could occur.

- Greenhouse gas emissions (which create climate change) - may be much higher than expected due to increased demand from Third World countries.
- Climate change impacts maybe more severe and more geographically spread than first envisaged.
- Human health may deteriorate, in general, due to the changing climate change conditions of the next century (poorer diets due to lack of foods and lack of access to appropriate drugs and clean water)
- Certain genetic pools maybe become more susceptible to different diseases in an unforeseen way as climate changes increase.
- Disease vectors may react differently and more radically than expected (in a warmer and wetter world).

It is clear that future climate may not mirror the past climate and as such will require special adaptive responses.

CLIMATE EFFECTS

The introduction of large quantities of greenhouse gas (carbon dioxide, methane and nitrous oxide being the principal ones) into the atmosphere since the industrial revolution has changed the overall concentrations to what some scientists are calling "dangerous levels" (1). Carbon dioxide (CO_2) concentrations have risen from pre-industrial levels of 280ppm to present day levels of 370ppm. Other greenhouse gases are rising at between 0.5-1%/annum. Clearly such emission levels are unsustainable, but the real question concerns the level at which it become dangerous. Does it lie at 450ppm, 550ppm or higher? A clear benchmark that is used by many scientists is two times (x2) pre-industrial-CO_2, which is nominally 550ppm (2).

The greenhouse effect, which is manifest on the very existence of greenhouse gases, allows heat to be trapped in the atmosphere for years or decades, depending on the gas concerned. Thus, not only is the actual level of greenhouse gas in the atmosphere important, but also the mixture of gases. If there is a preponderance of longer-living gases in the mix, then society is committed to a greater period of higher temperatures. This invariably depends on the fuel mix used to generate energy.

The heat that is trapped by the greenhouse gases warms the surrounding atmosphere that in turn allows the air to absorb and retain more water vapour (itself a potent greenhouse gas). As the atmosphere becomes more water-laden, the potential for more intense rainfall events (and hence floods) is increased. Equally, higher atmospheric temperatures will eventually lead to increased sea-surface and land temperatures. This in turn leads to the possibility of increased drought in those parts of the world that are already parched.

So, among the many climate change impacts the following main effects could be expected over the next century (3):-

- Temperature rise of between 1.4-5.8°C by 2100
- Sea-levels to rise by between 20-50cm

- Increased rainfall events
- Increased storminess in Northern hemisphere
- Potential for increased windiness

The United Nations Framework Convention on Climate Change (UNFCCC), which is the formal legal body charged with overseeing the global response to climate change, requires all parties to report on potential impacts to health in its annual reporting process. So far only eight countries have done so. The countries involved include:- Australia (4), Canada (5), Cuba (6), Czech Republic (7), Japan (8), The Netherlands (9), United Kingdom (10) and the United States (11).

CLIMATE FACTORS AND ASSIOCIATED HEALTH PROBLEMS

The climate change indicators mentioned in the previous section will generate quite specific environmental factors, that will in turn affect the health of populations through various mechanisms. Some of these mechanisms are direct, while others will operate in a more complicated and indirect manner.

The more specific environmental factors caused by climatic changes include the following range of effects.

Heat - Although the predicted range of temperature increases falls within the range of 1.4-5.8°C, this is only a global average. There could indeed be local variations in certain parts of the globe that will exceed this range.

In general, increased temperatures are a prelude to drought (when evaporation exceeds water absorption by the soil). Drought can have several direct and indirect effects on ecosystems that may lead to a variety of problems.

- Changes in vector abundance if that vector breeds in dry river beds (leading to a proliferation of a disease carrying species)
- Drier than normal conditions (leading to decreased crop production and famine and thence due to malnutrition)
- Reduction in the quality of water supply (leading to malnutrition and increased risk of infection due to water related illnesses)
- Displacement of people from regions of drought (leading to transmission of regional diseases to areas that have little/no immunity from those diseases)

Extreme weather events - Increased atmospheric energy will inevitably lead to a greater chance of more extreme weather events such as hurricanes, tornadoes, typhoons and storms in general. These events in themselves can cause local, regional and national problems simply from there sheer destructive nature (loss of infrastructure etc). In addition these large events can decimate crops instantly, exposing local and national populations to famine conditions and associated levels of malnutrition.

Hurricanes are large atmospheric disturbances that can span thousands of kilometres in width. When they form they have the ability to pull in the local atmosphere (from the central eye of the storm) and eject it many hundreds or thousands of kilometres away (at the outer edges of the storm system).

This very mechanism is thought to be a good way of spreading disease bearing organisms from one region to another in a sort of conveyor-belt/escalator mode. Thus, communities that have little or no immunity to a certain type of disease may be inadvertently exposed to new diseases from many thousands of kilometres away.

El Nino - When the specific climate event known as El-Nino operates (every 3-7 years) in the Pacific region, quite often an after-effect of this perturbation is an outbreak of cholera in the Peruvian coastline region. It seems that El-Nino (which is associated with a large mass of unusually warm water moving from central Pacific to north-eastern Pacific regions) allows the creation of masses of algae to move into coastal waters off Peru. The creation of the mass of algae allows the cholera bacteria to multiply in favourable conditions off the coast and invariably infects the local fishing communities, usually within six months of the onset of El-Nino (12).

Flooding - Climate change will inevitably bring about an increased chance of flooding by virtue of the fact that the atmosphere will be able to retain ever greater amounts of water, that when released may create flash-flood conditions in river flood plains. Damage by flood waters can be substantial and this can lead to the onset of disease such as typhus and cholera.

Mosquito populations can either be rapidly increased due to the wet conditions occurring in normally dry areas or in some cases can rapidly decrease, due to the destruction of breeding sites caused by violent floodwaters. Thus outbreaks of malaria and dengue fever are quite common after a major flood in the tropical regions of the world, where conditions are ripe for the breeding mosquitoes.

VECTOR-BORNE DISEASES

Malaria - Malaria is probably the most serious vector borne disease in the world. Transmission is via the mosquito and has a complex pathway. Climate change is foreseen to increase the spread of the mosquito to regions that do not yet experience it. Thus new populations with little or no resistance to malaria may well be exposed in the coming decades.

The regions that are most prone to malaria include lowland flood deltas. Climate change models show that due to increased precipitation, such areas will increase in size and move to new areas, currently not exposed. The most recent modelling data indicates that climate change will increase the current population at risk of malaria by 300-500 million by 2080. The models also show that a widespread increase in seasonal duration of transmission will occur in both current and new areas (13).

Dengue fever - Half the human population live in areas at risk of infection to dengue, transmitted, as with Malaria, by the mosquito. Some 50-100 million new cases present each year (14). It occurs mainly in urban towns and cities in the tropics and is exacerbated by inadequate sanitation in poor housing communities, as they provide perfect breeding grounds for the vector.

The latest global climate models show that the latitudinal and altitudinal range of dengue will increase in the future, as will the duration of the transmission season in temperate locations. The areas of greatest climate induced change will be found at the northern and southern limits of the virus (15). Presently it is widespread in Asia, Oceania, parts of Australia, the Caribbean, tropical America and parts of Africa.

Schistosomiasis - Schistosomiasis is caused by the flatworm which requires water snails as an intermediate host. Thus any landscape that involves water (rice paddies, inland lakes, rivers) will be able to host the vector for the disease. In addition, climate change will enhance the scarcity of water to populations (for drinking as well as crop irrigation) due to increased average temperatures. This will encourage increased irrigation systems to be produced, adding to the potential sites for the vector to multiply. Currently 600 million people worldwide are infected by schistomiasis.

Yellow Fever - Yellow fever is a viral infection limited to tropical climate zones outside Asia and the Pacific. Most cases are limited in number due to good vaccination procedures. However climate change may enhance the potential for outbreaks of the virus in such places as East Africa and the Americas. Higher temperatures will help the virus to multiply quicker. Generally most cases can be contained by good storage of potable water and adequate supplies of vaccines in an infected area. However of all the diseases, yellow fever is the one that will probably be of least concern in the future.

