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PREFACE

Annual Reports in Medicinal Chemistry continues to strive to provide 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 32 retains the familiar format of previous volumes, this year with 31 chapters. Sections I - IV are disease oriented and generally report on specific medicinal agents with updates from Volume 31 on antithrombotics, endothelin, neurokinin antagonists, cell cycle regulation, and obesity. As in past volumes, annual updates have been limited to only 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. To this end, chapters on topics not reported in at least five years include: migraine therapy, Alzheimer's disease, melatonin, bacterial resistance, bacterial genomics, antiretroviral resistance antifungals, angiogenesis, T lymphocyte potassium channel blockers, male contraception, psoriasis, selective PGHS2 inhibitors, and growth hormone secretagogues.

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 regulation of gene expression, blockade of TNF-α, and nuclear orphan receptors. Each of these areas is likely to lead to novel medicinal agents in the future. Chapters in Section VI, Topics in Drug Design and Discovery, reflect the current focus on mechanism-directed drug discovery and newer technologies. These include chapters on combinatorial mixtures as discovery tools, mass spectrometry of non-covalent adducts, nonpeptide agonists of peptide receptors, natural products, and cytochrome P-450.

Volume 32 concludes with To Market, To Market - a chapter on NCE introductions worldwide in 1996. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in volumes 1-32 of this series.

Over the past year, it has been my pleasure to work with 6 highly professional section editors and 70 authors, whose critical contributions comprise this volume.

James A. Bristol
Ann Arbor, Michigan
May 1997

SECTION I. CENTRAL NERVOUS SYSTEM DISEASES

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Chapter 1. Recent Advances in Migraine Therapy

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Introduction - The search for safe and effective treatments for migraine pain has accelerated in the pharmaceutical industry since the introduction of sumatriptan (1) in the marketplace. This chapter will highlight the proposed mechanisms of action of this acute therapy, the various approaches to improve both efficacy and safety, and future areas of study.

Mechanisms and Models of Migraine - The etiology of migraine is not certain although there are a set of hypotheses concerning various stages of the process of migraine. Migraine is thought to be initiated by a "trigger" of unknown origin. Environmental and physiological factors impinge upon this trigger to set in action the events leading to the migraine episode. It may be that migraineurs have an underlying "neural instability" which facilitates setting off the trigger (1). Once initiated, there are several components. The earliest phase may be a premonitory symptom the evening before a migraine headache. The next may be "aura" consisting of visual field disturbances which may be related to changes in cerebral blood flow; these blood flow changes may alter cortical perfusion. Ultimately, the headache appears. Headache pain has been attributed to several factors including dilation of the cerebral blood vessels (2), cerebral edema (3), neurogenic inflammation (4), and central transmission of pain in the brain itself (5).

The early animals models of migraine were based on the study of vascular changes in response to test compounds. The contraction of isolated blood vessels, such as the dog saphenous vein, was used to discover the activity of sumatriptan (6). Other vascular responses such as blood shunting through the arterial-venous anastomoses of the intact pig using radiolabeled microspheres have also been employed (7). More recently a model based on a "neurogenic" hypothesis has been widely explored (8,9). In this model, the trigeminal ganglion is stimulated electrically, which leads to release of neurotransmitters from the peripheral nerve terminals surrounding blood vessels in the dura. This leads to an increased permeability of the vessels which can be monitored by use of a tracer.

Serotonin Receptor Subtypes - Serotonin (5-HT) receptors have been divided into seven classes based upon pharmacological, structural and signaling properties (10). They are: five 5-HT1 receptor subtypes, three 5-HT2 receptor subtypes, one 5-HT3 receptor, one 5-HT4 receptor, two 5-HT5 receptor subtypes, one 5-HT6 receptor and

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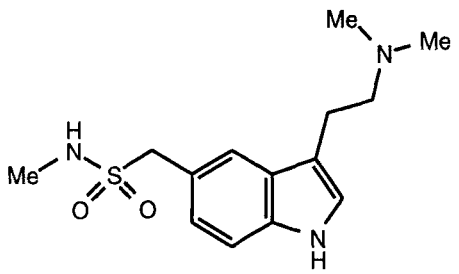
one 5-HT7 receptor. Splice variants or additional subtypes are likely for the 5-HT2C (11) and 5-HT2A (12) and have already been demonstrated for the 5-HT3 (13), 5-HT4 (14), and 5-HT7 receptors (15). The 5-HT3 receptor is a member of the ligand-gated ion channel superfamily (16). All other cloned 5-HT receptors are members of the G-protein coupled receptor superfamily. The pharmacological properties of these receptors have been extensively reviewed elsewhere (10,17). Only the 5-HT1 family has been implicated in the acute treatment of migraine. The five 5-HT1 receptors are termed 5-HT1A, 5-HT1B (formerly called 5-HT1Dβ), 5-HT1D (formerly called 5-HT1Dα), 5-HT1E and 5-HT1F (10,17) and they each contain comparable amino acid chain lengths (421, 377, 390, 365 and 366 respectively for the human sequences). These proteins all share a "homology cluster" displaying mutual conservation of greater than half of the amino acid residues in their transmembrane spanning regions which are likely to contain the ligand binding pocket (18,19). In contrast, the extracellular amino termini and loop regions and carboxyl termini lack significant homology that would enable prediction of their common intracellular coupling to second messengers. All 5-HT1 receptors couple to G-proteins which leads to inhibition of adenylate cyclase activity (10). The 5-HT1A (20), 5-HT1B (21), 5-HT1D (21), and 5-HT1F (22) subtypes also modulate intracellular Ca^{++} through PTX-sensitive G-proteins. In addition to these pathways, each of the 5-HT1 receptors, except 5-HT1E (23), has been shown to increase the turnover of inositol phosphates (21,22,24,25). Physiologically, these receptors may act through G-protein-gated channels such as Girk1 (26). The 5-HT1B, 5-HT1D and 5-HT1F all are thought to inhibit the release of neurotransmitters.

Role of 5-HT1B/1D/1-like Receptors - Sumatriptan (1) was developed using classical pharmacological methodology employing the contraction of the dog saphenous vein preparation (27). As modern receptor binding and classical pharmacological methods began to be reconciled, it appeared there was a relationship between the "5-HT1-like" receptor of the dog saphenous vein and the 5-HT1D receptor described in binding studies (28). Sumatriptan became a selective tool to study 5-HT1D receptors. The advent of molecular cloning further complicated interpretation of these data because two separate genes encoding 5-HT1D receptors were discovered (29), each with high affinity for sumatriptan. Further, in the rat and mouse, one the these receptors, 5-HT1Dβ, had a very different pharmacological profile and was known as a separate subtype, 5-HT1B. Molecular cloning demonstrated that the 5-HT1Dβ and 5-HT1B gene were species homologs (30,31). Their deduced amino acid sequences were 93% identical over the entire sequence and 96% identical in the transmembrane domains. In fact, a single amino acid difference pinpointed to TM VII (T355N) appears to be responsible for the disparity in the pharmacological profiles of these human and rat 5-HT receptor subtypes (32). The complications in the receptor nomenclature have led to a recent revision (32) and the 5-HT1Dβ receptor is now known as 5-HT1B. Both 5-HT1B and 5-HT1D receptors are distributed in the brain, where they appear to have primarily presynaptic localization (34). In several preparations, these receptors inhibit the release of serotonin from either terminal or somatodendritic processes (35). They also act as heteroreceptors, inhibiting the release of glutamate, acetylcholine and other neurotransmitters (36,37).



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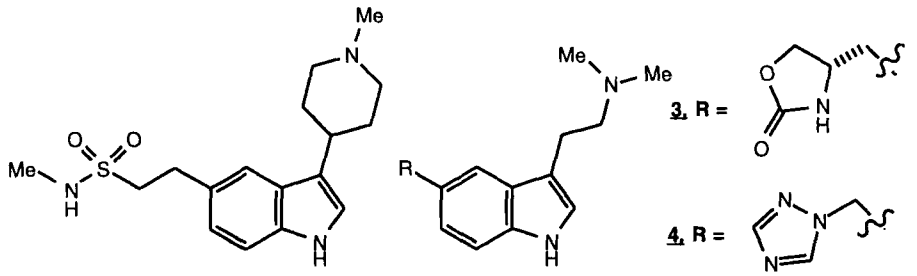
How do these receptors and the activity of sumatriptan and other 5-HT1 agonists described below relate to the models and mechanisms of migraine headache? It is clear that all 5-HT1 agonists that have been reported to be useful in the treatment of migraine, including naratriptan (2), zolmitriptan (311-C90, 3), and rizatriptan (MK 462, 4) have substantial affinity for both the human 5-HT1D and 5-HT1B receptor subtypes. It is possible that both activities are therapeutic in alleviation of the migraine episode. Several lines of evidence suggest that the 5-HT1B receptor may be the subtype most closely related to the dog saphenous vein 5-HT1-like receptor (34). Therefore, these agonists may act on 5-HT1B receptor on the smooth muscle of human cerebral blood vessels to cause vasoconstriction (similar to their activity in the dog saphenous vein). In addition, the 5-HT1-like agonists may act on 5-HT1D receptors on the terminals of the trigeminovascular system to inhibit neurotransmitter release and to thus stop the neurogenic inflammation (34,38). The clinical utility of compounds acting at only one of these two sites has not yet been reported although it has been suggested that a 5-HT1D-selective compound may be therapeutic and may reduce some of the cardiovascular liabilities of sumatriptan (38, *vide infra*).

Table 1. Affinities of Antimigraine Compounds at Cloned 5-HT1D and 5-HT1B Receptors.

Compound	pK_i 5-HT1D	pK_i 5-HT1B	Reference
Sumatriptan (1)	8.5	8.1	39
Naratriptan (2)	8.3	8.7	39
Zolmitriptan (3)	9.2	8.3	40
Rizatriptan (4)	7.7	7.3	39
CP 122,288 (5)	8.1	7.5	39
Avitriptan (8)	8.3	7.7	41
Alniditan (9)	9.0	8.7	42



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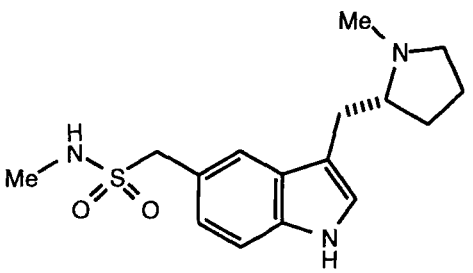
Designing an Improved Sumatriptan - While sumatriptan is effective as a migraine abortive agent, about 15% of patients fail to respond to treatment and up to 40% may suffer from recurrence of headache within 24 hours (43,44). Similar rates of headache recurrence have been observed with other antimigraine treatments. Further, the finding of decreased latency of onset of headache relief after subcutaneous *versus*

oral sumatriptan suggests a possible relationship between plasma drug concentrations and headache relief. A number of approaches for designing improvements beyond sumatriptan in migraine abortive treatment have resulted. These approaches include improvements in pharmacokinetics (45), central penetration, and binding profile for 5HT1D (46) and other receptors such as the 5HT1F (47,48).

Rizatriptan (4) has been demonstrated in preclinical studies to show both increased oral bioavailability and more rapid absorption compared to oral sumatriptan (49). These findings, supported by preliminary clinical pharmacokinetic studies, suggest that this agent might offer greater consistency of effect due to the increased bioavailability. Improvements in onset of efficacy might also occur as a consequence of the more rapid absorption (49).

The observation that sumatriptan fails to prevent headache development upon subcutaneous administration in the aura phase but shows effect after headache, possibly due to diminished integrity of the blood brain barrier during migraine episode, suggests that central penetration might be an important attribute of an effective antimigraine therapeutic (50). Zolmitriptan (3) is less hydrophilic than sumatriptan and shows both peripheral and central activities, with activity as a vasoconstrictor (peripherally mediated), as an inhibitor of trigeminally mediated neuropeptide release, and on the central components of the trigemino-vascular system within the nucleus caudalis (51). Preliminary studies suggest that oral zolmitriptan may be useful in preventing migraine when taken during aura (52), supporting the importance of central penetration.



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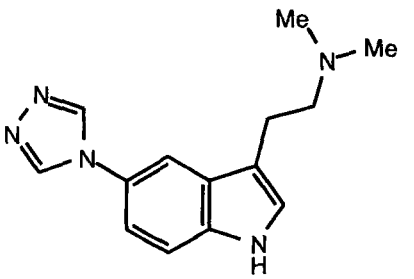

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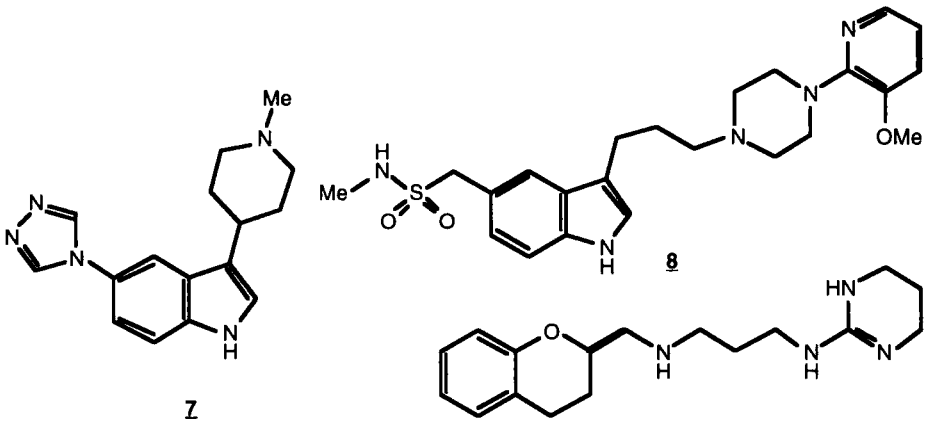
A conformationally restricted analog of sumatriptan, 5 (CP-122,288) selectively inhibits neurogenic inflammation in intracranial tissues at doses which lack vasoconstrictor effects in animal studies (53). With affinity comparable to sumatriptan for 5HT1D and 5HT1B receptors, 5 is 40,000 times more potent in blocking rat dural neurogenic plasma protein extravasation, while similarly potent to sumatriptan as a vasoconstrictor. The clinical experience with this agent should help to elucidate the relevance of each of these activities in migraine intervention. High oral availability is also found with the 5HT1D selective agonist 6 (L-741,604), which also shows reduced plasma clearance compared to either sumatriptan or rizatriptan in preclinical studies (54). The corresponding indolylpiperidine 7 (L-741,519) additionally has an increased *in vivo* half-life relative to the tryptamine analogs. This attribute might prove valuable in reducing the incidence of headache recurrence. Developed as a 5HT1B/1D agonist, avitriptan (BMS-180048, 8) selectively decreases corotid blood flow due to a reduction in arteriovenous anastomotic blood flow. This agent also potently constricts isolated human coronary artery with effects comparable to those of sumatriptan (55). Chemically unique among the serotonergic antimigraine agents, alniditan (9) is a non-

indole with high affinity for 5HT1B, 5HT1D, and 5HT1A receptors, and lacking significant affinity for the 5HT1F (*vide infra*) receptor (56).



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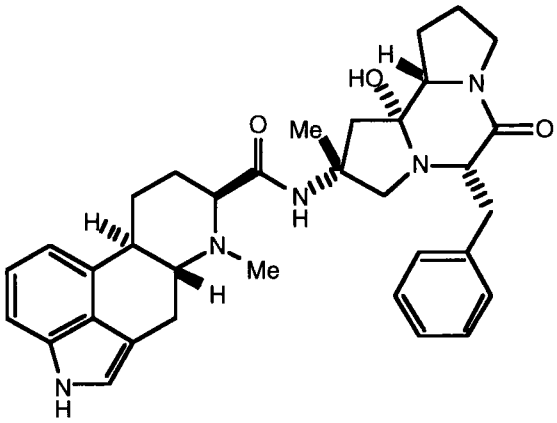
Novel Presynaptic Receptor Targets: 5-HT1F - In addition to the 5-HT1B and 5-HT1D receptors, a novel or "orphan" 5-HT receptor called 5-HT1F was cloned and characterized (57). This receptor showed many similarities to 5-HT1B and 5-HT1D receptors with respect to receptor binding properties, coupling to the inhibition of adenylate cyclase activity, and general distribution in the brain, although its abundance as reflected by the mRNA level for this receptors was relatively low. The pharmacological profile of this receptor was not easily related to any of the known pharmacological subtypes measured in native tissues. For example, the 5-HT1F receptor was characterized by the following rank order of agonist potencies: 5-HT> sumatriptan >> 5-CT, forming a unique pharmacological profile. The surprisingly high affinity and potency of sumatriptan for the 5-HT1F receptor subtype lead to speculation that sumatriptan might exert its anti-migraine action through this "orphan" subtype (57). Supporting this notion, the mRNA for this receptor was detected in the nucleus caudalis as well as in other central pain-processing nuclei (58) and was also demonstrated in the trigeminal ganglion (59).



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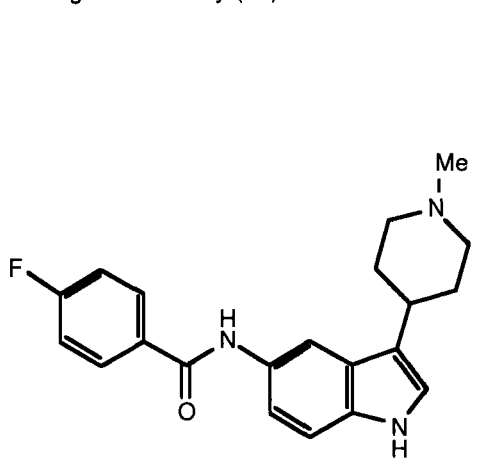
Further support for the 5-HT1F presynaptic mechanism came from a comparison of the activity of "5-HT1D/1B " agonists such as naratriptan, rizatriptan, zolmitriptan, and dihydroergotamine (DHE, 10) in human migraine and in migraine models with their affinities at the cloned human 5-HT1D, 5-HT1B, and 5-HT1F receptors (60,61). As

shown in Table 2, each compound had substantial activity at the 5-HT1F receptor in addition to affinity at the 5-HT1D and 5-HT1B sites.

Table 2. Affinities of Compounds at Cloned Human 5-HT1F Receptor (60,61).

Compound	**pK_i 5HT1F**
LY334370 (11)	8.8
LY302148 (12)	8.6
Naratriptan (2)	8.4
Zolmitriptan (3)	7.6
Sumatriptan (1)	7.6
DHE (10)	6.6
Rizatriptan (4)	6.6

Furthermore, comparison of these affinities to the potencies of the same compounds to inhibit plasma extravasation in the guinea pig neurogenic model indicated the best correlation was with the 5-HT1F receptor (60,61). Finally, selective 5-HT1F agonists were designed and evaluated in the cloned receptors as well as in the plasma extravasation model. The activity *in vivo* most closely matched predictions based on their 5-HT1F activity (47,48). Moreover, these 5-HT1F selective agonists were not potent stimulators of contraction of the rabbit saphenous vein, another of the preparations used to hunt for "5-HT1D/1B agonists" (60,61). The 5-HT1F agonist LY 334370 (11) is presently under clinical investigation and determine whether a serotonergic presynaptic receptor agonist devoid of vasoconstrictor properties has antimigraine efficacy (60).



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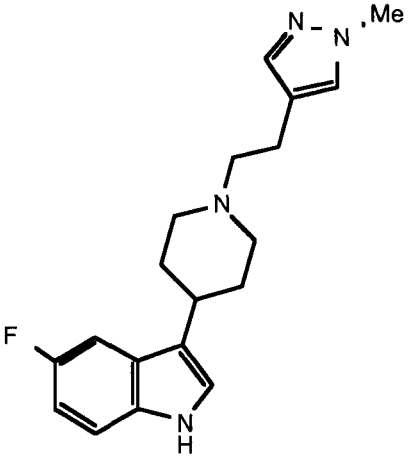

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Beyond Serotonin - Several additional pharmacological approaches to the treatment of migraine are in various stages of testing including substance-P antagonists, CGRP antagonists, somatostatin agonists, GABA A antagonists, and others (62). Although the clinical data accumulating on the tachykinin antagonists has not been promising

thusfar, additional clinical data are required to evaluate the potential of these other receptor-based therapies.

However, new insights into possible molecular neurobiological substrates which may underlie migraine pathology have been provided from the study of human genetics of a rare form of migraine, known as familial hemiplegic migraine (FHM). This condition is an autosomal dominant subtype of migraine with aura which is characterized by the occurrence of hemiplegia, or hemiparesis, during the period of aura (63,64). Due to the strong genetic basis for this disease, investigators have sought to find the possibly mutated FHM genes which may be involved in its pathophysiology. The frequency of migraine in patients suffering from another autosomal dominant neurological condition, CADISIL (Cerebral Autosomal Dominant Artereopathy with Subcritical Infarcts and Leukoencephalopathy), is extremely high (65), and this observation led to the hypothesis that the CADISIL gene might be responsible for FHM. Since the CADISIL gene had been mapped to chromosome 19, it was possible that FHM also mapped to this chromosome. Using genetic linkage analysis (66), it was demonstrated that the FHM gene resided within a chromosomal interval containing the CADISIL locus on chromosome 19. This set the stage for cloning efforts which have already yielded two interesting discoveries, a Notch protein and a calcium channel (*vide infra*).

In a recent report, a candidate gene from the critical region of the CADISIL locus was identified (67). This gene was shown to be a member of the Notch gene family, highly homologous to the murine Notch-3. It maps to human chromosome 19p 13.2-13.2. Ten different missense mutations occur in the sequence from over a dozen separate CADISIL families. The mutations are expected to result in an altered folding of the Notch protein because they are additions or mutations of cysteine residues. The functional consequences for the mutant Notch proteins remain to be elucidated. Interestingly, Notch signaling in *Caenorhabditis elegans* is affected by an interacting protein related to the mammalian presenilin genes, PS-1 and PS-2 which may be the keys to early onset familial Alzheimer's dementia (68). Coupled with the role of Notch proteins in Drosophlia development, these finding suggest that the mutations in the Notch-3 gene in CADISIL patients may be somehow responsible for the "adult-onset" aspect of this neurological condition thus as some kind of "clock". It is also interesting to note that nearly half of the CADISIL patients develop migraine as a first syndrome at about age 20, relatively late compared with the onset of symptoms in common migraine. The more severe aspects of the CADISIL syndrome often take another 20 years to develop (69).

Simultaneous with the discovery of the human Notch-3 gene in CADISIL another gene was isolated from the same chromosomal region, 19p 13. This gene encoded an alpha1 subunit of a type P/Q calcium channel (CACNL1A4, 70) which was mutated in FHM patients. This gene also maps to human chromosome 19p 13.1-p13.2. Four distinct missense mutations in five unrelated FHM families were found. One mutation may affect the voltage sensor (R129Q), a second may affect the ion selectivity filter (T666M), and two others are in S6 segments (V714A; I1811L). The role of these residues is as yet unknown although their absolute conservation in all calcium channel alpha1 subunits suggests a key role. Interesting, mutations at other loci in the same gene are observed in patients displaying episodic ataxia type 2 (EA-2) which is another episodic, autosomal dominant disorder with migraine as part of the syndrome. The mutations in these patients lead to truncated proteins which are unlikely to form functional calcium channels (70).

The discovery that a channel defect may underlie part of the syndrome in FHM and other genetic migraine states is intriguing. Changes in a P/Q type calcium channel such as those described may be expected to result in changes in the neural threshold for channel triggering by a variety of environmental or homeostatic mechanisms. Such an instability of calcium channels may result in the spurious release of substances from nerve terminals such as is mimicked in the "trigeminovascular" model of migraine. It would be interesting to determine whether the CALNL1A4 mRNA is expressed in human trigeminal ganglion neurons. The experimental use of Mg^{++}, which interferes with calcium channel function, in migraine prophylactics merits continued attention (71).

The molecular lesions of mutant stains of mice first identified over thirty years ago (72) have recently been discovered (73). The tottering (tg) mutation and the leaner (tg1a) mutation now appear to be related to homologous calcium channel defects as described or FHM and EA-2, respectively. The tottering and leaner mutations map to regions of the mouse chromosome that are syntetic with the human loci. The tissue distribution of these calcium channels are very similar in rat and human. In addition, the sequences are 99% identical in the regions which are critical for functional specificity. It will be interesting to compare the phenotypes of these mice with those produced by transgenic approaches in which the homologous mutations of the FHM and EA-2 can be constructed.

Summary - Research and development in receptor-based mechanisms for migraine therapy has proceed at a rapid pace over the last ten years. Several new products based on improvements of the prototype, sumatriptan, are being introduced into the market. Whether improvement in the pharmacokinetic parameters or in the degree of CNS penetration will affect rebound migraine or have a higher degree of efficacy awaits large population studies. Newer agents acting through neuronal mechanisms such as 5-HT1D selective or 5-HT1F selective await efficacy trials in humans. The role of additional receptor modalities beyond the tested serotonergic mechanisms and possible "channelopathies" in migraine will ensure a steady stream of new targets for possible pharmacotherapy of the disease.

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Chapter 2. Alzheimer's Disease: Recent Advances on the Amyloid Hypothesis

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Introduction - Alzheimer's disease (AD), the most common form of dementia, is characterized by large numbers of plaques and neurofibrillary tangles in brain regions responsible for higher cognitive function such as the hippocampus and association cortices. The plaques are primarily composed of aggregates of a 39-43 amino acid ß-amyloid peptide (Aß), of which the 42 amino acid peptide is the most prone to aggregation and, thus, plaque formation. Indeed, $Aß_{42}$ is found principally at the core of the plaques, leading many researchers to focus attention on the ratio of $Aß_{42}$ to $Aß_{total}$ as a guide in their pursuit of therapeutic agents aimed at modulating Aß production.

The "amyloid hypothesis" postulates that $Aß_{42}$ in particular is causal in the disease process (1, 2). This hypothesis is supported primarily by genetic evidence from individuals who greatly overproduce $Aß_{42}$ and possess disease-causing mutations in genes coding for either amyloid precursor protein (APP) or the recently discovered presenilin proteins (PS1 or PS2) which are described below.

A growing effort is being directed toward therapeutic strategies which target the effects of Aß as treatments for the causes of AD. These strategies are complimentary to symptomatic cognition-improvement therapies based on acetylcholinesterase (AChE) inhibitors and M1 agonists. The Aß strategies take three forms as discussed below: disruption of Aß production from its precursor protien, protection from the neurotoxicity of the Aß aggregates, and inhibition of the aggregation of Aß monomer into the neurotoxic aggregates that constitute the plaques. Recent developments in transgenic mouse models which develop AD pathology is expected to greatly assist the evaluation of potential agents for this devastating disease.

Aß-PEPTIDE PRODUCTION

Substantial effort has been directed at the mechanistic understanding and isolation of the relevant enzymes involved in the processing of APP and the production of Aß in an attempt to provide targets for intervention. Details of the processing of APP and the production of Aß-peptide have been extensively discussed (1-9) since the last report in this series (10). The presenilins and their effect on the levels of various Aß is an additional new arena for the development of therapeutic targets.

Enzymes Involved in Aß Production - APP is processed by a group of "secretases" labeled by their site of action as shown in Figure 1 (1-9). The ß- and γ-cleavages lead to the production of Aß-peptides ranging from 39-43 amino acids (with variation at the C-terminus). Research has focused on inhibiton of Aß production or secretion by various agents and the isolation and characterization of the ß- and γ-secretases.

Cleavage of APP to Aß has been shown to take place on membrane bound APP (11-15). Interestingly, the γ-cleavage sites are located in the membrane spanning region of APP. Evidence has also been presented that the ß- and γ-cleavages occur in separate cellular compartments, potentially allowing for movement of the cleavage site to the membrane surface as the partially processed protein is passed from one compartment to another (16).

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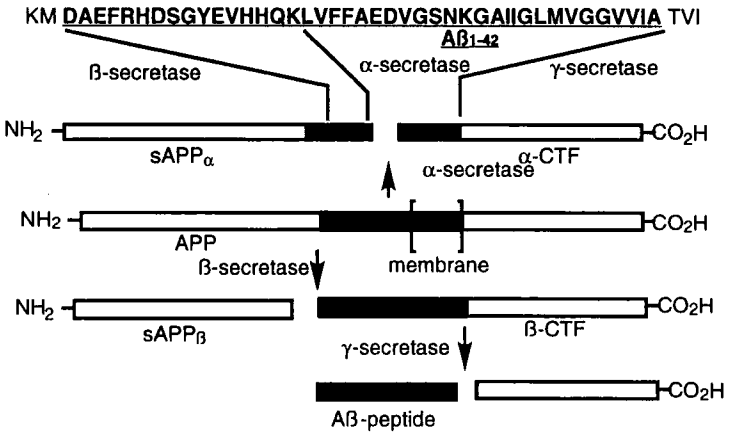
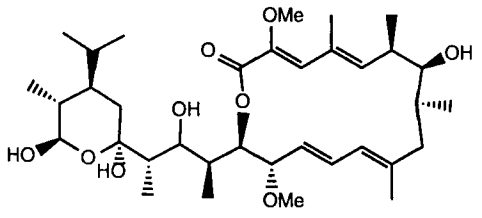


Figure 1. APP Processing.

In studies of the overall Aß biosynthesis process, the secretion of Aß-peptide is reported to be blocked by brefeldin A (16-19) and monensin (16, 19-21), both of which block intracellular trafficking at unknown sites. Further, bafilomycin A1 (baf A1), (1), a potent inhibitor of vacuolar H^+-ATPase, also blocks APP processing supporting previous observations that processing takes place in acidic compartments (19, 22, 23). Baf A1 has been shown to block the ß-secretase activity (24), and inhibited Aß production in cells transfected with the Swedish APP double mutation which replaces Lys_{670}-Met_{671} with Asn-Leu (25). However, in a wild type cell line an alternative ß-cleavage process led to Aß with either Phe-4 or Val-3 at the N-terminus.



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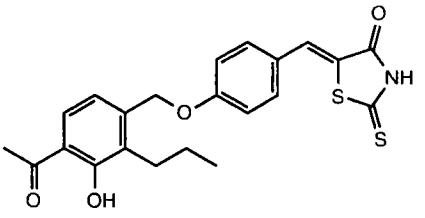

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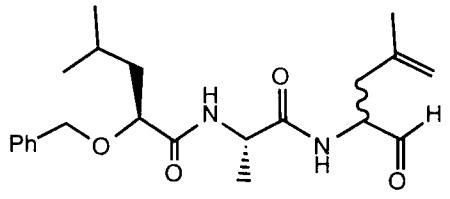
Isolation of ß- and γ-secretase has been the focus of considerable effort. Processing of APP by ß-secretase shows a high specificity for cleavage at the Met-Asp site (11, 24). There may be multiple ß-secretase activities which vary by cell type (11). A number of enzymes have been proposed as the ß-secretase, including Cathepsin G, Cathepsin D (Cat D), clipsin, endopeptidase 3.4.24.15 and prolyl endopeptidase (3, 26). However, none of these enzymes fully match the expected ß-secretase activity profile (27). Standard enzyme inhibitors have been unsuccessful in defining the ß-secretase mechanism of action. A patent application claiming the isolation of ß-secretase has recently published (27).

In the γ-secretase area, the varying chain lengths of the family of Aß-peptides are proposed to arise from different γ-secretases based on the selective inhibition in various cell lines of the production of $Aß_{40}$ over the more amyloidogenic $Aß_{42}$ (28, 29). The γ-secretase activity is also reported to be due to a nonspecific enzymatic cleavage of previously aggregated Aß (30). Cat D has been proposed as the γ-secretase, however, the secretion of Aß in a Cat D deficient (knockout) mouse does not support this possibility (31). A Cat D-like activity of γ-secretase is supported by pH and inhibitor profiles (32, 33). Rhodanine 2 has been described as a Cat D inhibitor which showed a reduction of Aß in APP transfected cells (34).