American trypanosomiasis (Chagas disease) - This disease is parasitic in nature and is transmitted via blood-feeding "kissing" bugs of the Triatominae family. Geographically, the disease is limited to the Americas and currently 100 million people are exposed to it. The most affected parts of the Americas include South and Central America. Currently the disease is undergoing a shift in its range due to ecological and demographic factors - caused by slum dwellings and blood transfusion practices. Higher temperatures associated with a future climate regime could present this parasite with an increased geographical distribution, both in latitude and altitude. Higher humidity as experienced in tropical forests in the region may also help the spread of this disease.

African trypanosomiasis (sleeping sickness) - Human African trypanosomiasis is also known as "sleeping sickness" and is transmitted by blood-feeding tsetse flies. Total numbers at risk in Africa are 55 million. The disease is usually fatal if untreated. The drugs used to treat the disease are expensive and cannot always be available in time for an epidemic (16). Although this disease affects lower numbers than other major diseases, it is substantial for the region due to the far-reaching economic consequences it creates. The geographical range of this disease is very susceptible to temperature and as such could be an increasing problem in Africa in the next century.

Cholera - Cholera is a water-borne disease caused by the bacillus Vibrio cholerae and is usually associated with faecal contamination of water supplies. There have been seven major pandemics usually originating in South East Asia (Bay of Bengal) or Latin America. Cholera outbreaks are associated with extremes of precipitation (drought and floods) and usually follow in the wake of a civilian emergency or disaster as sanitation systems fail and contaminate water supplies.

Climate change will bring about a change in precipitation patterns, rising average temperatures, increased likelihood of storms and reduced resilience of human populations. As such cholera is a prime candidate for increased activity in the next century. Seasonal diarrhoea may also be expected to increase as it is usually associated with wet and hot conditions.

POPULATIONS AT RISK

Climate change could have far reaching consequences for the human population and its health. Through the manifest ways that climate change operates (floods, storms, droughts) there will be an increase in the number of people exposed to natural perils with increased severity.

To place things in context it is worthwhile to review the death rate figures from natural perils in a typical year (17). Below in tables 1 and 2 are shown the break-down of deaths by peril and region for 1998.

Table 1. Deaths from natural disasters by region

AFRICA	578
AMERICA	14,995
ASIA	34,303
AUSTRALIA	2,231
EUROPE	743
WORLD	**52,850**

Table 2. Deaths by Natural Perils

EARTHQUAKE	9,510 (18%)
WINDSTORM	25,070 (47%)
FLOOD	13,741 (26%)
OTHERS	4,756 (9%)

By contrast the latest estimates for potential deaths by flooding related to sea-level rise associated with low-lying coastlines (by 2100) is **50-80 million** (18).

The potential numbers at risk of climate-related diseases is far harder to estimate due to the varied pathways that lead to the individual diseases. However a recent study by the World Health Organisation (19) is shown in table 3.

Table 3. Major tropical vector-borne diseases and the likelihood of change in their distribution as a result of climate change.

Disease Change	**vector**	**At risk (millions)**	**Infected (new cases/yr)**	**Distribution**	**Severity**
Malaria	Mosquito	2,400	300-500m	Tropics/sub	+++
Schistosomiasis	Water snail	600	200m	Tropics/sub	++
Lympatic filariasis	Mosquito	1,094	117m	Tropics/sub	+
African trypanosomiasis	Tsetse fly	55	250,000-300,000	Tropical Africa	+
Dracunculiasis	Crustacean (copepod)	100	100,000/yr	S.Asia/ M.East/ Central and West Afrcia	?

Leishmaniasis	Phlebotomine sandfly	350	12m infected, 0.5m new cases/yr	Asia/ South Europe/Africa/ Americas	+
Onchocerciasis	Blackfly	123	17.5m	Africa/Latin America	++
American trypanosomiasis	Triatomine bug	100	18-20m	Central-South America	+
Dengue	Mosquito	2,500	50m/yr	Tropics/sub	++
Yellow Fever	Mosquito	450	5,000 cases/yr	Tropical South America and Africa	++

The key components to be considered when assessing whether a vector-borne disease will increase its effect with the advent of climate change are as follows:-

- The current geographic distribution of the disease
- The range of non-human hosts and reservoirs (i.e. insect or mammal)
- Temperature related vector and parasite development, and adaptive processes pertaining to reservoir parasite interactions
- The current seasonality of transmission

Thus it can be clearly seen that the total numbers at risk of vector-borne diseases across the globe can be as high as 40% of humanity for a given disease (i.e. malaria and dengue). In addition, most climate changes will affect those populations that are already at risk of natural disasters and disease (tropics, sub-tropics, Africa, Latin America and South East Asia). Climate change will only exacerbate the total number of people at risk.

The clear conclusion from this analysis is that a distinct health crisis could occur in the coming decades, dependent on the extent of climate change. The responses of international agencies, governments and the pharmaceutical industry will be central as to whether this problem can be curtailed. If not, there maybe serious consequences for major parts of the globe and the associated economies.

WORLD SUMMIT ON SUSTAINABLE DEVELOPMENT

The recent World Summit on Sustainable Development held in South Africa in August 2002 set itself many targets in the health sector (20). Among the most notable targets were the following:-

- Halt by 2015 and begin to reverse the incidence of malaria and other major diseases

And more specifically:-

- At least 60% of those suffering from malaria have prompt access to and are able to use correct, affordable and appropriate treatment within 24 hours of the onset of symptoms

This set of targets could be adopted by the drug industry as a whole. This gives the major drug companies a little over a decade to research and develop new approaches to tackling many of these diseases.

MARSHALL PLAN ON CLIMATE RELATED DISEASES

To effectively address the looming problem of climate related diseases, a global strategy should be put in place. A "Marshall Plan" on Climate Related Diseases could be a good model to follow. If action is taken now by the large pharmaceutical sector, via active lobbying at the UN, and appropriate funding mechanisms put in place, there is a viable chance that the number of climate related diseases could be halted or reversed. Failure to adopt the WSSD targets of reversing the disease figures by 2015 will make the task almost impossible.

Conclusion - Climate change is with us and will grow increasingly important in this century. The predicted impacts will grow in intensity and severity as well geographically and climate change may not just affect the developing world. By virtue of its global reach and changing weather patterns, climate change may allow developing world diseases to visit the developed world in the coming century. The pharmaceutical industry, funding agencies and governments are in a unique position to find viable treatments for many of the leading diseases of the future.

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Chapter 35. Pharmaceutical Productivity – The Imperative for New Paradigms

George M. Milne, Jr – Boca Grande, Florida

Introduction – Despite dramatic increases in R and D investment, the promise of the genomics revolution, and the remarkable array of new technical tools available to the discovery scientist, the record of industry productivity over the past decade as measured by drug approvals has, if anything, declined (Figure 1). During this same period, the unpredicted rise of blockbusters and mega-blockbusters, a direct result of improved R and D/Marketing partnerships, has driven unprecedented revenue expansions, while the vigor of the generic industry has placed an exclamation point on sustainability. The expanding forward productivity gap, which has resulted, is painfully obvious to all of us who are actively engaged in the operation or leadership of biomedical R and D enterprises. Indeed, to maintain biopharmaceutical industry prospects for sustained growth and to meet the palpable healthcare needs of a global and aging population, increases in productivity on the order of 2 to 4-fold are required – and urgently. So why haven't we made more progress? Where might we go to seek the new concepts, the new assemblies of technology, different organizational concepts best suited to the creation of a breakthrough in R and D productivity? This is a core defining issue for the biopharmaceutical industry for the coming decade and the focus of this chapter.