Inhibitors of Aß-Peptide Production - Earlier reviews have covered the few examples of small molecules claimed to inhibit the production of Aß (1, 9). Recently, several peptide aldehydes have been examined *in vitro* as γ-secretase inhibitors. Calpain inhibitor I (CPI) (**3**), MG132 (**4**), calpeptin (**5**), MDL28170 (**6**) and the dipeptidyl aldehyde SIB1405 (**7**) have been shown to reduce the production of $Aß_{40}$ at micromolar to millimolar levels, however, **7** had little effect on the more amyloidogenic $Aß_{42}$ (28, 29, 35-37). Therefore, it may be possible to target $Aß_{42}$ without disrupting production of $Aß_{40}$.

3 Ac-Leu-Leu-Nle-H
4 Z-Leu-Leu-Leu-H
5 Z-Leu-Nle-H
6 Z-Val-Phe-H



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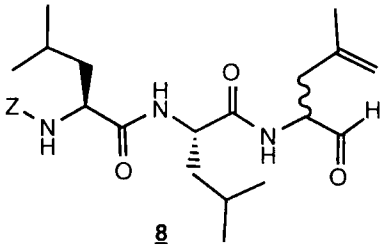

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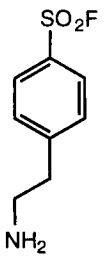
A series of aldehyde inhibitors closely related to CPI led to SIB1281 (**8**) which is proposed to be a ß-secretase inhibitor with an IC_{50} of 0.8 µM (37-39). Another compound suggested to be a ß-secretase inhibitor is the irreversible serine protease inhibitor AEBSF, (**9**), which inhibits Aß production and enhances $sAPP_{\alpha}$ secretion (40) in several cell lines, though at millimolar concentrations. Pyrrolidine **10** has also been reported as a ß-secretase inhibitor in a patent application (27).



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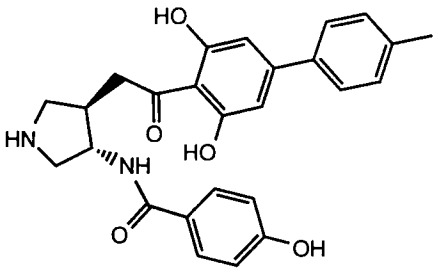

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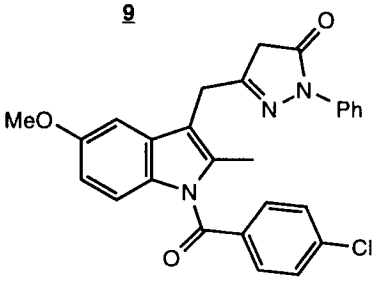

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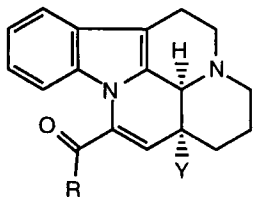
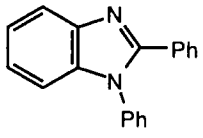


R = OH, Oalkyl, NR'R', etc.

12



13

From the patent literature, pyrazolones (41), some alkaloid natural products (42) and some benzimidazoles (43) are proposed as Aß reducing compounds. Representative examples from these patent applications are **11**, **12**, and **13**, respectively. A broad group of classical protease inhibitors have also been claimed to prevent amyloid production however no data were given (44).

Presenilins (PS) - The majority of known familial Alzheimer's disease (FAD) cases are associated with mutations of the *presenilin 1* (*PS1*) or *presenilin 2* (*PS2*) genes which are localized on chromosomes 14 and 1 respectively (9, 45-47). The secondary structure of the presenilin-1 protein (PS1) suggests an integral membrane protein with eight transmembrane domains where both the N- and C-termini are located in the cytosol (48-50). Disease causing mutations on PS1 and PS2 cluster both within and without the transmembrane regions (47). Molecular modeling based on the hydropathy of PS1 and PS2 has proposed a 3D structure where the transmembrane regions form a hydrophobic channel and the mutations cluster within this channel (46). The primary sequences of PS1 and PS2 share 67% homology implying that PS2 is also an integral membrane protein (9, 45-47). The majority of known PS mutations occur within the conserved regions of PS1 and PS2, further supporting similarity of function (47). Interestingly, PS1 has been found to share homology with *Caenorhabditis elegans'* SPE-4 (31%), which is involved with protein transport, and SEL-12 (48%), a transmembrane domain protein (47-52).

The native functions of *PS1* and *PS2*, and the specific roles that they play in FAD are currently unknown. The ratio of $A\beta_{42}/A\beta_{total}$ isolated from skin fibroblast cells and plasma from patients bearing either *PS1* or *PS2* mutations has been observed to increase by as much as eight fold (53-55). Subsequently, a similar increase in $A\beta_{42}/A\beta_{total}$ has been demonstrated in the brains of patients bearing a *PS1* mutation (54). More recently, an increase in the $A\beta_{42}/A\beta_{total}$ ratio has been reported in double-transfected 293 human embryonic kidney cells containing the Swedish APP mutation and mutant *PS1* or *PS2*, and also in double-transgenic mice bearing the wild-type APP_{695} gene and mutant *PS1* (55). Data also suggest that the *PS* mutations either directly or indirectly alter the activity of γ-secretase(s), but not α- or ß-secretase (55). These results support the idea that *PS* mutations produce a dominant gain in function and may induce AD by enhancing $A\beta_{42}$ production and promoting cerebral ß-amyloidosis (55).

Aß-PEPTIDE NEUROTOXICITY

Recent Biology - The mechanisms underlying neuronal degeneration in AD were recently reviewed (56). Currently, no consensus biochemical mechanism has emerged for the role of Aß in causing neuronal toxicity. It is generally accepted that the Aß peptide is neurotoxic *via* apoptotic mechanisms *in vitro*, however, the debate continues as to whether the peptide is toxic *in vivo*.

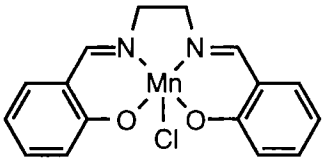
The major hypotheses involving Aß neurotoxicity continue to focus on the general areas of disruption of cellular calcium homeostasis, the role of reactive oxygen species (ROS) and free radical oxidation, and the involvement of Aß in general membrane perturbation. It is likely that these hypotheses are interrelated in a temporal fashion, i.e. membrane perturbation leads to disruption of calcium homeostasis, which in turn leads to generation of ROS.

The field is complicated by the known aggregation properties of $A\beta_{40}$ and $A\beta_{42}$ and the need to "age" fresh lots of the peptide to generate a toxic species. Many researchers circumvent these problems by using the $A\beta_{25-35}$ peptide, but whether $A\beta_{25-35}$ toxicity is related to $A\beta_{40}$ toxicity is unclear. In addition, $A\beta_{25-35}$ cannot be isolated from AD brain. Researchers also report differences in toxicity in neuronal cell lines versus primary cultures. Finally, determining Aß neurotoxicity using LDH release or MTT reduction as a measure of cell viability is also a point of debate (57,58).

One of the earliest reported effects of Aß *in vitro* involved the disruption of cellular calcium homeostasis and the resulting cell vulnerability to excitotoxicity (59). A recent review (60) enumerates the various effects of $A\beta_{40}$, $A\beta_{25-35}$, and $A\beta_{39}$ on activation,

increase of conductance, and even selective inhibition of calcium, potassium, or nonselective cation channels in neuronal cultures, various cell lines and artificial lipid bilayers. Another report indicates no effect on $A\beta_{40}$ induced neurotoxicity in hippocampal cultures upon treatment with various voltage- or ligand-gated calcium channel antagonists (ω-conotoxin, nifedipine, verapamil, APV, or MK-801) (61).

The role of oxidative damage in various neurodegenerative diseases was recently reviewed (62, 63). There have been reports providing histological evidence for (64, 65) and against (66) oxidative damage in AD patients. It has been reported that *in vitro* Aß spontaneously generates radicals (67) and that Aß activates microglia leading to an inflammatory response (68). This response is thought to result from the release of ROS as well as a variety of cytotoxic agents such as proteolytic enzymes, cytokines and complement proteins from microglia (69). Antioxidants such as vitamin E and propyl gallate and other ROS scavengers protect against Aß neurotoxicity (70). The formation of superoxide through interaction of Aß with endothelial cells on blood vessels has been reported (71). A radical scavenger EUK-8 (**14**) also prevented Aß-induced free radical formation and lipid peroxidation and was shown to protect against $A\beta_{40}$ and $A\beta_{42}$ toxicity at 25 µM in hippocampal cultures (72).



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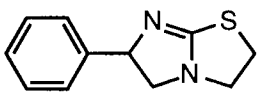
Reports of the ability of Aß to bind to two cell-surface receptors on neurons and microglia, the "receptor for advance glycation end products" (RAGE) (73) and class A scavenger receptors (SR) (74), have appeared. Both reports provide compelling evidence that Aß adheres to the respective receptors and leads to the generation of ROS. The relevance of these receptors to Aß neurotoxicity has been questioned (75). It has been shown that *in vitro* Aß selectively disrupts membranes containing acidic phospholipids as evidenced by dye leakage (78). The pH dependence of this interaction is consistent with disruption of endosomal membranes. The contribution of these membrane disruptions to toxicity, ion homeostasis and ROS generation needs to be elucidated.

The potential to generate ROS from the arachidonic acid cascade has been reported. $A\beta_{42}$ and $A\beta_{25\text{-}35}$ have been shown to activate phospholipase A_2 (PLA_2) *in vitro* at concentrations that induce toxicity (76). The use of PLA_2 inhibitors to treat Aß induced neurodegeneration has been reported (77).

Recent Chemistry - The chemical patent literature is just now disclosing areas for therapeutic intervention. One recent patent discloses arylimidothiazole analogs of levamisole **15** as inhibitors of alkaline phosphatase and Aß toxicity (79). At 100µM, levamisole protected HT4 neurotumor cells against $A\beta_{25\text{-}35}$ induced toxicity.

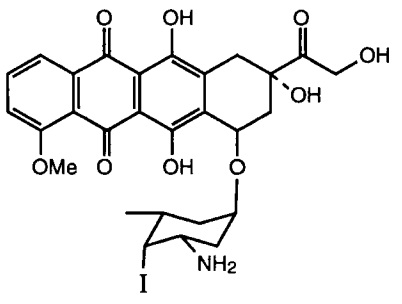


15

Aß-PEPTIDE AGGREGATION

The Aggregation Process - The aggregation of amyloidogenic peptide fragments into the classical ß-sheet structure of amyloid deposits is a characteristic of several diseases such as AD, systemic amyloidosis (SAA), and amyloidosis caused by monoclonal Ig light chains (AL) (80). The crucial phenomena of aggregation are not well deciphered at this time. The steps involved in the aggregation of monomers to oligomers and subsequent growth into fibrils and precipitation/deposition are under extensive study, though without conclusive chemically useful information at this time. Assays are available to follow the aggregation of synthetic peptide and subsequent deposition (81). Studies have shown that mutations of the hydrophobic region 17-21 of Aß result in significant loss of aggregation ability of the peptide (82). From work first using 10-mers spanning the Aß peptide, and then refining with 5-mers in the 11-20 region, has come the determination that the peptide sequence with the highest aggregation potential is the 17-21 (LVFFA) sequence, a particularly hydrophobic region. Unfortunately, this work refers only to inhibition of $Aß_{40}$ aggregation with no comment on $Aß_{42}$ results. However, $Aß_{42}$ and fragments containing the $Aß_{42}$ C-terminus have been shown to be significantly more prone to aggregation than corresponding $Aß_{40}$ fragments (83). There is also the suggestion that complement binding protein C1q promotes the nucleation phase of aggregation, raising the prospect of external agents triggering aggregation (84).

Inhibitors of Aß aggregation - There has been little published on aggregation inhibitors in AD. The clinical observation that IDOX (**16**) can induce amyloid resorption in AL suggests that amyloid disaggregation may be a viable target (85). IDOX binds to all classes of amyloid fibrils, including Aß fibrils, with a high affinity and inhibits the *in* vivo growth of the fibrils to larger deposits (85). Thus, inhibition of the progression of Aß to fibril to plaque has been proposed as an approach to treating AD (80, 86) and represents a non-cellular target.



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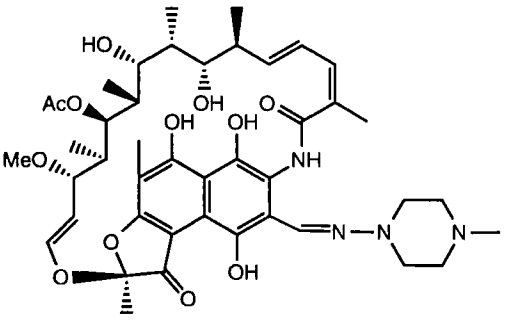


17

Rifampicin (**17**), a macrocyclic naphthalene-1,4-diol anti-leprosy drug, has been shown to inhibit the aggregation of $Aß_{40}$ in a concentration dependent manner and completely at 100µg/mL (87, 88). Similar results with hydroquinone, quinone and 1,4-dihydroxynaphthalene suggest that scavenging of hydroxyl radicals is involved in the inhibition of aggregation and neurotoxicity by these compounds (87).

An inhibitor of the aggregation of $Aß_{40}$, AcQKLVFF-NH2 (**18**), has been prepared (based on the 16-21 region studies described above) which binds to the peptide and stabilizes it against fibril formation (82). Two other groups have developed molecules attaching a highly aggregating sequence from Aß to a "disrupter unit" so that upon adhesion to Aß, a non-aggregating complex is created. In one case, an oligolysine

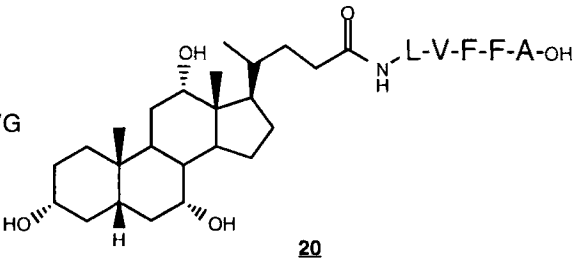
hexamer attached to the C-terminus of the 15-25 sequence (**19**) has led to a protein like structure that adheres to $A\beta_{39}$ but prevents aggregation and fibril growth (89). Another group used shorter sequences and non-peptide groups to produce PPI-368 (**20**) and related compounds (90, 91).

The construction of "anti-ß-sheet" molecules by the insertion of proline into a sequence designed for similar hydrophobicity to the 15-25 region of Aß gave the 11-mer iAß1 (RDLPFFPVPID) (**21**) which not only reduced aggregation of native $A\beta_{40}$ (and to a lesser extent $A\beta_{42}$), but also caused disaggregation of preformed aggregates of $A\beta_{40}$ (92). A shorter sequence (LPFFD) showed equivalent inhibition. Also, a D-amino acid replacement version of iAß1 showed similar inhibition and substantial resistance to proteolytic degradation.

18 Ac-QKLVFF-NH_2

19 KKKKKK-G-QKLVFFAEDVG

21 RDLPFFPVPID



20

ANIMAL MODELS

Several transgenic mouse models that overexpress human APP develop a number of pathological hallmarks associated with AD (93-95). The overproduction of Aß, the formation of amyloid plaques and the development of other significant neurodegenerative features of the disease provide important tools for testing the efficacy of the "amyloid hypothesis"-based inhibitors. In particular, the PDAPP mouse which overexpresses mutated $APP_{717(V->F)}$ develops extensive neuritic dystrophy, gliosis and synaptic loss. The extent of the neurodegeneration in this model supports the primacy of Aß-peptide in the pathogenesis of AD.

Conclusions - While much recent progress has been made in the genetics of AD, the primary chemical targets arising from this research remain to be elucidated. Research in APP processing, Aß induced neurotoxicity and Aß aggregation have reached a level of maturation where clinical evaluation of the "amyloid hypothesis" is anticipated. The development of transgenic mice which demonstrate much of the AD pathology should accelerate the development of amyloid based therapies.