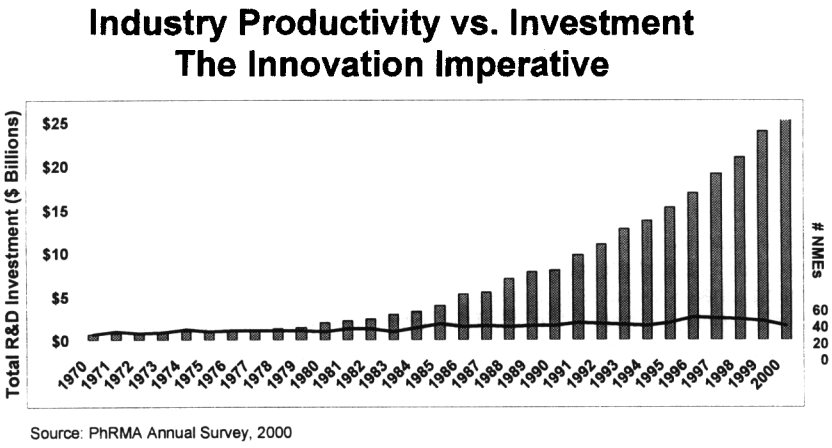


Figure 1. Industry NCE Productivity

The source of the answer lies, as we all know, in overcoming the unrelenting attrition statistics we experience as we go from therapeutic hypothesis to hit, to lead, to drug candidate, and, finally, to man. The attrition statistics have not improved despite a number of incremental advances and in many cases have been eroded due to ever more stringent regulatory hurdles and the increasing numbers of unprecedented targets emerging from genomics (Figure 2). With the unrelenting expiration of the patents on key products, the challenge is both compelling and urgent. The challenge is also quantal which should free us intellectually from just working harder on more conventional approaches to productivity since the risk of failure from persevering along the current path is arguably absolute. This gives us the opportunity and, indeed, the mandate to venture forth along unproven paths - to

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challenge conventional thinking while doing what motivates all of us – making a difference in health.

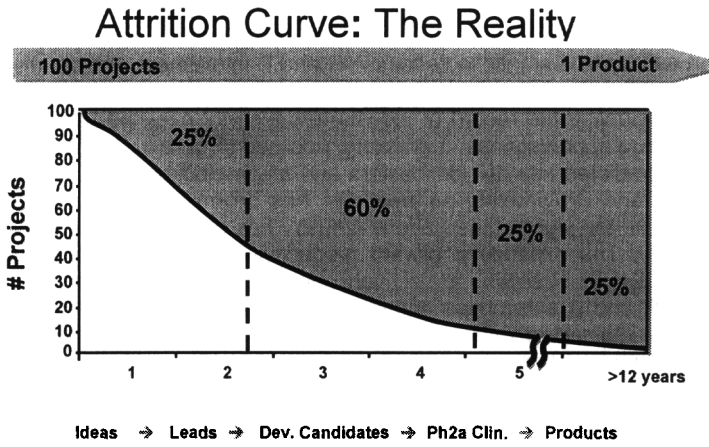


Figure 2. Project to Product Attrition Curve

The success rate for a specific therapeutic lead is the product of the odds that the target will be valid as a point of therapeutic intervention – often referred to as confidence in rationale – and the odds that the selected chemical will prove to react favorably with not just the desired molecular target, but with related targets, sites of toxicity, its own metabolism and the metabolism of other drugs, distribution, absorption and stability, etc., i.e. does the series confer true chemical drugability. As

SUCCESS RATE = (confidence in rational) X (chemical drugability)

outlined above the overall industry statistics for success is about 1 in 100 for programs starting at the hypothesis level. Only about one quarter of the time do we identify true candidate quality leads or strengthen the hypothesis during the screening phase which still leaves a survival rate from preclinical candidate to approved product of about 1 in 25, The component reflecting the odds of confirming the confidence in rationale for a nominated candidate averages about 25%, but can vary from unity for highly precedented targets to infinity for an unproven orphan receptor. However, by far the largest routine hit to survival (75%) comes because we have simply selected a chemical or chemical series that is not truly drugable. Indeed, if we return to the many screens that fail to yield viable leads, the contribution of chemistry to the outcome exceeds 85%. While chemistry must take a leadership role, it is important to emphasize with HTS displaying reproducibility in the 30- 70% range for some tests, the final solution will lie with the disciplined application of highly discriminating chemistry and biological test systems.

As we will discuss below, this low level of precision and overall success is unacceptable given the paucity of great targets. This dissection of the current hurdles for success serves as the outline for the four themes that are explored in this essay: 1. expanding the number of high confidence in rational targets, 2. a central role for chemistry and chemical diversity, 3.enhanced lead identification and parallel optimization of hits, and 4. creating connectivity – target hopping and data mining. An important sub theme is that while success will require that we push existing technologies, the real breakthroughs will come from fundamental challenges to how we assemble the technology and to the culture and practices that are so embedded

in our upbringing that they are invisible – and that chemistry and chemists are at the heart of both the opportunity and the challenge.

EXPANDING THE NUMBER OF HIGH CONFIDENCE-IN-RATIONALE TARGETS

The revenues of the pharmaceutical industry are based on a relatively small number of unique molecular targets and to that only 2 or 3 new targets are added each year, a fact highlighted by a recent review (1). This reality stands in stark contrast to the promise of potential drug targets emerging from the genomics advances of the last 5 years which some have estimated to be in the 5-10,000 range (2). The demonstration that not all of these are suitable therapeutic targets is already dampening our enthusiasm for the promise implied from large numbers alone. This is hardly surprising since transcriptional genomics and proteomics reflect the natural outcome of the reductionist approaches to biomedical research favored over the last two decades. While powerful and essential to progress, these tools are unable to discriminate between cause and effect (3).

In an industry that has succeeded based on 2 to 3 new proven targets per year, the challenge and the opportunity are how to efficiently identify the small percentage which represent high confidence in rationale/quality targets. Clearly this is a point of enormous competitive leverage, since the identification of only 10 such new targets a year would more than triple the raw innovative capacity of the biomedical enterprise. At the same time the increase in certainty of the rationale would lift success rates during development from their current levels that reflect the current industry mix of precedented and unprecedented programs. While best in class, fast follower approaches will allow companies to participate and hence need to be part of the chemical armamentarium, it is clear that innovation leaders need to develop approaches and alliances that drive the availability of new high probability targets for their enterprise.

This challenge of expanding the number of high confidence in rationale targets has begun to attract specific attention. All of these approaches rely in one form or another on the importance of phenotypic, more integrated read outs versus the targeted molecular approaches that have dominated screening in the recent decade. For those who have been in the industry for several decades this will remind of the messy, but effective pharmacology and physiology that served as the basis of so many of the current drug armamentarium. The approaches being undertaken reflect a rich mix of chemical, biological and empiric perspectives. Chemical genetics is based on a very old pharmacological concept that to understand a system you need to perturb it. This offers a central role for chemistry, but one that is conceptually and managerially distinct from the targeted therapeutic programs that dominate biopharmaceutical thinking. The challenges associated with creating the diverse libraries required to probe the genome have been recently been reviewed (4,5). He makes the point that diversity oriented synthesis requires a new type of strategic planning by the organic chemist – that the tools and concepts must permeate their thinking. This is an arena where in silico enforced diversity may be a critical ally for the chemist (6). In a repeated theme for this whole chapter, the importance of common data standards and high quality are emphasized as essential to data comparability and recognizing new patterns from complex, integrated data sets (7).

In the biological arena, the work by Zambowicz and Sands illustrates an approach to what they refer to as treatment vs. disease genes using selective gene knockouts (1). Their approach is to identify the phenotype of the desired therapeutic result vs. mimicking the disease phenotype and to use the heterozygous animals to show the ability to modulate the effect as a precursor to engaging in therapeutic research. In a nice example of scholarship, they have correlated their approach with

gene knocks outs related to the top 100 drugs as a form of retrospective validation. The use of siRNA has recently also attracted a lot of attention as another means for efficiently dissecting the functional role of specific gene products (8). As discussed in the third section, expanded high confidence target opportunities can also be created by applying high throughput tools of structural biology and chemical interrogation to gene super families with the proven ability to generate drugs.