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Chapter 3. Obesity: Leptin - Neuropeptide Y Interactions in the Control of Body Weight

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Introduction - To cope with variability in energy supplies, mammals have developed the capacity to store excess energy in adipose cells as triglycerides. These energy deposits are highly regulated by a number of mechanisms and can be rapidly mobilized during periods of intense physical exercise or nutrient deprivation. Obesity represents the end result of more energy ingestion than is utilized by the body. This positive energy balance is then stored in the form of body fat. Fat storage in obesity is affected by three parameters, energy ingestion, metabolism or energy expenditure and genetic susceptibility to excess fat storage. It may be that the genetic predisposition to obesity is facilitated by nutrient availability and a low level of physical activity. It is difficult to determine a single root cause of obesity since, in some patients, the cause is clearly caloric intake while, in others, this does not seem to be a predominant factor. What is clear is that clinically relevant obesity is now at epidemic proportions in the United States with a third of the population being affected (1, 2). Obesity results in a number of adverse health effects including increases in morbidity and mortality from non-insulin dependent diabetes mellitus (NIDDM), hypertension, hypercholesterolemia, sleep apnea, and other medical conditions (3). In the U.S., the most direct link is the comorbidity of NIDDM and obesity with more than 80% of the NIDDM patients being affected (4). The insulin resistance seen in NIDDM is believed to contribute to cardiovascular disorders such as dyslipidemias, atherosclerosis and hypertension. Weight loss can result in a significantly lower risk for these endocrine and cardiovascular disorders (5, 6).

Historically, pharmaceutical research focused with very limited success on metabolism and appetite suppression as strategies to treat obesity. Through these efforts, it is becoming increasingly clear that regulation of food intake and energy expenditure is coordinated though the hypothalamus. It is likely that the adipose tissue communicates with the hypothalamus to determine adiposity appropriate for the environmental circumstances. In turn, the hypothalamus regulates adiposity through food intake, metabolism via the sympathetic nervous system and endocrine function. Several endocrine factors and neurotransmitters appear to be involved in this complex network designed to maintain body weight at a prescribed "set point". Dysregulation of this network undoubtedly contributes to obesity.

Recent advances in the biology of obesity has rekindled the interest of the pharmaceutical industry in this area. The recent approval and subsequent sales of dexfenfluramine in the United States has helped convince opinion leaders that obesity is a treatable disease. Also, the identification of genetic defects in obese animals has been an essential element of this renewed interest. Perhaps the most important contributer was the cloning of the *ob* gene and discovery of its encoded gene product, leptin, an endocrine peptide that is secreted from the adipose tissue (7, 8). Subsequently, it was found that several other obese animals such as the *db/db* mouse (9-11) and Zucker rat (12, 13) had defects in the gene encoding the leptin receptor. As suggested by the title of this review, one of the important actions of this peptide is the suppression of the synthesis and release of neuropeptide Y (NPY). NPY is one of the most potent orexigenic agents and is the only endogenous neuropeptide that is capable of inducing obesity in experimental animals (14, 15). Most animal models of obesity exhibit increased concentrations of hypothalamic NPY and mRNA encoding

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NPY. Therefore, it is presumed that leptin and NPY will play important and interdependent roles in human obesity. A comprehensive overview of the pharmacotherapy and pathophysiology of obesity was the subject of a recent review (16), therefore, we will focus on what appear to be two of the key components in the coordinate regulation of body weight, leptin and NPY .

NPY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-amide
PYY	YP**I**KP**EA**PGEDA**S**P**E**E**LN**RYY**AS**LRHY**L**NL**V**TRQRY-amide
PP	**A**P**LE**P**VY**PGD**N**A**T**PE**Q**MAQY**AAD**LRR**Y**IN**ML**TR**P**RY-amide

Figure 1. Sequences of the human NPY family of peptides. Sequences of the known peptides in the NPY or PP-fold peptide family. Amino acid residues that differ from NPY are indicated by the bold lettering.

ROLE OF NEUROPEPTIDE Y IN FEEDING AND METABOLISM

Neuropeptide Y is a 36 amino acid peptide discovered by investigators searching for C-terminally amidated peptides in extracts of porcine brain (17, 18). The name results from the presence of a C-terminal amidated tyrosine. In mammals, it is a member of a family of mammalian peptides that includes pancreatic polypeptide (PP) and peptide YY (PYY) that are also 36 amino acid peptides all distinguished by a C-terminal tyrosine. The amino acid sequence is tightly conserved (Figure 1) across the three peptides, particularly in the last five amino acids that comprise a majority of the resides required for interaction with the receptor (19). The peptides also share a common structural motif consisting of two anti-parallel helices, an N-terminal polyproline helix (residues 2-8) and a long amphipathic alpha-helix (residues 14-32) connected by a beta-turn (20, 21). While PP and PYY are considered to be endocrine peptides, NPY is one of the most abundant peptides in the central and peripheral nervous systems (22-25). In many nerve terminals, it is stored in synaptic vesicles with classical neurotransmitters such as norepinephrine (NE) (25). The NPY amino acid and DNA sequences are highly conserved throughout evolution (26) suggesting this peptide is important to survival. The conservation of NPY, its abundance and potent biological effects have convinced numerous investigators of its importance as a pharmaceutical research target.

NPY is found in a variety of brain regions including areas involved autonomic and endocrine function. Of particular interest is the prominent localization of NPY containing nerve fibers throughout the hypothalamus. The most prominent pathway in this region originates in the arcuate nucleus of the hypothalamus, a structure that contains a dense cluster of NPY immunoreactive cell bodies. These cell bodies project to several brain regions including the paraventricular nucleus of the hypothalamus, a region known to be important in the regulation of food consumption. Release of NPY in this region appears to be regulated by the feeding status of the animal, increasing during periods of starvation and decreasing during feeding (27). Thus, NPY appears to play a role in the normal maintenance of appetite and body weight. In a number of animal models of obesity, hypothalamic levels of NPY are increased (28-33) suggesting that elevated levels of NPY may contribute to the hyperphagia and adiposity seen in obese animals. Particularly interesting is the substantial increase in NPY mRNA and peptide concentrations in the *ob/ob* mouse, an animal model of obesity that lacks functional leptin (see below). The obese Zucker rat which lacks a functional leptin receptor also exhibits increases in NPY and NPY mRNA (31, 34). This increase is observed as early 6- to 9-day-old animals which precedes the development of the obese state (35).

Central administration of NPY results in a profound increase in feeding in a variety of species. The effect has been best characterized in rodents where the peptide produces a preferential increase in carbohydrate consumption (36). Hypothalamic injections of the peptides are particularly effective in eliciting this

behavior, while the most sensitive region appears to be the perifornical region (37), a part of the hypothalamus that is lateral to the paraventricular nucleus. Interestingly, NPY is the only known endogenous substance that can induced obesity in experimental animals after chronic administration (14, 38, 39). In an important study, NPY was administered to rats for seven days while restricting food intake to control levels in some of the animals (40) . Surprisingly, the animals still exhibited a weight increase indicating that NPY has additional metabolic and endocrine effects that contribute to the development of the obese state. In addition, the animals exhibited hyperglycemia and hyperinsulinemia, two hallmarks for NIDDM and obesity. In a more recent study, the paraventricular nucleus of the hypothalamus was implicated as a region of the brain mediating the feeding and metabolic effects of NPY, while perifornical injection resulted only in increased feeding (41). Consistent with the thesis that NPY produces changes in both food intake and metabolism, NPY is capable of reducing sympathetic activity to brown adipose tissue (42). While NPY is a critical central element in the control of body weight, it is less clear how this is mediated. Much of the recent research effort on NPY has been focused on the elucidation of the receptor subtype that mediates the feeding and metabolic response.

Table 1. Subtypes of receptors for NPY and related peptides.

Receptor Subtype	Other names	Peptide Potency
Y1		**NPY=PYY=Pro^{34}-NPY>PP>NPY13-36**
Y2		**NPY=PYY=NPY13-36>Pro^{34}-NPY>PP**
Y3		NPY=Pro^{34}-NPY=NPY13-36>>PYY
Y4	**PP1**	**PP>>NPY=PYY=LP-NPY>NPY13-36**
Y5		**NPY=PYY=Pro^{34}-NPY>NPY13-36>PP**
Y6*	**PP2, Y2b**	**NPY=PYY=Pro^{34}-NPY>NPY13-36>PP**
PP Receptor "PYY Preferring"		PP>>PYY=NPY PYY>NPY>>NPY13-36>>Pro^{34}-NPY

Receptors in bold lettering have been cloned.
*Full length protein not expressed in humans.

Receptors for the NPY Family of peptides - Historically, the receptors for NPY were divided into postsynaptic Y1 and presynaptic Y2 based on the effects of the fragment, NPY 13-36 in isolated smooth muscle assays (43). Recent advances in the molecular biology of NPY receptors has led to an elucidation of a number of additional receptors. Despite these advances, there appear to be several receptors that can be distinguished by pharmacological means that are still to be cloned. The "state of the art" for NPY receptor subtypes is summarized in Table 1. The first receptor to be cloned was the Y1 receptor, which can be distinguished by high affinity for both NPY and PYY as well as Pro^{34}-substituted analogs (44-46). This receptor has relatively low affinity for C-terminal fragments and PP. The Y1 receptor appears to mediate some of the peripheral cardiovascular and central anxiolytic effects of NPY. While initial pharmacological studies implicated the Y1 receptor as the receptor mediating the feeding response, subsequent studies indicated a "variant" may be responsible (47, 48). Several Y1 selective nonpeptide antagonists are available (49) such as BIBP3226 (50) and SR120819A (51), however these molecules do not effectively cross the blood-brain barrier. Therefore, many of the brain actions of NPY thought to be mediated by the Y1 receptor have yet to be documented by blockade using a specific Y1 antagonist. The Y2 receptor was recently cloned by several groups (52-54) and appears to mediate the many of the NPY effects on neuronal excitability (55). This receptor has high affinity for NPY, PYY and C-terminal fragments of those peptides. The receptor has relatively low affinity for PP and Pro^{34}-substituted analogs of NPY and PYY. To date, no Y2 antagonists have been described in the scientific literature. The Y3 receptor has not been cloned but can be observed

pharmacologically in adrenal chromaffin cells (56), distal colon preparations (57) and the rat brainstem (58, 59). This receptor exhibits higher affinity for NPY when compared to PYY. The Y3 receptor also exhibits appreciable affinity for Pro^{34}-NPY.

The first of several novel receptors was cloned based on homology with the Y1 receptor. The resulting receptor was called Y4 (60) based on its genetic relationship to the Y1 receptor or PP1(61) due to its high affinity for PP. This receptor has extremely high affinity for PP as well as high affinity for PYY, NPY and Pro^{34}-substituted analogs of NPY and PYY. Little is known about the functional role of this receptor, though it does appear to be pharmacologically distinct from the PP receptor seen in limited brain areas (62). Recently, a Y5 receptor was discovered using expression cloning techniques from a rat hypothalamus library (63). This receptor has some of the pharmacological properties of the receptor that mediates the effects of NPY in the hypothalamus. In addition, considerable expression of this receptor can be observed in the rat hypothalamus. Y5 has relatively high affinity for NPY, PYY, Pro^{34}-substituted analogs of NPY and PYY and long C-terminal fragments of these peptides (63). The receptor also has considerable affinity for PP. The Y6 receptor was cloned from the mouse and was originally called the Y5 receptor (64). The clone was renamed Y6 to avoid confusion in the scientific literature. From limited information, this receptor has similar pharmacology to the Y5, but is truncated in humans and rats so that the gene does not encode for a full length receptor (65). Therefore it is unlikely to mediate the effects of NPY in the rat or human.

One of the more interesting studies has used the transgenic "knock out" methodology to eliminate NPY expression from the mouse (66). In these studies, little change was noted in the feeding behavior of the animals and the mice exhibited normal responses to leptin. However, when the NPY deficient mice were cross bred with the ob/ob strain, the obesity was attenuated (67). These results would suggest that NPY is not important in the normal regulation of body weight in mice, but it plays a critical role in the maintenance and, perhaps, development of obesity. Animals deficient in the receptor subtypes should be available soon and it will be very interesting to evaluate the importance NPY and leptin in these strains. Another interesting research avenue is the use of antisense oligodeoxynucleotides to reduce translation of the receptors. This has been used successfully with the Y1 receptor where intracerebroventricular infusion of an antisense oligodeoxynucleotide reduced the expression (68). These animals exhibited a behavior consistent with an anxiogenic response while their feeding was unaffected. Direct injection of antisense to Y1 into the ventral medial hypothalamus resulted in reduced feeding (69) while injections into the amygdala resulted in increased feeding (70). The key advance in the understanding of NPY biology will be the availability of selective antagonists for the receptor subtypes that penetrate the blood-brain barrier. Considering the potential importance of NPY in a variety of disorders including feeding and metabolism, these compounds may be useful therapeutic tools as well.

The NPY neurons for this complex system of receptors receive afferent input from other neurons as well as peripheral hormones such as insulin and leptin. As such, leptin is the most recent member of the endocrine family and its discovery redefines adipose tissue as an endocrine gland that stores and regulates the storage of lipid. Considering the importance of NPY in the regulation of body weight and composition, one of the first interactions explored by scientists was the role of peripheral leptin on the synthesis and release of NPY in the hypothalamus.

ROLE OF LEPTIN IN FEEDING AND METABOLISM

Body Fat Transducer - An adipocyte factor transducing the quantity of adipose to a hormonal message that targets the hypothalamus has been postulated for many years. In 1973 it was demonstrated that *db/db* mice were resistant to such a circulating satiety factor and thus the mice were hyperphagic and obese (71). Further,

ob/ob mice were sensitive to this satiety factor but apparently did not secrete it. Recently, a gene that encodes this protein was identified (8). The 16-kD protein product of this gene was named leptin, derived from the Greek root "leptos", meaning thin (7). Administration of a recombinant leptin to *ob/ob* mice replaces deficient levels and corrects their obesity (7, 72, 73). However, similar treatment of *db/db* mice, which turn out to be deficient in leptin receptors, is ineffective (7, 74).

These findings suggested that adipose tissue performed important biological functions beyond simply the storage of fat. In fact, adipose tissue functioned very much like other endocrine tissues, providing leptin to the brain to modulate the availability of nutrients and metabolic signals. To fully satisfy requirements for a hormone, evidence was presented to demonstrate that leptin is secreted from cultured adipocytes (75) and is detected in blood of rodents (7, 76) and human (76, 77). Studies in men and women indicate that circulating leptin values correlate very closely with quantity of body fat (reviewed in (78)). Thus, most types of human obesity are not associated with insufficient leptin levels as in the *ob/ob* mouse but are characterized by elevated circulating leptin levels, suggesting leptin resistance.

Leptin Transducer - The hormonal message encoded by plasma leptin levels is further transduced to neuronal signals by the hypothalamus. Using autoradiographic techniques, it was observed that leptin bound to choroid plexus and expression cloning was used to identify the leptin receptor gene (11). Others (9, 10) used genetic mapping and genomic analysis to search for a mutation in the *db* and *fa* genes. Collectively, these data indicate that spliced variants of the leptin receptor are located in many tissues including hypothalamus, choroid plexus, heart, lung, liver, skeletal muscle, and kidney. Functions for these variants are unknown but may involve transport of leptin from blood to its target neurons directly or via diffusion from cerebral spinal fluid (79, 80). A mutation in the *db* locus results in a spliced transcript that is proposed to be incapable of signal transduction, leads to leptin resistance, and is likely the cause of severe obesity and diabetes reported in these animals. The wild-type leptin receptor gene has a long intracellular domain that is thought to be crucial for intracellular signal transduction. This receptor closely resembles class I cytokine receptors and is only located in hypothalamus. Direct injection of leptin into the lateral cerebral ventricle of *ob/ob* mice generated a potent anorectic effect that could only be matched by much higher doses of the protein when it was administered subcutaneously, suggesting the principle leptin target to be central (81).

Like other models of obesity, *ob/ob* mice are characterized by high expression of hypothalamic NPY (82). Chronic administration of leptin to *ob/ob* mice decreased NPY mRNA levels in the arcuate nucleus when compared to that observed in lean counterparts (81). This decrease in NPY biosynthesis could be observed after shorter treatments of 5 days (83) or even 1h (84). In addition to regulating NPY biosynthesis, leptin inhibits NPY release from hypothalamic explants (81). Such data demonstrating a rapid action of leptin to inhibit release of NPY are consistent with the recent demonstration that leptin inhibits excitatory postsynaptic membrane potentials in NPY neurons of the arcuate nucleus (85).

Considerable evidence advocate NPY neurons of the arcuate nucleus as a major center for regulating fuel homeostasis and its associated adaptive endocrinology. These NPY neurons project to other areas of the hypothalamus that regulate feeding behavior and coordinate neuroendocrine secretion (86). Careful mapping of NPY stimulated feeding has been reported (37). Fasting and starvation are powerful stimuli while feeding is inhibitory to these neurons and their synapses. Similarly, hormones responsible for metabolic homeostasis are regulated by NPYergic transmission. Activation of NPY neurons is associated with increased insulin secretion (87), activation of the hypothalamic-pituitary-adrenal axis (88), and inhibition of the thyrotropin-releasing hormone-thyroid axis (89). In addition, both episodic basal and cyclic release of luteinizing hormone-releasing hormone (LHRH) is stimulated by

NPY and thus is essential for fertility (90). Prolonged stimulation by NPY, however, inhibits pituitary-gonadal function (91) that is very dependent on precise intermittent signaling. Thus, it is not surprising that a signal of energy imbalance would be associated with infertility (92) and central NPY is associated with reduced sexual function in rats (29, 38, 93, 94). The postsynaptic NPY receptor at each of these sites is unknown (Figure 2) but likely includes several of those discussed above.

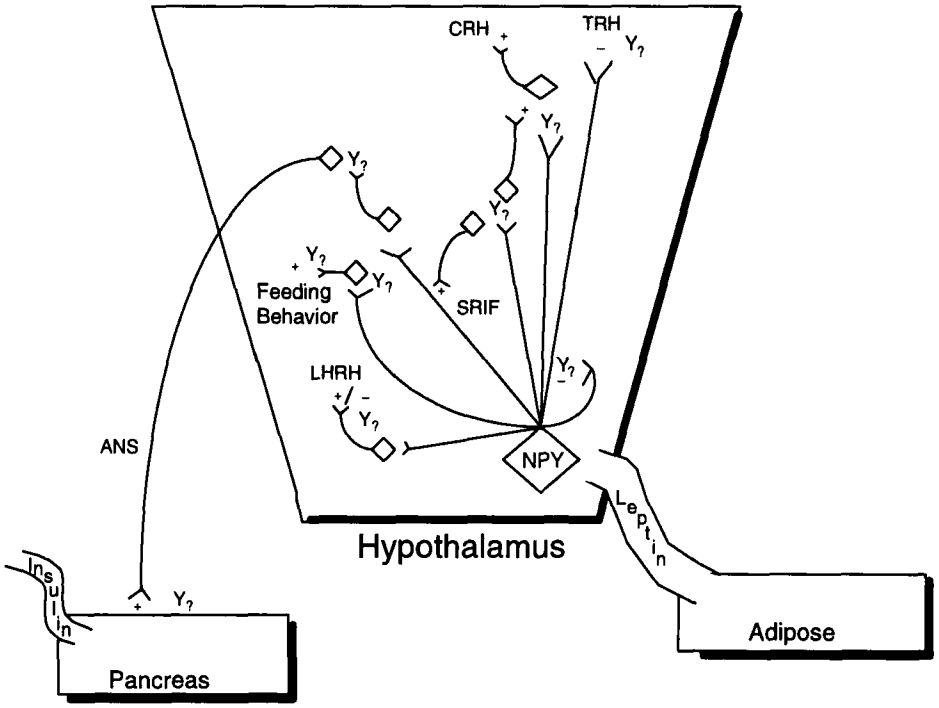

Figure 2. Transducing an adipose signal to a neuroendocrine message. Lepin, secreted by adipose tissue, is carried to its hypothalamic target by blood. Both synthesis and secretion of NPY are inhibited by the adipocyte hormone. In turn, NPY neurons in the arcuate nucleus synapse with TRH, CRH, SRIF, and LHRH neurons. NPY neurons also synapse with nuclei regulating feeding behavior and insulin secretion. The specific postsynaptic NPY receptor at each of these neurons is currently unknown and designated by Y?. NPY synaptic transmission results in a decreased response (-), increased response (+), or both (- / +) depending on physiological state. Since leptin is inhibitory to NPY neurons, such transmission is reduced.

The leptin deficient *ob/ob* mouse is incapable of transferring a signal from adipose tissue reflecting the level of lipid storage. Neuronal activity of NPY arcuate nuclear neurons is increased as are feeding, plasma corticosterone and plasma insulin levels. Further, these mice are infertile. Administration of exogenous leptin corrects hyperphagia and resulting obesity (7, 72, 73), decreases NPY expression and release (81, 83, 84), as well as ameliorates the hypercortism (81), hyperinsulinemia (81) and establishes fertility (95). Fasting in normal mice rapidly decreases the leptin signal from adipose (96). The physiological response to this signal of decreased energy balance is an increase in hypothalamic NPY mRNA levels, increased plasma levels of ACTH and corticosterone, decreased plasma thyroxine values, and decreased plasma testosterone. Centrally administered NPY serves to increase many of these endocrine parameters. It has long been known that the obesity seen in

Zucker rats can be reversed or attenuated by adrenalectomy (97-99). In adrenalectomized animals, NPY does not stimulate feeding (97-99) while glucocorticoid replacement restores the response (100). Blockade of CRF receptors or destruction of the CRF-containing neurons in the paraventricular nucleus results in an enhance feeding response to NPY (101, 102). NPY also increased the levels of CRF (103) and mRNA encoding CRF (104) in the hypothalamus suggesting a reciprocal interaction of NPY and CRF. Such regulation influences endocrinology, behavior and physiology for replenishing energy stores. Administration of leptin attenuated the physiological responses to fasting (96). Thus, a decrease in plasma leptin levels transmit a signal from adipose to a "central processing unit" for energy homeostasis, the arcuate nuclear NPY neurons, that lipid stores are depleted beyond a certain set-point. In turn, these stimulated neurons dispatch signals to other areas in the hypothalamus as well as nuclei outside of the hypothalamus likely by synapses with neurons containing one of several receptors. Finally, it may be that leptin also decreases food intake and body weight by additional mechanisms since it is an effective inhibitor of feeding when administered to NPY-deficient mice (66).

Leptin and NPY as therapeutic targets - Since leptin and NPY appear to be key and interdependent players in the control of body weight, they are of great interest for development of pharmaceuticals. Mimicking the action of leptin is the most straightforward of the strategies and several pharmaceutical companies are developing leptin or leptin analogs for human use. Based on the data available to date, most human obese subjects appear be leptin resistant and the addition of exogenous leptin may be of little consequence (reviewed in (78). Other strategies will target the signaling pathway for leptin (105) in an effort to increase leptin sensitivity. Since NPY appears to be a key player in leptin signaling in the hypothalamus and hypothalamic NPY concentrations are elevated in obese animals, specific receptor antagonists (49) will be an important strategy to understand the importance of this peptide and its interrelationships with leptin. The challenge in this area will be the targeting of the appropriate receptor subtype(s) to block the diverse effects of NPY on feeding, metabolism and endocrine responses (Figure 2). Dexfenfluramine, a recently approved anorectic agent, has been reported to reduce hypothalamic NPY (106, 107) though several other investigators saw no change (108, 109). Similarly, fenfluramine is reported to either suppress (110) or not effect NPY-induced feeding (111). These differences may relate to the different animal models and experimental paradigms used in these studies and suggest that the NPY system may be affected directly by appetite suppressants under certain circumstances. Finally, the role of other neuropeptides that effect feeding such as corticotropin releasing hormone, urocortin, cholecystokinin, opioids and galanin in the leptin-NPY axis is just beginning to be understood. Many of these substances may represent attractive drug discovery opportunities as well.

Conclusions - Energy balance is essential for life and thus must be carefully regulated by complex neuroendocrine systems that have necessary backups. This energy balance is undoubtedly maintained around a fixed set point that is determined genetically. Considerable evidence indicates much of the regulation involves the modulation of the NPY containing hypothalamic arcuate neurons. Receptors at these synapses that transmit the NPY signal to other neurons have been cloned and are in the process of being defined in terms of their physiological role. Key to this definition will be the discovery of potent and selective antagonists for each receptor subtype. Recent discoveries in leptin biology point to this hormone as a key signal from the adipose tissue to the hypothalamus to regulate adiposity. Several points along this pathway present themselves as attractive targets for pharmaceutical discovery. Understanding the relationship of leptin with key central nervous system transmitters such as NPY will be an important advance in understanding the etiology and treatment of obesity.

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Chapter 4. Melatonin Receptor Ligands and Their Potential Clinical Applications

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Introduction - Melatonin (*N*-acetyl-5-methoxytryptamine), first isolated from bovine pineal gland extracts in 1958 (1), is present in all mammalian species. The biosynthesis of melatonin takes place primarily in the pineal gland (2, 3) and occurs *via* rate limiting *N*-acetylation of serotonin and subsequent O-methylation. Melatonin is synthesized in a circadian manner, with peak levels in all species occurring during the period of darkness. In humans, melatonin synthesis and secretion increase near the onset of darkness, peak during the middle of the night, and gradually decline during the later half of the night. Mammalian melatonin rhythms are generated by a circadian clock in the suprachiasmatic nucleus of the hypothalamus (SCN), which is entrained to the 24 hour day by the light/dark cycle. The circadian nature of melatonin secretion has led to numerous studies of the role of melatonin in modulation of the sleep-wake cycle and circadian rhythms in humans. While recent data indicates melatonin may have *bona fide* effects on human circadian rhythms and sleep, other physiological effects of melatonin are less well elucidated. Molecular cloning data suggests melatonin exerts its effects through pharmacologically specific, high affinity, G-protein coupled receptors (mel_{1a}, mel_{1b}, and mel_{1c}). However, the lack of structurally diverse melatonin agonists and antagonists which can be used as pharmacological tools, combined with extensive species differences in the distribution of melatonin binding sites/receptors has prevented a full elucidation of the role melatonin and its receptors play in human physiology and pathophysiology.

POTENTIAL THERAPEUTIC UTILITIES OF MELATONIN RECEPTOR LIGANDS

Disorders of Circadian Rhythms - Radio-iodination of melatonin produces [^{125}I]-iodomelatonin, a high affinity, high specific-activity radioligand suitable for the discrete localization and pharmacological characterization of melatonin binding sites (4). Soon after the demonstration that daily injections of melatonin to rodents entrained wheel-running activity rhythms (5), efforts began in earnest to elucidate the role of melatonin in modulation of human circadian rhythms. The target of melatonin's actions is thought to be the SCN, "the biological clock", since an intact SCN is necessary for melatonin to exert an effect on rodent circadian rhythms (6). 2-[^{125}I]-Iodomelatonin binding sites are present in the SCN of both rodents and man (7, 8). In the rat SCN slice preparation, melatonin causes a phase-advance of the peak in neuronal firing rate, demonstrating an EC_{50} similar to that obtained for functional responses at cloned human melatonin receptors (9). Melatonin agonists, interacting selectively with melatonin receptors in the SCN, may provide a novel approach for the treatment of sleep disorders associated with disruptions in circadian rhythms. In contrast to the currently prescribed benzodiazepines and barbiturates, the melatonergic approach may be free of the deleterious side effects of the classical sedative-hypnotics.

Disorganization of human circadian rhythms may result from jet lag, blindness, shift work, or delayed/advanced sleep-phase syndromes (10). Melatonin and melatonin agonists have efficacy as chronobiotics in animal models of circadian rhythm disorders, including resynchronization of severely disrupted circadian rhythms, entrainment of free running circadian rhythms, treatment of delayed sleep phase syndrome and acceleration of re-entrainment after phase shifts (5, 11-14). Several studies have shown melatonin may also play a role in organization of human circadian rhythms, and melatonin possesses chronobiotic efficacy in several different paradigms of disrupted circadian rhythms. Melatonin alleviated jet lag by increasing

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daytime alertness, decreasing sleep latency, improving sleep quality, and reducing fatigue, while accelerating the resynchronization associated with transmeridian travel (15-18). Melatonin advances sleep onset patterns in patients with delayed-sleep phase syndrome (19-21). Administration of exogenous melatonin to blind humans with free running circadian rhythms was found to improve sleep, and phase advance and entrain circadian rhythms (22, 23). Melatonin treatment may also be beneficial to shift workers (10, 24). While administration of melatonin advanced the onset of endogenous of melatonin secretion (25), melatonin administration also improved subjective sleep, alertness, and performance, associated with phase shifts, while facilitating the readaptation of the melatonin rhythm (26).

If melatonin modulates human circadian rhythms and thereby the sleep-wake cycle, alterations in the phase or amplitude of melatonin levels should be observable in disorders of circadian rhythms, which are ultimately manifest as sleep problems. A generalized decrease in secretion of melatonin was observed in patients diagnosed with primary insomnia (27), which could be the result of either a phase shift in melatonin secretion resulting in a delayed peak or an overall lowering of melatonin output. In patients suffering from chronic primary insomnia with frequent nighttime awakenings, secretory patterns of melatonin in plasma were found to increase earlier in the evening and remain significantly lower throughout the night, with the most impaired rhythms seen in patients with at least a five year history of insomnia (28).

Melatonin as a hypnotic - While the above studies provide convincing evidence that melatonin possesses chronobiotic efficacy in several different types of disrupted circadian rhythms, melatonin may also possess hypnotic properties. Early human studies using pharmacological doses of melatonin (approximately 50 mg) have shown melatonin induces sleep (29). Recently, lower doses of daytime melatonin (0.1-10 mg), which produced "physiological" peak serum melatonin levels within the range of normal nocturnal levels, were found to significantly decrease sleep-onset latency, oral temperature and vigilance in normal healthy volunteers (30). Similar results were obtained when melatonin was administered in the early evening, prior to the nocturnal rise of endogenous melatonin (31). Comparably low doses of melatonin administered later in the evening, closer to the time of release of endogenous melatonin and initiation of sleep onset, also produced decreased sleep onset latency without affecting sleep architecture in both young and middle-aged subjects (32-34). While the mechanism behind the hypnotic properties of melatonin has not been elucidated; neither the hypnotic nor the hypothermic effects of melatonin on daytime sleep were blocked by flumazenil, suggesting benzodiazepine receptors are not involved in these processes (35). Synthetic melatonergic agonists will ultimately be useful to investigate the mechanism and potential hypnotic properties of melatonin.

Reports of insomnia increase with age (36). Complaints in the elderly include difficulty in initiating and maintaining sleep, less total sleep time, and daytime drowsiness (37). Melatonin levels are decreased in the elderly (38, 39), and are inversely proportional to the prevalence of sleep disorders (40). Therefore, increasing night-time levels of melatonin in the elderly might improve sleep. Studies in the elderly aimed at sustaining circulating levels of melatonin over the course of the night, found that controlled release of melatonin improved the efficiency, initiation and maintenance of sleep (41, 42). Whether elevated levels of melatonin are required throughout the night to improve sleep parameters is unclear, as 0.3 mg melatonin given orally provided similar benefits in elderly insomniacs (43). While these studies are intriguing, additional studies using larger sample sizes to determine the optimal patient population and treatment regimen, are warranted. Furthermore, issues surrounding toxicity and long term effectiveness need to be resolved. The consequences of activating melatonin receptors outside the SCN, or activating multiple subtypes of melatonin receptors by non-selective agents must also be addressed. Nonetheless, the willingness of the public to self-medicate with melatonin, thus participating in largely uncontrolled clinical studies, demonstrates the need for additional safe, effective hypnotic agents.