In the screening laboratories of the industry, there is a recognition that one can no longer work on isolated, single hypotheses, but rather one must create approaches that capture entire systems (9). There is increased reliance on whole cell systems that capture the activity of targets in their appropriate physiologic context (10). In the university setting, there is also an encouraging move toward integrative biology departments. This is exemplified by MIT's recently announced Computational and Systems Biology Initiative (11). The aggressive mining of results from feedback loops built on connections with academic researchers, clinical trials (ones own and others) and on the conjoint application of multiple biological techniques is becoming more and more critical to the early and effective identification of superior targets. It is also becoming increasingly clear that it is important to view the approach to screening as a discipline in its own right and not just a step to be gotten through expeditiously on the way to hits and leads. It is clear that we need also to move away from the seduction of the in vitro assay with highly purified proteins which produces very clean SAR, to phenotypic assays reminiscent of screening in the spontaneously hypertensive rat of 50 years ago – high in content, but lower in intrinsic precision exactly because they integrate multiple steps.

What is clear from the math is that increasing the confidence in rationale for the targets we screen against is a critical priority- that it could easily double our net productivity and deserves targeted attention before setting out on the Discovery path.

Some implications/suggestions for consideration: 1. Make the identification of new, more highly validated targets an internal and collaborative priority. 2. Look to see how to organize and reward teams to achieve this goal in ways that are distinct from the targeted phases of drug discovery. 3. Seek out new technologies that accelerate the phenotypic characterization of targets and build them in at the very outset of programs. 4. Insist on rigorous data standards and quality and 5. Expand your web of collaborative networks creating opportunities for integrated insights from deeply invested university or biopharmaceutical partners.

A CENTRAL ROLE FOR CHEMISTRY AND CHEMICAL DIVERSITY BASED APPROACHES

This segment is based on two key observations: 1. the fact that, even with a validated target, the chemical properties of the candidate determine more than 85% of the attrition as the target molecule interacts with various biological systems during development and 2. the empiric observation that, while individual companies have made very little progress on success rates, the industry as a whole succeeds with well precedented targets more than 90% of the time. This begs the question as to why individual companies have been unable to capture industry success rates despite growth to several multiples of their former size. The hypothesis which will be elaborated is that the reason may lie in a failure to achieve industry levels of chemical diversity at the outset of the Discovery process - the screening hit to lead level. One can further speculate that this could be exacerbated through management practice and by deeply entrenched psychology within the chemical community. Figure 3 below offers a schematic that captures industry experience with a typical target.

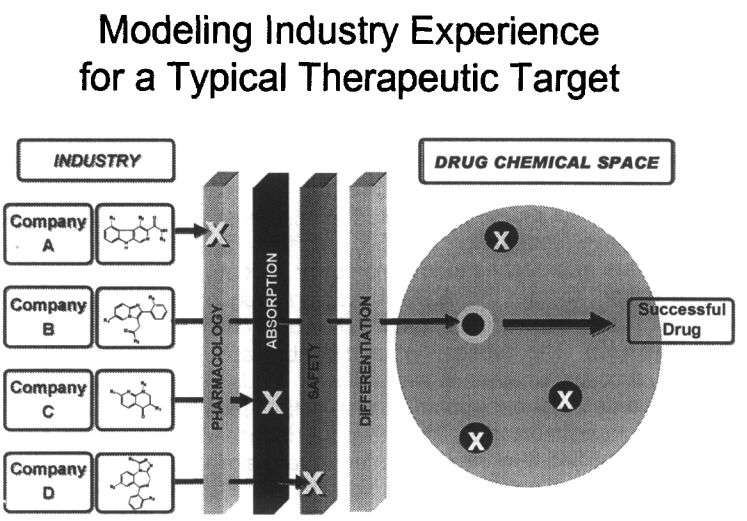


Figure 3: Model Industry Schematic

Drug chemical space, as defined by individual companies, often results in 4 or 5 distinct chemical families with high intrinsic potency for a specific molecular target as reflected in the patent literature. However, as illustrated above, typically only one of these families, or in rare cases two, ever produce a successful drug. What is obvious from the cartoon is that companies A, C, and D were "doomed from the start," but only discovered that through costly, failed development. The first part of the solution is obvious and that is the creation of a diverse library that starts to match that of the industry in size, diversity and quality, as illustrated in Figure 4.

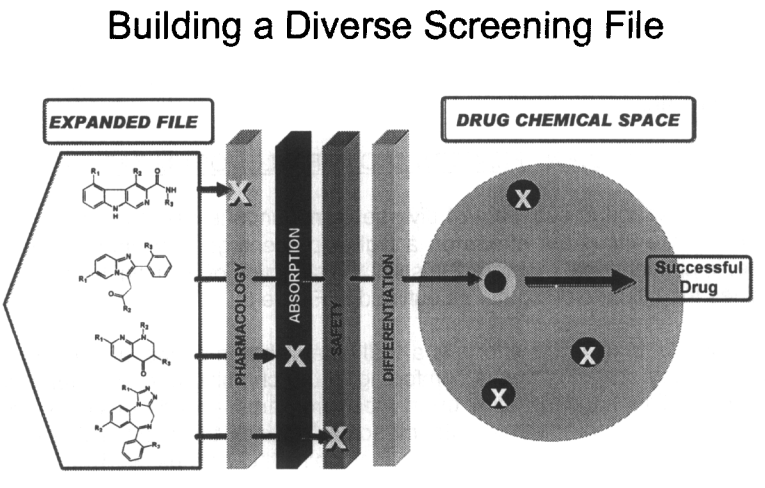


Figure 4: Expanded Screening Library Creation

Advances in combinatorial chemistry enable the preparation of specifically designed libraries with interesting scope and adherence to the rule of five (12, 13).

Computational programs with the capacity to enforce targeted diversity are increasingly in active use (14). In addition, the creation of library and screening paradigms is the subject of some excellent experimentally based scholarship. For example, in a study of over 100 screening assays within a family of greater than 0.85 Tanimoto similarity, the screening of only a single member yields a greater than 70% chance of missing an active, while the inclusion of 10 members reduces that to 3% (15). This has obvious implications for the building of screening libraries. Scholarship to rule out promiscuous hits has also been exemplified (16). A number of companies have undertaken to build scaffolds and the chemical technology required to create approaches to low molecular weight, conformationally restrained, rule of five based libraries capable of probing drug relevant biological space. In another report it has been argued that much robust chemistry exists to be applied to this challenge (12). The creation of a drugable and diverse starting point is a condition for success whether in fast follower mode or not. The investment in a series of candidates with orthogonal attrition risks needs to be a core competence – an institutional investment that lifts the performance of all traditional therapeutic teams who will not find it in their self interest to make these changes on their own account. The other challenge is that data quality in the lead generating screens must be very carefully managed and since the benefits of a great and diverse library will be quickly eroded away by screens with low levels of precision and reproducibility. We must not take comfort in large numbers at the expense of precision. Again this requires institutional and disciplinary attention at what seems like the most distant point but, as will be repeatedly emphasized, the point that, counterintuitively, most determines final success or failure.

Implications/Suggestions for consideration: 1. Give library creation to one of your most compelling leaders; 2. Make the creation of a large, diverse combinatorial library a focused managerial and scientific priority with significant resourcing; 3. Study your statistics so that with each round of screening and analysis you have the opportunity to lift the quality of your game to a new level, 4. Specifically look at reproducibility and signal window to estimate what you may have missed and drive the system to 90% reproducibility, 5. Build the infrastructure and technology for screening a large library and for rapid chemical follow up and expansion of hits and leads and 6. Build quality into the entire system, not just diversity of library or purity of samples. Understand stability of samples, precision of assays and reproducibility of data over time.