Other Potential Uses - The scientific literature, as well as the lay press, contains reports of numerous beneficial effects of melatonin. Few are as well-documented as circadian rhythms/sleep. A potential role for melatonin in the treatment of epilepsy was reported, although early rodent studies required quite high doses of melatonin (44). A more recent study showed considerably lower doses of chronically administered melatonin (25 μg for 13 weeks), reduced the number and severity of seizures induced by the convulsant pentylenetetrazol (45). These promising animal data have prompted at least one preliminary study of the effects of melatonin on seizures in humans (46). Given the involvement of melatonin in modulation of circadian rhythms, it is not surprising a role for melatonin is postulated in seasonal or winter depression. This hypothesis is supported by the finding that extension of the photoperiod with bright artificial light reverses seasonal depressive symptoms, while administration of melatonin exacerbates the symptoms (47, 48). While an early report suggested administration of melatonin in moderate to severely depressed patients resulted in exacerbation of dysphoria (49), the weak melatonin partial agonist/antagonist, luzindole (see below), displays antidepressant-like activity in the mouse behavioral despair test (50). At pharmacological doses, both *in vitro* and *in vivo*, melatonin acts as an intracellular scavenger of hydroxyl and peroxyl free radicals (51). However, the concentrations required do not support a high affinity melatonin receptor-mediated event. Consideration should be given to the concentration (physiological *vs* pharmacological) of melatonin required to elicit these effects. Ultimately, the complete molecular characterization of the melatonin receptor family and the development of chemically diverse agonists and antagonists selective for each of the subtypes of melatonin receptors will allow further studies into the role of melatonin and its receptors in human physiology.

MOLECULAR BIOLOGY OF MELATONIN RECEPTORS

A high-affinity melatonin receptor was first cloned from frog dermal melanophores in 1994 (52). To date, 20 distinct full-length or partial melatonin receptor DNA sequences from a variety of species have been reported (53-57). Phylogenetic analyses of the predicted amino acid sequences of melatonin receptors support their division into three subtypes: $mel_{1a,}$ mel_{1b}, and mel_{1c} families. However, a fourth family, typified by a partial *Xenopus laevis* cDNA clone, may exist (58). In addition, a highly related orphan receptor, bearing 45% homology to the $mel_{1a,}$ mel_{1b} and mel_{1c} receptors has been cloned from humans, sheep and rats (59). Interestingly, while the overall sequence homology in the transmembrane domains is 55%, the melatonin-like orphan receptor does not bind melatonin or related compounds. Analysis of the full-length receptor sequences predicts the presence of seven hydrophobic segments, consistent with the architecture of G protein-coupled receptors (GPCRs). Even though melatonin is derived by metabolism of serotonin, sequence analysis shows that the melatonin receptors bear no homology to serotonin receptors and further that the melatonin receptor family forms a distinct group within the larger GPCR superfamily.

Expression Patterns, Pharmacology and Signal Transduction - Expression patterns of various melatonin receptors has been studied by mRNA *in situ* hybridization, reverse transcription-polymerase chain reaction (RT-PCR), and receptor autoradiography using the radioligand 2-[^{125}I]-iodomelatonin. Experiments to date suggest considerable species diversity with respect to exact locations of receptor subtype expression. However, it must be emphasized that these studies are not complete. Some of the problems that have complicated such analysis are the lack of selective radioligands for autoradiography (2-[^{125}I]-iodomelatonin binds with high affinity to all three subtypes of melatonin receptors) and low levels of mRNA encoding some of the receptors (for example, mel_{1b} receptor mRNA has never been visualized by *in situ* hybridization). These caveats aside, some general conclusions can be drawn. Messenger RNA encoding the mel_{1a} receptor subtype has been detected by *in situ* hybridization in the SCN and pars tuberalis of all non-human mammals examined to date, implicating the mel_{1a} receptor in the circadian and seasonal reproductive actions of melatonin (52). *In situ* hybridization studies localized the mel_{1a} receptor to the pars

tuberalis in sheep, mouse, and hamster brain, the SCN of the hamster, mouse and rat, and the human cerebellar granule cells (53, 57, 60). Additional studies using RT-PCR, identified mel_{1a} transcripts in human cortex, thalamus and hippocampus (60). Results of RT-PCR amplification studies indicate the mel_{1b} receptor is highly expressed in retina and, to a lower extent, in whole brain and hippocampus, prompting the suggestion that the mel_{1b} subtype mediates the effects of melatonin in the retina (54). To date, the mel_{1c} receptor has been detected only in nonmammalian vertebrates (55). Transcripts corresponding to the melatonin-like orphan receptor have been detected by *in situ* hybridization and Northern hybridization in human pituitary, and hypothalamus (59).

Displacement of 2-[^{125}I]-iodomelatonin at the cloned human mel_{1a} receptor yields the following pharmacological profile (K_i, nM): 2-iodomelatonin (0.018) > melatonin (0.23) > 6-chloromelatonin (2.0) > 6-hydroxymelatonin (2.0) >> *N*-acetylserotonin (170) >>> serotonin (>10,000). Interestingly, the pharmacological profiles of the cloned human mel_{1b} and chicken mel_{1c} receptors are currently indistinguishable from the mel_{1a} receptor, with the singular exception of 6-chloromelatonin, which is equipotent to melatonin at each of these receptors (54, 55). The signal transduction properties of the mel_{1a}, mel_{1b}, and mel_{1c} receptors have been studied in stably transfected heterologous cell lines. These experiments show that melatonin causes a concentration-dependent attenuation of forskolin-stimulated cyclic AMP accumulation at each of the cloned receptors, which is eliminated by treatment with pertussis toxin. These results are consistent with the coupling of the melatonin receptors to inhibitory G-proteins, and are furthermore consistent with signal transduction studies done in native tissues, where melatonin inhibited cyclic AMP accumulation in sheep and hamster pars tuberalis (61, 62), rat pituitary (63), and rabbit parietal cortex (64). There is, however, recent evidence the mel_{1a} receptor may also be involved in modulating signal transduction pathways other than those employing adenylyl cyclase (65).

In contrast to the cloned high affinity G-protein-coupled melatonin receptors, an apparently distinct melatonin binding site has been described in hamster hypothalamus (66) and RPMI-1846 Syrian hamster melanoma cells (67). This ML_2 binding site is characterized by lower affinity for 2[^{125}I]-iodomelatonin (1-3 nM), rapid association and dissociation rates for the radioligand and increased affinity at low (0° C) temperatures (68). Pharmacological experiments have confirmed the rank order of potency of compounds at the ML_2 binding site is different from that seen at any member of the cloned melatonin receptor family. Surprisingly, the precursor for melatonin, *N*-acetylserotonin (NAS) displays higher affinity for the ML_2 binding site than melatonin, which has led to speculation that NAS may represent the endogenous ligand for the ML_2 binding site (67). Furthermore, the α-adrenergic antagonist prazosin, but not phentolamine, displaces 2-[^{125}I]-iodomelatonin with nanomolar affinity, resulting in a unique pharmacologic profile (66). The ML_2 binding site is apparently not affected by guanine nucleotides (68), but was reported to couple to activation of phosphoinositol hydrolysis (69-71), an apparent contradiction that may be resolved only by cloning of the ML_2 binding site. Recent development of the ML_2 selective radioligand [^{125}I]-methoxycarbonyl-amino-*N*-acetyltryptamine (72) and additional novel ML_2 selective compounds (73) should provide useful tools to study localization and function of this novel binding site.

Molecular Modeling - The large number of melatonin receptor sequences available has prompted efforts to model receptor tertiary structure and to identify amino acid residues involved in ligand binding (74-76). All of the models are based on the identification of key amino acids which can participate in hydrogen bonding to the methoxy group and to the amide of melatonin, both of which have been identified as critical interaction points for the molecule based upon SAR studies as described further below. While agreeing in certain respects, these models differ in others. One reason for disagreement may be that the models are constructed on different templates. Only one melatonin receptor model is based on a low-resolution electron projection map of rhodopsin, the GPCR retinal light receptor (74). The other models are built on the three-dimensional crystal structure of bacteriorhodopsin (75), a seven

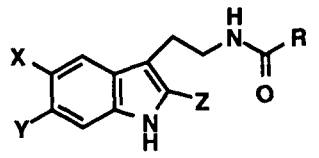
transmembrane bacterial protein which has no primary sequence homology with GPCRs. Significantly, residues predicted to participate in melatonin binding by the rhodopsin-based model are conserved among all known melatonin receptors, while at least several of those proposed in bacteriorhodopsin-based models are not. Interestingly, of the five amino acids that are predicted in the rhodopsin-based model to participate in melatonin binding, only one is not conserved in the orphan melatonin-like receptor (a serine in the seventh transmembrane domain), so it would seem that the simple presence of these residues is not sufficient to account for high-affinity melatonin binding. Site-directed mutagenesis of the receptor will almost certainly advance our understanding of the structure of the melatonin binding pocket.

STRUCTURE ACTIVITY RELATIONSHIPS

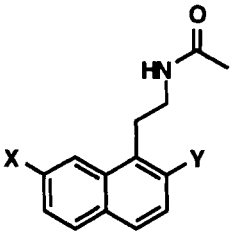
Describing the SAR for melatonergic compounds is complicated by the fact that different researchers have used different tissue preparations for binding studies and the exact nature of the melatonin receptor subtype(s) present in these tissues was not determined. The available data, however, indicate the major tissue types commonly used in SAR studies, including whole chicken brain, ovine pars tuberalis, *Xenopus* melanophores, and chicken retina, are very similar in their binding profiles and reflect binding by a high affinity ML_1-type receptor. In fact, a recent study utilizing a number of structurally diverse ligands was unable to differentiate between the native melatonin receptors found in several of these tissues (77). Thus, data from these sources was combined to provide a composite SAR for the ML_1-type receptor. SAR for the low affinity, ML_2-type binding site has also been described.

ML_1-type Receptor - SAR studies on derivatives of melatonin (**1**) have elucidated several key interactions between the ligand and the ML_1-type receptor. Importantly, both the methoxy group and the amide functionality are critical to melatonin's affinity. *N*-acetyltryptamine (**2**) possesses over a thousand-fold lower affinity (K_i = 730 nM) for the receptor in chicken brain compared to melatonin (K_i = 0.24 nM) while 5-methoxy tryptamine (K_i = 2528 nM) exhibits no significant affinity for the ML_1-type receptor (75). Both the methoxy group and the amide group are therefore presumed to be involved in critical hydrogen bonds to the receptor recognition site. While the methoxy group is a major factor in binding melatonin to its receptor, it is not a necessary criteria for agonist activity (78) as had been suggested previously (79).

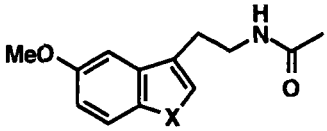
Additional features of the ML_1-type receptor include a small, hydrophobic pocket which can accommodate some increase in the size of the group, R, attached to the amide carbonyl. Increasing the length of R, up to three carbons (**3**), increases affinity for the receptor in ovine pars tuberalis but groups longer than three carbons, or those with branching, are not well-tolerated and result in a significant decrease in receptor affinity (80). Substituents on the indole ring are well-tolerated at both the 2- and 6-positions. 6-Chloromelatonin (**4**) demonstrates comparable affinity (K_i = 0.58 nM) to melatonin for the receptor in chicken brain (75). Substitution at the 2-position of the indole by halogen, methyl or phenyl enhances receptor affinity, either by influencing the conformation of the ethylamido sidechain or accessing an auxiliary binding site (81, 82). The increase in affinity associated with substitution at the 2-position can partially offset the decrease in affinity which results from removal of the 5-methoxy group. Thus, *N*-acetyl 2-phenyltryptamine (**5**) possesses a K_i of 100 nM at the receptor in chicken brain (81).



1 R=Me X=OMe Y=Z=H
2 R=Me X=Y=Z=H
3 R=nPr X=OMe Y=Z=H
4 R=Me X=OMe Y=Cl Z=H
5 R=Me X=Y=H Z=Ph



6 X=OMe Y=H
9 X=Y=OMe
10 X=H Y=OMe

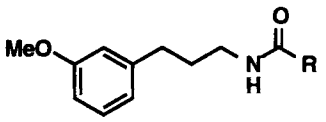


7 X=O
8 X=S

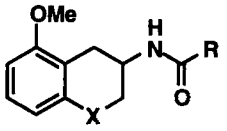
The indole ring of melatonin appears to be a scaffold which, in and of itself, is not critical for melatonin receptor recognition. For instance, the naphthalene ring system substitutes completely for the indole ring of melatonin. The naphthalene derivative **6** (S-20098) possesses essentially equivalent affinity (K_i = 0.035 nM) to melatonin, for the receptor in ovine pars tuberalis (83). Compound **6** is currently the only structurally novel melatonin agonist for which significant data is available. This compound is nonselective, displaying high affinity for both the human mel_{1a} and mel_{1b} melatonin receptors (54). It was selected prior to the cloning of the melatonin receptor family on the basis of its high affinity for the melatonin binding site in sheep pars tuberalis, and its melatonin-like efficacy in the inhibition of forskolin-stimulated cyclic AMP production in sheep pars tuberalis cells (84). *In vitro*, compound **6** is equipotent to melatonin in eliciting a phase advance in the peak of the neuronal activity in isolated rat hypothalamic SCN brain slices. Furthermore, compound **6** demonstrates *in vivo* melatonin agonist activity in free-running rats maintained in constant darkness, rats exposed to an eight hour phase advance, and in rats demonstrating a negative phase angle delay, three different animal models commonly used to predict chronobiotic efficacy. Compound **6** is currently undergoing clinical trials for utility in sleep/wake cycle dysfunctional depression/anxiety patients (85).

The indole ring of melatonin can also be replaced with a benzofuran (**7**) or benzothiophene (**8**) group but these bio-isosteric replacements result in a slight loss in affinity (86). However, replacement of the indole ring with a benzimidazole ring dramatically attenuates activity (86). Further SAR studies in the naphthalene series have identified an additional binding site which can be exploited by the addition of a second methoxy group (87). In ovine pars tuberalis, compound **9** (K_i = 0.1 nM) showed an order of magnitude increase in affinity over the monomethoxy derivative **10** (K_i = 2.68 nM). This auxiliary binding site may be the same as the one occupied by 2-substitution of the indole ring of melatonin.

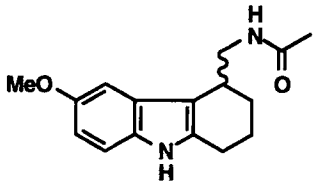
The minimal structural features necessary for receptor recognition were elucidated in an SAR study of a series of simple phenylalkyl amides (88). Variations in aromatic substitution pattern and the length of the linking alkyl chain identified compound **11** which demonstrated remarkably good affinity (K_i = 63 nM) for the receptor in chicken brain despite its simplicity. The affinity can be further improved by optimization of the amide group. Thus, the butanamide **12** shows a ten-fold increase in affinity (K_i = 5.5 nM). The affinity of compound **11** implies it possesses the minimal critical elements which are necessary for receptor recognition. In fact, this structural motif is embedded in all melatonin receptor ligands reported to date.



11 R=Me
12 R=nPr

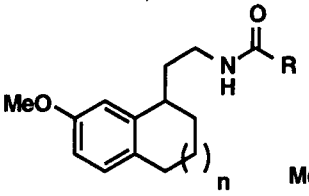


13 X=CH_2 R=Me
14 X=O R=nPr

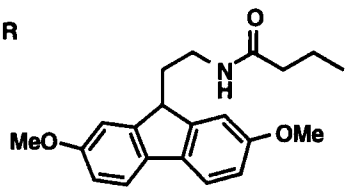


15

A number of different ring systems, which conformationally restrict the active structural elements identified above, are melatonin ligands. The amidotetralin **13** (K_i = 46 nM) and methoxychroman **14** (K_i = 94 nM) demonstrate modest affinity for the melatonin receptor in chicken retina and chicken brain, respectively (89, 90). The aminomethyltetrahydrocarbazole **15** was reported to possess comparable affinity (K_i = 0.97 nM) to melatonin for the receptor in chicken brain (91). All of the affinity for the receptor was later shown to reside with the (-) enantiomer (92). The tetrahydronaphthalenes **16** were described generally as equipotent to the corresponding naphthalenes which, as discussed above, are excellent melatonin ligands (93). A related indane derivative, **17**, is a preclinical lead (94). Fluorene derivatives including **18** were reported to possess good affinity (IC_{50} < 10 nM) for the cloned human mel_{1a} receptor (95). Finally, a series of phenylenes (*e.g.* **19**, K_i = 0.7 nM) were shown to be good melatonin ligands at the receptor in chicken brain (96). The affinity of these compounds, however, is an order of magnitude poorer than the corresponding naphthalene derivatives, perhaps reflecting an inability of the molecule to attain the optimum conformation due to the highly rigid structure.