ENHANCED LEAD IDENTIFICATION AND PARALLEL OPTIMIZATION OF HITS

While an expanded substrate of diverse compounds is essential, it does not, in itself, ensure we focus our efforts on a higher percentage of winners. This is true because, in conventional programs, it is still the selection of an initial lead series that determines the ultimate outcome, as outlined in Figure 5.

The drive to focus one's efforts is a well established managerial concept and in itself can convey progress that is comforting to the chemist, to project team and to management. This happens early on in most programs – made easier by the fact that we most often start with a potency screen to pick the most active hits and, thereafter, a preferred lead series. At that point the serious work of Discovery usually begins in a multiyear, series of iterative steps as we seek acceptable levels of bioavailability, genetic toxicity, pharmaceutical properties, drug metabolism and so on for our chosen series. As has been elaborated by Lipinski and his coworkers, the rise in the observed molecular weight, increased lipophilicity and decreased solubility of initial screening leads has made the task of lead to drug conversion far more difficult (13). The provocative observation is that this degradation in the chemical starting point for recent Discovery programs coincides with our move away

from pharmacological screens to in vitro molecular screens. Drugability has been the victim and increasing candidate selection criteria will not rescue a suboptimal early choice. This suggests that to change the productivity of the system we need to move beyond a focus on increasing the hurdle rate to fundamental changes in our processes for selecting and developing leads. If we do not change, it seems likely that even with vastly larger files we will still "select to fail."

Imagine that we have created an industry like screening file as indicated in Figure 5, and that we have access to all the leads previously represented by Companies A – D. What would we do? In general, the practice and the instinct is to focus in relatively quickly on one or two series based on initial probes to see which series most quickly delivers a strong SAR based on potency measures. Most typically after several months of work we would have a mid-nanomolar lead from one of these series. But is it the "right" one? Psychologically, there is great comfort from a nanomolar lead which makes it very difficult for any chemist to turn back to a micromolar hit even if it has superior drug potential as measured by molecular weight and other elements captured by the rule of five, In fact, many of us have learned that getting nanomolar potency is seductively easy compared to getting all the properties of drugability built in up front. We also often have all too convenient confidence in the ability of our colleagues in pharmaceutical sciences to rescue us downstream. The problem may also owe part of its origins to the compounds in our libraries and to the precision of our screening practices.

Historically the progress of the sciences has most often been linked to advances in the quantitative and qualitative precision of the analytical "eyes" we bring to bear on a problem. This is certainly true of the contribution of analytical chemistry to organic chemistry. There is a comparable and clear opportunity to push for the same end to end QA/QC if the screening and lead generation process. For example, by setting our screening criteria so that we achieve a manageable hit rate an unintended consequence is often that highly desirable 300 MW scaffold hits may never even reach our attention. Also because of the comfort that comes from large numbers we may not work as hard on the making sure that we understand the dynamics of our screening conditions. As one illustration, the impact of different procedures on hit identification is highlighted by the work of Sills and coworkers (17). Screening for tyrosine kinase inhibitors by three different methods they showed surprisingly little overlap in the compounds identified as leads. More investment in quality thinking and experimentation at the front end of the process is a shared chemistry and biology opportunity to improve the outcome.

The ultimate trap is the same one we experience with energy minimization procedures i.e. once you are in a well (or a hole) it is very hard to get out and detect the presence of other potentially superior minima and yet is hard not to keep digging deeper. If we express our scheme and sequenced series of hurdles in this way you can see a graphical representation of the initial selection of a series is determinative in an iterative, serial process (Figure 5).

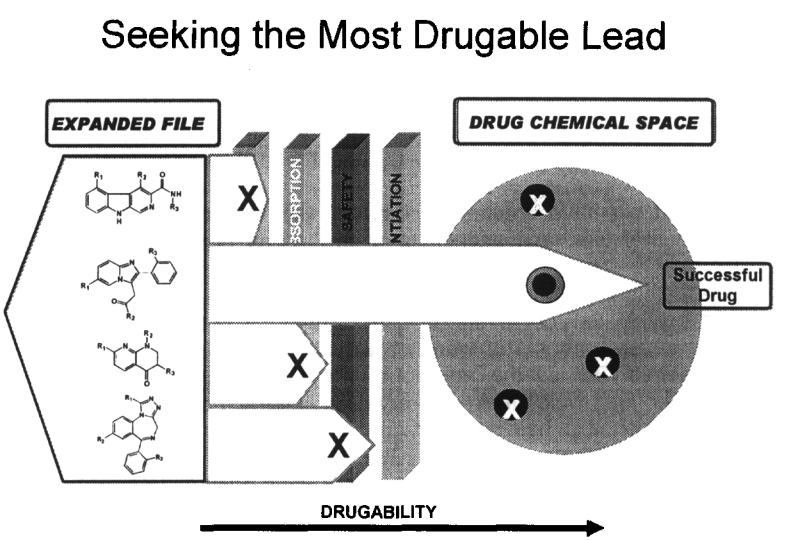


Figure 5.

Rather than focus in, this reasoning suggests that we need to set about to enhance lead identification through an open and parallel, not serial, optimization of as many hits as we can handle. We should engage processes that for a time force us downstream – to solve the 5 or 6 key hurdles of successful drug development in parallel 6 dimensional space (Figure 6). We need to resist the pressure to pick and

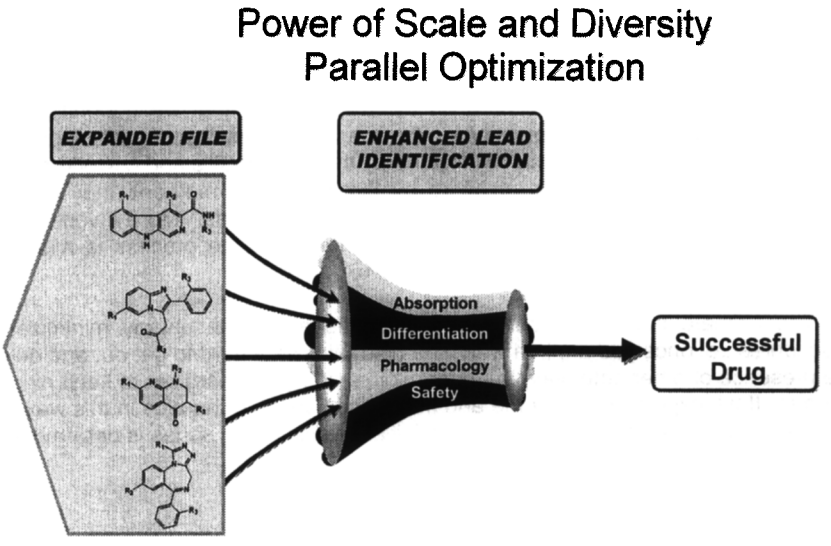


Figure 6: Parallel Optimization Vortex

thereby create an intellectual vortex that opens up the opportunities to create a deeper and interconnected experimental base – improving our chances of identifying the truly drugable series for final elaboration. The benefit of starting with multiple chemotypes is that it gives the Discovery team maximum flexibility to recover from unforeseen problems along the development path. In a targeted form, rapidly expanding chemotype space is also key to generating patentable new prototypes

capable of meeting the high hurdles for best-in-class, fast follower approaches Clearly, this focus on high capacity parallel optimization requires new tools and, in particular new ways of working and managing this most key step in the discovery process.

Early stage parallel optimization requires the assembly of a series of ultra high capacity technologies including an emphasis on rapid combinatorial expansions of hit series (18-21). It requires new partnerships between Discovery scientists and their colleagues in Drug Safety Evaluation and Pharmaceutical Sciences (formulation, stability, physicochemical properties) in developing well validated new assays which allow one to build in drugability at the stage of hits and early leads. It requires the development of visualization tools which enable us to enhance lead identification though a process of parallel optimization where closed- loop learning, parallel processing and data mining meet to create a vastly richer array of choices leading up to the selection of a lead series (22, 23).

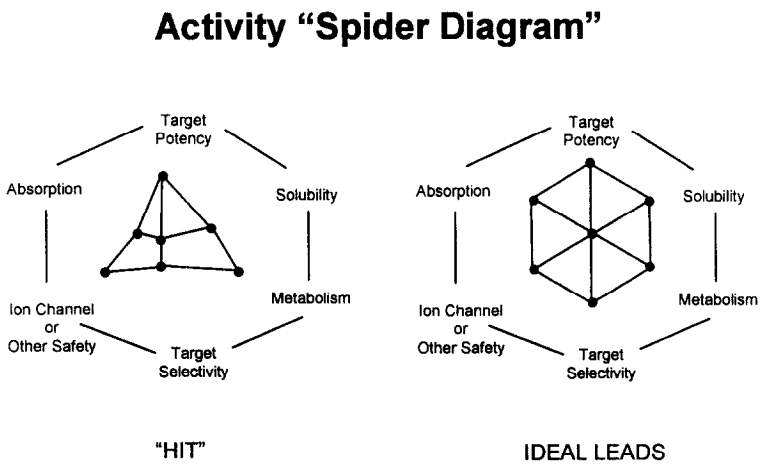


Figure 7: Multidimensional Optimization of Hits and Leads

By creating the kind of "spider diagram" (Figure 7) for each of the hits and by expanding a dozen or more hits through several cycles of computer aided diversity expansions; by keeping an eye on the rule of 5, we should be far more likely to settle in on a series with maximum drugability. This is a testable hypothesis, but even if we pick wrong we are in a position to rapidly reload based on intellectual property already created! In addition, with time this investment in high quality multifactorial data sets should also begin to elaborate new and more powerful prospective approaches to building in drugability and the preparation of more targeted, "biased libraries.

Some implications/suggestions for consideration: 1. Change what we measure as progress in early discovery so that it supports the discomfort of not choosing prematurely; 2. Develop combinatorial chemical processes and infrastructural capacity that allow rapid and diversity driven expansions of a dozen or more hit series through several iterations; 3. Invest in high capacity molecular toxicology AD/ME and pharmaceutics assays and the informatics to enable parallel evaluation of potential to surmount the 5 or 6 most likely hurdles to successful and speedy development; 4. Employ your experience to develop your own scholarship on the effectiveness and productivity of specific tactics and 5. Be specific about the

technical implications of the new paradigm and, as a priority, engage webs of collaborators in their solution.

CREATING CONNECTIVITY – TARGET HOPPING AND DATA MINING

The elaboration of entire families and sub families of genes for targets with proven potential to yield drugs opens an added opportunity to increased productivity based on the genomics advances of the last decade. The disease targeted efforts described under this paradigm generate staggering amounts of data and yet the reality is that only about 0.1% of the screening results provide specific positive data for a specific target. The challenge and the opportunity is to use some of our new found technical nimbleness and take a systems approach to mining and harvesting the larger data set – data which may hold elusive clues for other targets. The idea would be to harvest this data in search of clues to new leads for disease X or Y or perhaps to learn how to solve the problems of drug metabolism lability or hepatoxicity or to create the tools required to explore the function of some new gene in collaboration with an external partner who also holds part of the puzzle (Figure 8).

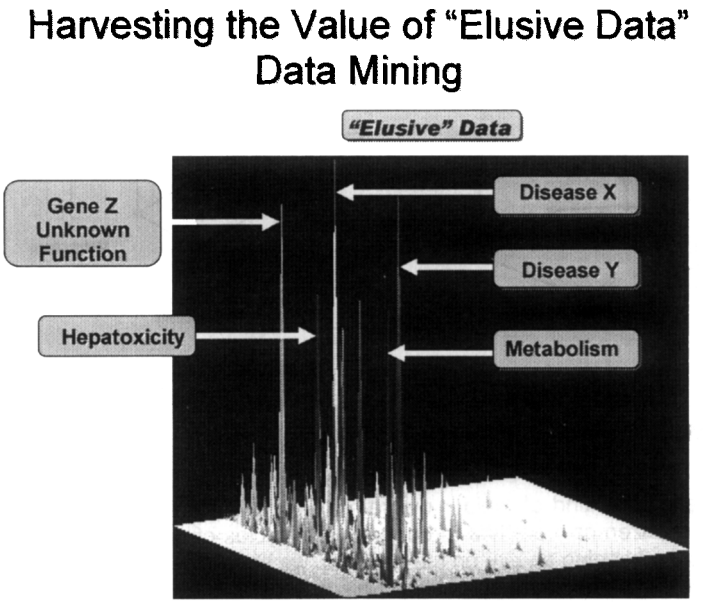


Figure 8.

This concept has the ability to accelerate progress particularly when working within proven families of drugable targets where the screening of a dozen or more members yields rapid information about how to achieve selectivity and provides the tools to understand added therapeutic applications in a very targeted way as illustrated in Figure 9 below and as anticipated by chemical genomics. Absolutely key to this approach is high data quality from screening runs conducted at very high capacity – a key area of study exemplified in a study of caspases in microfluidic environments (19).

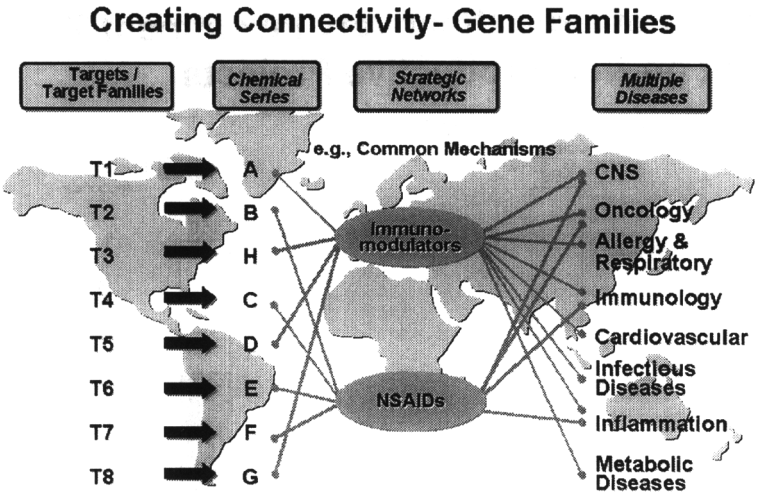


Figure 9.

Some implications/Suggestions for consideration: 1. Make an organizational investment in gene families that can leverage the therapeutic scope of your organization and its web of collaborators; 2. Build the informatics tools and that allow the mining of elusive data and the identification of connections that form the basis of an integrative biology approach which builds on islands of data and knowledge; 3. Build up technologies that allow full access to similarly configured assays and inhibitor x-ray crystal structure determinations across different families and selective hits so that progress is accelerated, and 4. Leverage contacts with the academic and venture communities in probing for the physiologic role of unproven genes using the chemical genomic tools developed by this approach

CAN WE AFFORD TO FRONT LOAD THE HIT TO LEAD SEGMENT OF DISCOVERY – WILL IT TAKE TOO MUCH TIME?

The levels of dollar investment in failed candidates are enormous, as illustrated in Figure 10 (24). Any changes that promise a two- or even four-fold improvement in success rates can easily justify tens and even hundreds of millions of dollars of investment. As to time cost, it only feels costly to take extra time at the outset to refine the scope of our hits. If we sum the time lost in serial attempts to enhance drugability and the time cost in reloading after failures, extra months spent at the front end will actually save time when viewed from a business systems perspective. The time savings from speedier development of the resulting highly drugable leads will more than make up for the up front investments in hit diversity and elaboration and the ability to return to a broad, well developed IP base in the event of the still inevitable failures. These kinds of benefits are not beyond our reach if we use our new found scale, capacity and chemistry and new assemblies of biological screens, as outlined in this chapter.