16 R=Me n=1
17 R=CF_3 n=0



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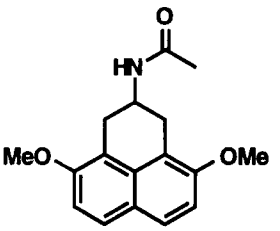

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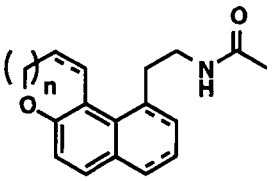
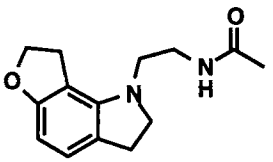


20 n=0 or 1
21 n=1 all possible double bonds present

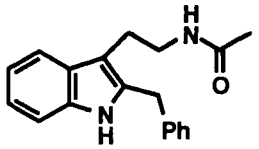


22

Recently, tricyclic compounds **20-22** were reported which incorporate the critical aromatic methoxy group into a ring (93, 97, 98). A number of these derivatives demonstrate excellent affinity for the ML_1-type receptors. For example, the dihydropyran derivative **21** possesses comparable affinity (K_i = 0.1 nM) to the corresponding methoxynaphthalene derivative **6** for the receptor in ovine pars tuberalis (93). The indoline **22** possesses better affinity (K_i = 0.42 nM) than melatonin (K_i = 6.3 nM) for the receptor in chick retina. Such compounds provide an indication of the necessary directionality of the hydrogen bond between the receptor and the methoxy group of melatonin.

ML_1-Antagonists - Potent, selective antagonists for the ML_1 type receptors are still lacking. Although a few compounds are functional antagonists (**23-27**), their affinity

for ML_1 receptors is modest (68, 84). Luzindole, *N*-acetyl-2-benzyltryptamine (**23**) antagonizes the melatonin-induced inhibition of dopamine release from rabbit retina (99). A recent report has demonstrated an excellent correlation between the mel_{1b} binding affinity of compounds **24**-**26** and their activity in the same functional assay (100). The naphthalene derivative **27** blocks the inhibitory action of melatonin on forskolin-stimulated cyclic AMP accumulation in sheep pars tuberalis cells and reverses the melatonin-induced pigment aggregation in *Xenopus* melanophores (101).



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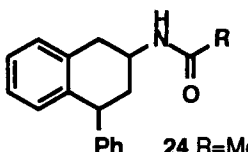
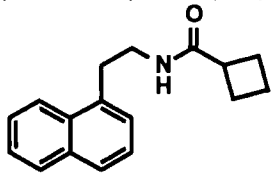
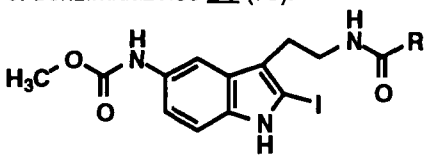


24 R=Me
25 R=Et
26 R=CH_2Cl

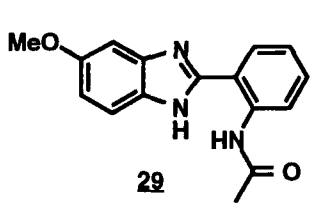


27

ML_2-binding site - SAR data for the ML_2-binding site is limited. In simple derivatives of melatonin, lengthening the group on the carbonyl increases affinity for the ML_1 receptor, but decreases affinity for the ML_2 site, thereby increasing selectivity for the ML_1 site over the ML_2 (102). Compounds which demonstrate good selectivity for the ML_2 site include, 2-iodo-5-methoxycarbonylamino-*N*-acetyltryptamine **28** (72), and a series of benzimidazoles **29** (73).



28



29

Future Directions - Molecular characterization of a family of melatonin receptors has provided the foundation for new avenues of exploration. While the receptor proteins have been expressed in heterologous expression systems, data regarding precise localization of these receptors throughout the CNS and periphery and how localization varies with species, is limited. The interest in melatonin receptors as a substrate for drug discovery will ultimately provide the chemically novel pharmacological tools necessary to probe the role of each melatonin receptor subtype in normal and abnormal physiological processes. Also, while numerous physiological actions are attributed to the endogenous ligand melatonin, these melatonin receptor specific ligands can then be utilized in an effort to determine the role melatonin receptors play in these events. Lastly, these novel ligands should be used in rigorous clinical studies to validate the potential of melatonin receptors as targets for novel CNS medicines.

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Chapter 5. Corticotropin-Releasing Hormone (CRH) Receptors and the Discovery of Selective Non-Peptide CRH_1 Antagonists*

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Receptors for corticotropin releasing hormone (or factor, CRH or CRF) have become targets for drug design since dysfunction in the hypothalamus - pituitary - adrenal (HPA) axis has been correlated with various diseases (1-3). CRH is the prime regulator of the HPA axis, coordinating endocrine, autonomic, behavioral and immune responses to stress and it is therefore very important in general physiology and the etiology of several human diseases. Hypersecretion of CRH in the central nervous system (CNS) has been proposed to underlie depression and a spectrum of anxiety-related disorders (1, 4). Hyposecretion of CRH in the brain may be linked to Alzheimer's disease or Parkinson's disorder (2, 5).

Previous reports have described two known subtypes of CRH receptors in rats, one of which has splice variants, and a CRH binding protein (6,7). The receptor subtypes are G-protein coupled receptors, which are distinguished by the rank order of potencies of peptide agonists in a CRH-coupled adenylate cyclase assay and by their anatomical localization. The CRH_1 receptor, which consists of 415 amino acids, is positively-coupled to adenylate cyclase and is primarily expressed in the rat cortex, hypothalamus, amygdala, cerebellum and pituitary. It is the predominant CRH receptor subtype in the pituitary. The $CRH_{2\alpha}$ receptor, a 411 amino acid protein, which is very similar to the CRH_1 subtype (71% overall homology), is also positively coupled to adenylate cyclase. $CRH_{2\alpha}$ receptors are found primarily in the rat lateral septum, the ventromedial hypothalamus, amygdala and entorhinal cortex, but not in the cerebellum, cortex and pituitary. A 431 amino acid splice variant, the $CRH_{2\beta}$ receptor, is primarily expressed in rat heart, lung and skeletal muscle. Finally, there is also a CRH binding protein, CRH-BP, which is present in the human CNS and plasma and which has high avidity for CRH (7). The main function of the peripheral CRH-BP is to counteract the hypersecretion of CRH during the last trimester of pregnancy; the role of central CRH-BP has not been elucidated.

In the past two years, since the last review on CRH in this series (8), significant progress has been made in defining the roles of CRH receptor subtypes and the binding protein in disease through studies on knockout and transgenic mice as well as the discovery of peptidic and nonpeptidic antagonists, some of which may become therapeutic agents. This report summarizes the recent developments in CRH molecular biology, pharmacology, clinical data and drug design studies.

HUMAN PATHOPHYSIOLOGY

Clinical data suggesting a role for CRH in major depression has been accumulating over the years. Early studies demonstrated that patients suffering from major depressive disorder have elevated concentrations of cortisol in their plasma and supranormal concentrations of CRH in their cerebrospinal fluids (CSF) relative to normal volunteers (1, 9). However, there are data suggesting that this finding of elevated CRH concentrations may be limited to certain subtypes of depression (10). Some studies suggest a positive correlation between reduction of CRH concentrations and reduction in the severity of symptoms with treatment for depression. Administration of fluoxetine, a selective serotonin reuptake inhibitor, to patients with major depressive disorder led to significant decreases in the concentrations of CRH in the CSF in seven out of nine patients, which was accompanied by a corresponding

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improvement in depression rating scores (11). A parallel decrease in arginine vasopressin concentrations was also discovered in all the patients of this same study. An anecdotal subchronic study (one female patient, 16 days) of the effects of electroconvulsive shock therapy (EST) on biochemical parameters revealed a decline in urinary free cortisol and a parallel decrease in CSF concentrations of CRH (12). Moreover, there was a positive correlation between the improvement in symptoms and the decline of CSF concentrations of CRH. Finally, postmortem immunohistochemical studies on depressed patients, who received antidepressant medication during the last month of their lives, revealed a fourfold increase relative to controls in the number of CRH-expressing neurons in the paraventricular nuclei (PVN) of the hypothalami of these patients (13). The above data provide additional support for the hypothesis that CRH is hypersecreted in major depression.

Suggestive data for a role of CRH in other neuropsychiatric disorders has recently been reported. CSF levels of CRH are elevated in patients suffering from anorexia nervosa (14), obsessive compulsive disorder (15), post-traumatic disorder (16), but not panic disorder (17). However, in the last case, a blunted effect of intravenously administered CRH on ACTH levels has been reported, suggesting that pulsatile hypersecretion of CRH may occur in this condition.

PRECLINICAL BIOLOGY

Whole Animal Studies - Experiments in laboratory animals indicate a role for CRH in behavioral responses to stress, which are likened to the behavioral manifestations of anxiety states in humans (1, 3, 18). Thus, intracerebroventricular (icv) injection of CRH in rats leads to freezing in a novel environment, enhanced fear responses, increases in conflict behaviors and decreases in sleeping, feeding and sexual behaviors. These CRH-mediated behaviors as well as the behavioral consequences of stress can be attenuated by a peptide CRH antagonist, α-helical CRH (9-41) (19-21). The animal data suggest that alterations in the CRH system may, in part, underlie the clinical symptomatology of anxiety disorders, depression and anorexia nervosa.

The transgenic mouse line CRH-Tg^{+} was produced to further understand the impact of increased central CRH levels on the physiology and behavior of laboratory animals (22). These animals express a chimeric CRH transgene containing a mMT-1 promotor; they continuously over-produce CRH in the CNS and have increased plasma ACTH (adrenocorticotropin hormone) and corticosterone levels. Symptoms similar to those found in Cushings disease, such as muscle atrophy, thin skin, fat accumulation and female infertility are also observed. The locomotor activity of these animals in a novel environment is suppressed, a response observed in animals receiving icv CRH injections (23). In the elevated plus-maze paradigm, an animal model of anxiety, the CRH-overproducing transgenic mice spend less time in the open arms of the maze indicating a heightened state of anxiety (22). These data suggest that increased continuous endogenous production of CRH leads to alterations related to Cushing's disease and anxiety-related behaviors.

A large subset of individuals, who experienced some form of trauma during childhood, such as parental loss or abuse, present symptoms of affective disorders as adults (24-26). Recent preclinical studies have provided data which suggests that alterations in central CRH circuitry may be triggered by childhood trauma. Thus, adverse rearing conditions in macaques leads to persistent elevations of CRH concentrations in the cisternal CSF (27). Short term maternal deprivation in rats has been shown to cause long lasting alterations in the response of animals to stress and in biochemical parameters related to central CRH neuronal systems (28). Immature rats, isolated from dams 6 hrs daily from post-natal days 6 through 20, have elevated basal and stress-induced levels of plasma ACTH as adults at 3 months of age. Moreover, increases in immunoreactive CRH levels are found in the median eminence and parabrachial nucleus. Alterations in central CRH receptors are also found. The

results from both the non-human primate and rat studies provide evidence of long-lasting changes in central CRH parameters as a result of adverse rearing conditions. These changes might play a part in the development of affective disorders in humans exposed to early-life stressors.

CRH Receptor Subtypes and Novel CRH Peptide Homologs - Two CRH receptors, CRH_1 and CRH_2, encoded by two distinct genes were identified several years ago (29-32). The existence of splice variants of the CRH_2 receptor, termed $CRH_{2\alpha}$ and $CRH_{2\beta}$, are also observed in the rat. While initial studies failed to demonstrate additional splice variants beyond the $CRH_{2\alpha}$ sites in humans, recent work confirmed the existence of $CRH_{2\beta}$ sites in human tissues (33). The potencies of some peptide agonists have been defined for these subtypes (Table1) (37, 47).

Table 1. Peptide CRH Receptor Agonists

Peptide	Sequence	Mean Adenylate Cyclase EC_{50} (nM) CRH_1	$CRH_{2\alpha}$
Urocortin	DNPSLSIDLTFHLLRTLLEL-ARTQJQRERAEQNRIIFDSV	0.8	0.18
Urotensin I	NDDPPISIDLTFHLLRNMIEMA-RNENQRFQAGLNRKYLDEV	6.3	1.5
Sauvagine	EGPPISIDLSLELLRKMIEIEK-QEKEKQQAANNRLLLDTI	3.4	1.4
r/hCRH	SEEPPISLDLTFHLLREVLEM-ARAEQLAQQAHSNRKLMEII	3.5	13.2
o-CRH	SQEPPISLDLTFHLLREVLE MTKADQLAQQAHSNRKLLDIA	9.7	61.9

Ligand binding techniques using the cloned CRH_1 receptor and [^{125}I]-0-tyr-rat/human CRH (r/hCRH) has been employed to characterize the interaction of peptides, such as r/h CRH, α-helical CRH and sauvagine (the counterpart of CRH in frogs), with the receptor (34, 35). Assays used to identify and to optimize small molecule antagonists have been developed using endogenous rat brain CRH receptors as well as cloned $hCRH_1$ receptors expressed in several cell lines (35, 36). Differential interactions of compounds at CRH_1 and CRH_2 receptors were initially detected using a functional assay which measures the production of the second messenger cAMP (29, 32). Receptor binding was not employed since [^{125}I]-0-tyr-r/hCRH has relatively low affinity for CRH_2 receptors and it was therefore an unsuitable radioligand. More recently, the peptide ligand [^{125}I]-0-tyr-sauvagine, which has higher affinity for CRH_2 sites, was used to develop an assay for CRH_2 receptor occupancy in CHO, LN and HEK293 cell expression systems (37, 38). This assay provides a possible tool to identify small molecule ligands for CRH_2 receptors.

Localization of CRH_1 and CRH_2 receptors has been studied using *in situ* hybridization methods (39). CRH_1 receptors are widely distributed in brain mainly in neocortex, cerebellum and other areas. Additionally, the CRH_1 receptor is by far the most abundant CRH receptor found in the pituitary. The localization of CRH_1 receptors defined using *in situ* hybridization techniques mirrors well the distribution of CRH receptors found by labelling with [^{125}I]-0-tyr-r/hCRH (40, 41). CRH_2 receptors are found in the vasculature and heart as well as in brain. In contrast to central CRH_1 receptors, CRH_2 receptors are found predominantly in subcortical structures in brain

such as the septum and hypothalamus [39]. Recent use of [^{125}I]-0-tyr-sauvagine in combination with a CRH_1-specific small molecule antagonist in receptor autoradiography, a method to specifically study CRH_2 sites, confirmed the localization of CRH_2 receptors to areas demarcated by *in situ* techniques (42).

Antisense oligonucleotides can be used to reduce the expression of peptide and proteins, including receptors, in both cell lines and in anatomically discrete areas in whole animals (43). It was shown that antisense knockdown of CRH_1 receptors in the amygdala of the rat has anxiolytic effects. This suggests that CRH_1 receptors mediate the anxiogenic effects of CRH.

Recent reports on small molecule antagonists specific for CRH_1 receptors (*vide infra*) have also suggested the importance of CRH_1 receptors in fear and anxiety responses. These molecules have been shown to be effective in fear-potentiated startle [44], social interaction (45) and elevated plus-maze (46) rodent models. Further experiments with these small molecule antagonists will shed additional light on the functions of CRH which are mediated by CRH_1 receptors. To date, no small molecule CRH_2 antagonist, which might be used to study CRH_2 functions, has been identified.