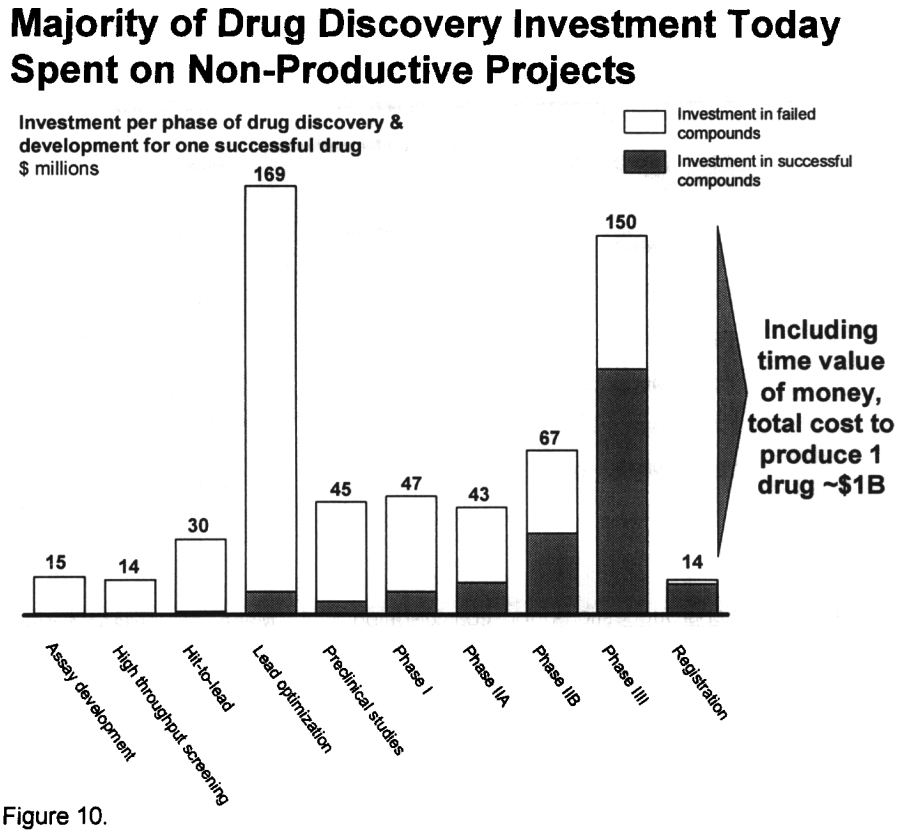


Figure 10.

Even modest systemic improvements in success rates at each of the stages have an enormous cumulative impact (Figure 11). By building a superior screening file we might conservatively expect to increase hit rates for leads from 25 to 50%. By starting with more leads from multiple chemotypes and by vortexing them using parallel optimization, there is every possibility of improving the lead to candidate development yield from 60 to 80%. The industry aggregate experience tells us that exploratory development success rates can rise sharply whenever we work with diverse families of leads. In addition, it is intrinsically obvious that this also creates a strong intellectual property estate and gives us flexibility and speed in reacting to clinical findings. As we build in a closed feedback loop between chemistry and biology and as we work within gene families, we can expect confidence in rational to rise – although that will come slowly and the impact will be modest and less certain early on. The consequence of the change in trajectory at the earliest stage in the discovery process would be a five fold increase in productivity.

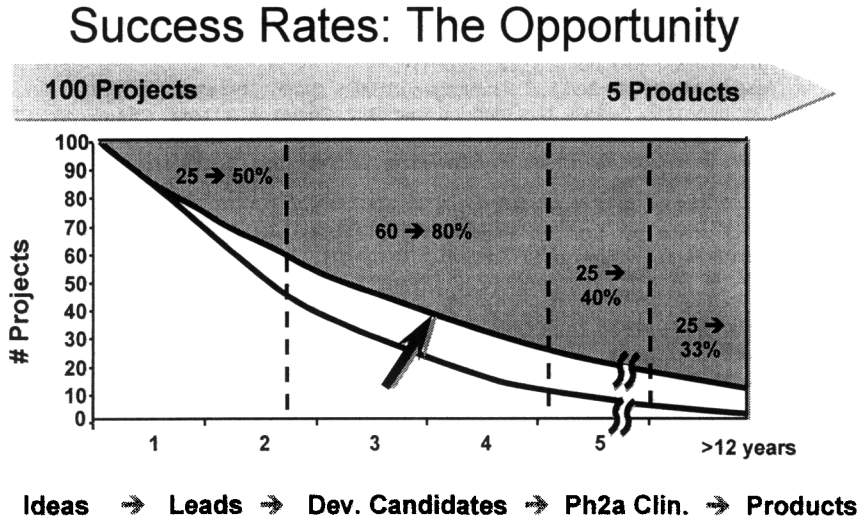


Figure 11.

Conclusion: While the productivity of the biomedical enterprise has not yet met the rising expectations of our investors or of the patients and their families who need to see real advances in medicine, the technologies developed during the last two decades exist or can be adapted to make the required advances in the pace of new discovery. The key hurdle lies not with technology but with how we organize to implement new paradigms so as to challenge the fundamental statistics of the pharmaceutical R and D enterprise. To achieve this quantum jump in productivity will require the sort of management attention that normally is reserved for development candidates. It will require investment, alliances and most of all a change in both culture and organization. Strong chemistry leadership and a willingness to challenge existing conventions is required. It will undoubtedly also require creative investments in new people and technology, but to yield the increase in new product flow required to sustain the biomedical enterprise. The author believes that the answers are now palpable - in our technology and in our experiences - to discover and to experiment our way to the required advances. The penalty for not setting out to try is certain failure.

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abacavir sulfate	antiviral	1999	35, 333
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
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
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
alitretinoin	anticancer	1999	35, 333
alminoprofen	analgesic	1983	19, 314
almotriptan	antimigraine	2000	36, 295
anakinra	antiarthritic	2001	37, 261
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
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

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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
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
atorvastatin calcium	dyslipidemia	1997	33, 328
atosiban	preterm labor	2000	36, 297
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 314
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
barnidipine HCl	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
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
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

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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
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
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
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

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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
cevimeline hydrochloride	anti-xerostomia	2000	36, 299
chenodiol	anticholelithogenic	1983	19, 317
CHF-1301	antiparkinsonian	1999	35, 336
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325
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
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
clopidogrel hydrogensulfate	antithrombotic	1998	34, 320

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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
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
defeiprone	iron chelator	1995	31, 340
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
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
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

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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
dutasteride	5α reductase inhibitor	2002	38, 353
duteplase	anticougulant	1995	31, 342
ebastine	antihistamine	1990	26 302
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
edaravone	neuroprotective	2001	37, 265
efavirenz	antiviral	1998	34, 321
efonidipine	antihypertensive	1994	30, 299
egualen sodium	antiulcer	2000	36, 303
eletriptan	antimigraine	2001	37, 266
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
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
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
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
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
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
etoricoxibe	antiarthritic/analgesic	2002	38, 355
exemestane	anticancer	2000	36, 304

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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
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	5α-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
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosinopril sodium	antihypertensive	1991	27, 328
fosphenytoin sodium	antiepileptic	1996	32, 308

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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
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
indinavir sulfate	antiviral	1996	32, 310
indobufen	antithrombotic	1984	20, 319
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-1α	antineoplastic	1992	28, 332
interferon, β-1a	multiple sclerosis	1996	32, 311
interferon, β-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
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
latanoprost	antiglaucoma	1996	32, 311
lefunomide	antiarthritic	1998	34, 324
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
levocabastine HCl	antihistamine	1991	27, 330

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
miglitol	antidiabetic	1998	34, 325
milnacipran	antidepressant	1997	33, 338
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
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
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
mycophenolate mofetil	immunosuppressant	1995	31, 346
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
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
neflinavir mesylate	antiviral	1997	33, 340
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
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
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
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
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
pegaspargase	antineoplastic	1994	30, 306
pemirolast potassium	antiasthmatic	1991	27, 331
penciclovir	antiviral	1996	32, 314
pentostatin	antineoplastic	1992	28, 334

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
pivagabine	antidepressant	1997	33, 341
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
pramipexole HCl	antiParkinsonian	1997	33, 341
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pranlukast	antiasthmatic	1995	31, 347
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
prezatide copper acetate	vulnery	1996	32, 314
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
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
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
rapacuronium bromide	muscle relaxant	1999	35, 347
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
risedronate sodium	osteoporosis	1998	34, 330
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342
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
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin HCl	antibacterial	1992	28, 335
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin HCl	hyperphenylalaninemia	1992	28, 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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
setraline HCl	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
sibutramine	antiobesity	1998	34, 331
sildenafil citrate	male sexual dysfunction	1998	34, 331
simvastatin	hypocholesterolemic	1988	24, 311
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
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorivudine	antiviral	1993	29, 345
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	β-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
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
talipexole	antiParkinsonian	1996	32, 318
taltirelin	CNS stimulant	2000	36, 311
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	β-lactamase inhibitor	1992	28, 336
tegaserod maleate	irritable bowel syndrome	2001	37, 270
teicoplanin	antibacterial	1988	24, 311
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
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
valsartan	antihypertensive	1996	32, 320
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
xamoterol fumarate	cardiotonic	1988	24, 312
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
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

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GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
lanreotide acetate	ACROMEGALY	1995	31, 345
nitrefazole	ALCOHOL DETERRENT	1983	19, 322
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
azelaic acid	ANTIACNE	1989	25, 310
betotastine besilate	ANTIALLERGIC	2000	36, 297
emedastine difumarate		1993	29, 336
epinastine		1994	30, 299
fexofenadine		1996	32, 307
nedocromil sodium		1986	22, 324
olopatadine hydrochloride		1997	33, 340

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ramatroban		2000	36, 311
repirinast		1987	23, 341
suplatast tosilate		1995	31, 350
tazanolast		1990	26, 309
lodoxamide tromethamine	ANTIALLERGIC	1992	28, 333
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
dirithromycin		1993	29, 336
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
trovafloxacin mesylate		1998	34, 332
meropenem	ANTIBIOTIC, CARBAPENEM	1994	30, 303
panipenem/betamipron		1994	30, 305
mupirocin	ANTIBIOTIC, TOPICAL	1985	21, 330
nadifloxacin		1993	29, 340
alemtuzumab	ANTICANCER	2001	37, 260
alitretinoin		1999	35, 333
arglabin		1999	35, 335
bexarotene		2000	36, 298
denileukin diftitox		1999	35, 338
exemestane		2000	36, 304
fulvestrant		2002	38, 357
gemtuzumab ozogamicin		2000	36, 306
ibritumomab tiuxetan		2002	38, 359
letrazole		1996	32, 311
OCT-43		1999	35, 345
oxaliplatin		1996	32, 313
raltitrexed		1996	32, 315
SKI-2053R		1999	35, 348
tasonermin		1999	35, 349
temozolomide		1999	35, 350

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
topotecan HCl		1996	32, 320
valrubicin		1999	35, 350
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
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
citalopram		1989	25, 311
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
glimepiride		1995	31, 344
insulin lispro		1996	32, 310
miglitol		1998	34, 325
nateglinide		1999	35, 344
pioglitazone HCl		1999	35, 346
repaglinide		1998	34, 329
rosiglitazone maleate		1999	35, 347

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
dolasetron mesylate	ANTIEMETIC	1998	34, 321
granisetron hydrochloride		1991	27, 329
ondansetron hydrochloride		1990	26, 306
nazasetron		1994	30, 305
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
tiagabine		1996	32, 320
topiramate		1995	31, 351
centchroman	ANTIESTROGEN	1991	27, 324
caspofungin acetate	ANTIFUNGAL	2001	37, 263
fenticonazole nitrate		1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
lanoconazole		1994	30, 302
micafungin		2002	38, 360
naftifine HCl		1984	20, 321
oxiconazole nitrate		1983	19, 322
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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

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cilazapril		1990	26, 301
cinildipine		1995	31, 339
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
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
valsartan		1996	32, 320
zofenopril calcium		2000	36, 313

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
nabumetone		1985	21, 330
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, INTESTINAL	1984	20, 318
osalazine sodium		1986	22, 324
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21, 323
aminoprofen		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
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

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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
gemcitabine HCl		1995	31, 344
idarubicin hydrochloride		1990	26, 303
imatinib mesylate		2001	37, 267
interferon gamma-1α		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

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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, TOPICAL	1992	28, 333
miltefosine		1993	29, 340
dexfenfluramine	ANTIOBESITY	1997	33, 332
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
tazarotene		1997	33, 343
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29, 346

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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

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misoprostol		1985	21, 329
nizatidine		1987	23, 339
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
cidofovir		1996	32, 306
delavirdine mesylate		1997	33, 331
didanosine		1991	27, 326
efavirenz		1998	34, 321
famciclovir		1994	30, 300
fomivirsen sodium		1998	34, 323
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
neflinavir 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
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

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zidovudine		1987	23, 345
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
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
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,	1995	31, 339

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	INJECTABLE		
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
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

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alglucerase	ENZYME	1991	27, 321
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
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
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
malotilate	HEPATOPROTECTIVE	1985	21, 329
mivotilate		1999	35, 343
buserelin acetate	HORMONE	1984	20, 316
goserelin		1987	23, 336
leuprolide acetate		1984	20, 319

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nafarelin acetate		1990	26, 306
somatropin		1987	23, 343
zoledronate disodium	HYPERCALCEMIA	2000	36, 314
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
loprazolam mesylate		1983	19, 321
quazepam		1985	21, 332
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
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
modafinil	IDIOPATHIC	1994	30, 303

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	HYPERSOMNIA		
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
gusperimus		1994	30, 300
mizoribine		1984	20, 321
muromonab-CD3		1986	22, 323
mycophenolate mofetil		1995	31, 346
pimecrolimus		2002	38, 365
tacrolimus		1993	29, 347
defeiprone	IRON CHELATOR	1995	31, 340
alosetron hydrochloride	IRRITABLE BOWEL	2000	36, 295
tegasedor maleate	SYNDROME	2001	37, 270
sulbactam sodium	β-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
interferon ß-1a	MULTIPLE SCLEROSIS	1996	32, 311

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interferon ß-1b		1993	29, 339
glatiramer acetate		1997	33, 334
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
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
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
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

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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
tiludronate disodium	PAGET'S DISEASE	1995	31, 350
temoporphin	PHOTOSENSITIZER	2002	38, 367
verteporfin		2000	36, 312
beraprost sodium	PLATELET AGGREG. INHIBITOR	1992	28, 326
epoprostenol sodium		1983	19, 318
iloprost		1992	28, 332
sarpogrelate HCl	PLATELET ANTIAGGREGANT	1993	29, 344
trimetrexate glucuronate	*PNEUMOCYSTIS CARINII* PNEUMONIA	1994	30, 312
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
finasteride	5α-REDUCTASE INHIBITOR	1992	28, 331
surfactant TA	RESPIRATORY SURFACTANT	1987	23, 344
dexmedetomidine hydrochloride	SEDATIVE	2000	36, 301
kinetin	SKIN PHOTODAMAGE/	1999	35, 341

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	DERMATOLOGIC		
tirilazad mesylate	SUBARACHNOID HEMORRHAGE	1995	31, 351
APSAC	THROMBOLYTIC	1987	23, 326
alteplase		1987	23, 326
balsalazide disodium	ULCERATIVE COLITIS	1997	33, 329
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
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