Another experimental approach to the elucidation of the physiological functions of CRH receptor subtypes is based on identification of mammalian homologs of CRH itself. CRH is a member of a family of peptide hormones found in several species. Related peptides such as sauvagine and urotensin have been isolated from frog and fish respectively [35]. Recently a new mammalian member of the CRH peptide family has been cloned and named urocortin (47). Its anatomical localization in rat brain has been defined and its gene is found on human chromosome 2 (48). Urocortin binds with high affinity to both CRH_1 and CRH_2 receptors and is effective in eliciting ACTH release [47]. However its higher affinity for CRH_2 receptors over CRH_1 receptors and its regional co-localization with CRH_2 receptors suggests that urocortin is an endogenous ligand for CRH_2 receptors in mammalian species. Urocortin is more potent than CRH in suppressing appetite (49). On the other hand, it is less potent than CRH in producing anxiety-like effects. Doses as low as 10 nanograms injected icv were shown to be effective in decreasing food intake in rats (49). These results suggest that urocortin may be responsible for the effects of stress on appetite. The fact that urocortin appears to be an endogenous CRH_2 ligand with effects on appetite and not anxiety would imply that CRH_2 receptors have a greater relevance to feeding behaviors than to anxiety.

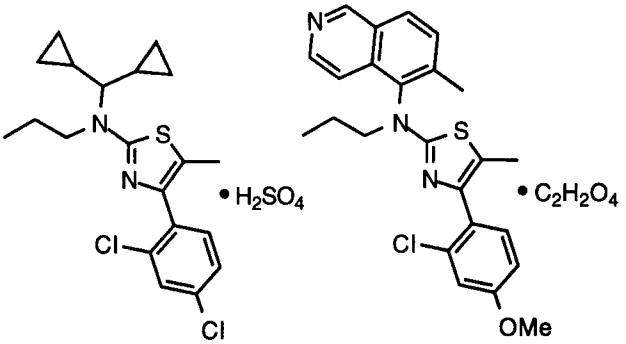
The CRH Binding Protein (CRH-BP) - Another protein involved in CRH-related physiology is the CRH binding protein (CRH-BP) (50-52). This protein was first identified in human plasma and has been proposed to sequester CRH, thereby reducing its functional concentration. This would be particularly important during pregnancy since the placental production of CRH is substantial. The CRH-BP has also been found in brain. In contrast to the free form found in plasma, the CRH-BP in the brain is membrane bound (53). However, its function in brain, to sequester CRH, has been proposed to be simlilar to its plasma counterpart. The binding protein itself has been suggested as a drug target in disease states such as Alzheimer disease (AD) and obesity in which an increase in CRH tone is thought to have potential benefit (5). A binding protein inhibitor would serve as an indirect agonist that would increase the effective concentrations of CRH by blocking its interaction with the binding protein. Recently, positive cognitive effects in rats were observed after icv administration of human CRH (6-33), a selective CRH-BP ligand (5).

However, there is a question regarding the specificity of the CRH binding protein for CRH. There are many regions of the brain where the CRH-BP is found with little or

no overlap with CRH. Evidence has been found for additional novel CRH binding protein ligands in extracts of sheep brain and in synovial fluid collected from the joints of arthritic patients (7]. It has been speculated that these novel CRH-BP ligands could be the peptides responsible for many of the roles currently assigned to brain, peripheral, or immune CRH.

SELECTIVE CRH RECEPTOR LIGANDS

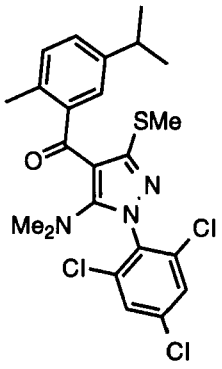
Peptide CRH Antagonists - Astressin (cyclo(30-33)[D-Phe^{12}, Nle^{21,38}, Glu^{30}]r/hCRH (12-41)) is one of the most potent peptide antagonists of $hCRH_1$ receptors reported to date (54). Systematic conformational restriction studies on linear CRH antagonist sequences led to the discovery of this cyclic lactam derivative, which has high affinity (mean K_i = 2 nM) for the cloned human receptor expressed in CHO cells, but no significant affinity for the CRH-BP. Astressin strongly antagonizes r/hCRH-induced secretion of ACTH from rat anterior pituitary cell cultures (mean IC_{50} = 1.0 nM vs. 374 nM for α-helical CRH (9-41)). Intravenous administration of astressin (30 or 100 mg/kg) reduced ACTH secretion (> 50% at 15 min postdose) in adrenalectomized rats and it reduced electroshock-induced ACTH secretion in male rats (> 75% reduction at 10 and 30 min post-stress with a 0.3 mg/kg (iv) dose vs. approximately 50% and 10% reductions 10 and 30 min post-stress respectively with a 3.0 mg/kg iv dose of the linear peptide antagonist, D-Phe^{12}, Nle^{21,38}-r/h-CRH (12-41)). Superior solubility in neutral aqueous solutions, high binding affinity and high antagonist potency make astressin an attractive agent to further explore CRH receptor pharmacology.



1 **2**

Non-Peptide CRH Antagonists - Thiazole analogs represented by compound **1** were previously reported in a patent application to inhibit the binding of ^{125}-I-0-tyr-o-CRH to rat cortical membranes at concentrations ranging from 0.01 mM to 10 mM (55). No specific *in vitro* or *in vivo* biological data were reported; however, five compounds, such as **1**, were specifically claimed. A followup patent application has published, with an expanded scope for the sidechain substitution on the thiazole ring, e.g. compound **2** (56). No specific biological data were provided.

Several patents on fused and non-fused pyrroles, pyrazoles, pyrimidines or pyridines represented by formulae **3** through **8**, as well as novel processes for their syntheses and utility claims, have been published (57-67). All these compounds were reported to have binding affinity at concentrations ranging from 0.2 nM to 10 μM in a rat CRH receptor binding assay using cortical homogenates. The pharmacology of one of these compounds, **6** (CP154526-1) has been described in great detail (44). This compound has high affinity for CRH receptors from multiple species (mean K_i = 2.7 nM



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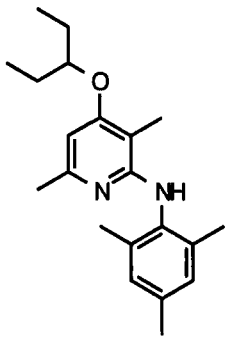

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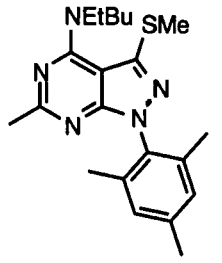

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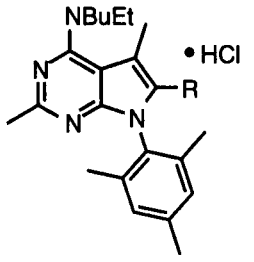
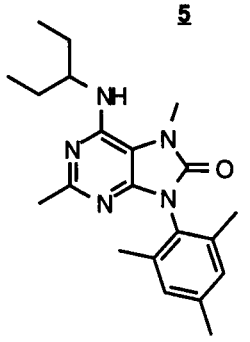


6 R = H, CP154526-1
7 R = Me, antalarmin



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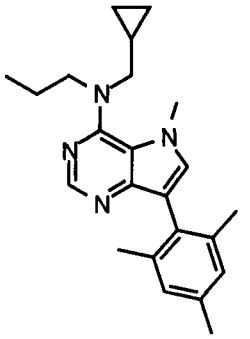
for human CRH_1 receptors expressed in the IMR32 neuroblastoma cell membranes or 1.4 nM in rat receptors in pituitary homogenates), but 6 has no affinity for CRH_2 receptors. The compound blocks in a dose-dependent manner adenylate cyclase activity stimulated by 100 nM o-CRH in rat cortical membranes, without perturbing basal or forskolin-stimulated adenylate cyclase activity. Compound 6 inhibits physiological effects of CRH *in vivo*. Ovine CRH (o-CRH)-induced increases in rat plasma ACTH levels are blocked by 6; its ID_{50} is 13 ± 1.5 mg/kg (sc) when it is administered 30 min after an iv dose of o-CRH (4 μg/kg), while the peptide antagonist, α-helical CRH(9-41) has an ID_{50} = 3 mg/kg (iv) when given concurrently with the agonist. Furthermore, CRH-induced excitation of neuronal firing in the the rat locus coeruleus is antagonized by this compound (ID_{50} = 2 mg/kg (iv) vs. r/hCRH (3.0 μg in 6.0 μL, icv), 5-10 min pretreatment time). Finally, 5 antagonizes some behavioral effects of CRH in rats. It blocks the r/hCRH-enhanced (1 μg in 2 μL, icv) acoustic startle responses of rats (partial blockade at 5.6 mg/kg (ip), complete blockade at 17.8 mg/kg (ip)). For comparison, the peptide antagonist D-Phe-CRH (12-41) (3.3 μg, icv) completely blocks this effect when co-administered with the agonist. Compound 6 also antagonizes the fear-potentiated acoustic startle response in rats (complete blockade at 17.8 mg/kg (ip)). Recently, 6 was evaluated in the rat learned helplessness test, a putative model for depression. Rats were exposed to inescapable foot shocks for three consecutive days. Compound 6 (10-32 mg/kg, ip, 60 min pretest) appeared to reverse the effects of this "helplessness training" on acute administration (significance vs. controls was achieved only at the highest dose) (78). In comparison, acute treatment with the tricyclic antidepressant imipramine hydrochloride (17.8 and 32 mg/kg, sc) had no significant effect. The effects of 6 on behavior suggests a potential anxiolytic or antidepressant indication for CRH_1 antagonists. No oral pharmacokinetic or efficacy data have been presented for this compound.

Antalarmin, compound **7**, which is the 6-methyl homolog of **6**, has been studied for its antagonism of CRH-mediated central and peripheral effects (79). This compound displaced [^{125}I]-o-CRH from rat pituitary, cerebellum and frontal cortex homogenates in a dose-dependent manner (K_i = 1.9 nM, 1.3 nM and 1.4 nM respectively), but not from rat heart tissue. The regional selectivity of the binding inhibition of this compound suggests that it may be a CRH_1-selective ligand. Pretreatment with antalarmin (20 mg/kg, ip, 90 minutes) reduced ACTH release induced by icv injection of r/hCRH (1 nmole) in rats. Furthermore, the compound suppressed increases in the leukocyte concentration found in a subcutaneous exudate in the carrageenan-induced rat inflammation model (79).



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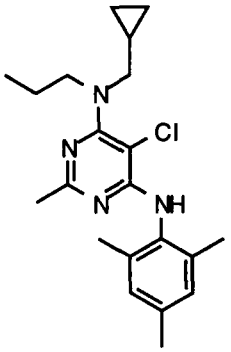

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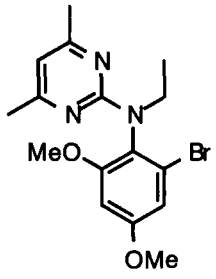

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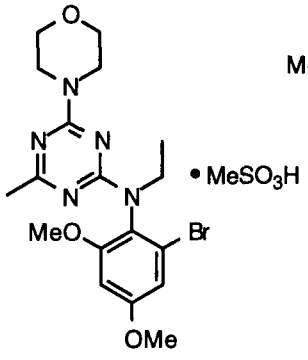

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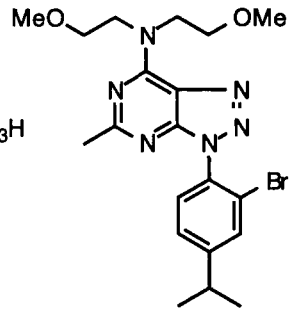

13

Additional structures have been reported to be CRH_1 receptor ligands. Deazapurines represented by compound **9**, which are regioisomeric to some of the bicyclic structures described above, have been described as highly selective partial agonists or antagonists of CRH_1 receptors (68). Minimal biological data have been published on these structures, e.g. compound **9** has an IC_{50} equal to 0.11 μM in a rat receptor binding assay. Several aminoheterocycles have also been described as CRH_1 receptor ligands (69-71). One of these, compound **10**, has very high affinity for human CRH_1 receptors (mean K_i = 1.7 nM vs. 20 nM for D-Phe-CRH (12-41)) and it selectively blocked CRH-stimulated adenylate cyclase activity in COS-7 cell membrane preparations containing the human receptors with no effects on basal cyclase activity. Finally, 2-anilino-pyrimidines / -triazines and triazolopyrimidines represented by structures **11**, **12** and **13** respectively, have been reported to be high affinity CRH_1 antagonists (72-76). Compounds **11**, **12**, and **13** (XQ771, SA627 and SC241 respectively) have high affinity for $hCRH_1$ receptors expressed in HEK293 cells

(mean K_i = 12, 32 and 5 nM respectively). All selectively block CRH-stimulated adenylate cyclase activity (e.g. mean IC_{50} = 12 nM for compound **13**) without altering basal or isoproterenol-stimulated adenylate cyclase activity. Compound **13** has no affinity for $hCRH_2$ receptors expressed in HEK293 cells (K_i > 10000 nM vs. ^{125}I-0-tyr-sauvagine) nor did it have affinity for for a variety of biogenic amine receptors, including dopamine D_1, D_2, serotonin $5HT_1$, and $5HT_2$ receptors. The pharmacokinetic profile of this compound has been studied in dogs. Compound **13** (1 mg/kg, iv, po) has a long iv elimination half life (31.3 h) as well as high plasma levels relative to its K_i and bioavailability after oral administration (C_{max} = 307 nM , T_{max} = 0.67h, oral bioavailability = 30%) (77).

FUTURE CHALLENGES

There is a serious unmet medical need for new neuropsychiatric drugs, which are mechanistically distinct from existing therapeutic agents. Large patient subpopulations do not respond well or tolerate well current anxiolytics or antidepressant drugs. The need is more urgent for other related affective disorders, such as obsessive-compulsive disorder, anorexia nervosa, post-traumatic stress disorder and drug withdrawal. The primacy of CRH as regulator of the HPA axis suggests that agents acting at CRH receptors or the CRH binding protein may have profound therapeutic effects in these disorders. The multiplicity of CRH receptor subtypes and the specificity of their anatomical localization suggests that certain CRH-mediated effects may be occur via certain subtypes. The available data suggest that subtype-selective CRH modulators may become therapeutic agents with novel pharmacological profiles.

Three key challenges face researchers in CRH receptor research: 1) the discovery of CRH_2 subtype-selective agents to define the physiological roles of these sites; 2) the clinical evaluation of CRH_1-selective ligands with optimized pharmacokinetic profiles to confirm the preclinical hypotheses on their functions and 3) the discovery of subtype-selective nonpeptide radioligands to define better the anatomical distribution of these receptors. The recent developments reviewed above open a new era in CRH receptor research in which these challenges may be met and new medicines may emerge.

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Chapter 6. Recent Advances in Neurokinin Receptor Antagonists

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Introduction - The mammalian tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) serve as the preferred ligands for the neurokinin$_1$ (NK_1), NK_2, and NK_3 receptors, respectively. These peptides have been localized to capsaicin-sensitive primary afferent neurons (C-fibers), to enteric sensory neurons and to numerous pathways within the CNS. The NK_1 receptor is widely distributed throughout the CNS and peripheral tissues, while the NK_2 receptor is found mainly in smooth muscle of the gastrointestinal (GI), respiratory and urinary tracts, with considerably lower levels present in the CNS (1). In contrast to NK_2, the NK_3 receptor is expressed mainly in the brain and spinal cord, with modest but well-defined expression in peripheral tissues such as the GI tract (2).

Neurokinin receptors have been proposed to play a significant role in a diverse array of clinical syndromes. The ability of SP and NKA to provoke a number of responses in the airway, including bronchoconstriction, vasodilatation, inflammatory cell infiltration, mucus secretion and plasma protein extravasation has led to suggestions that these mediators and their cognate receptors may be involved in the initiation or exacerbation of asthma and other inflammatory airway disorders (3,4). Since the NK_1 receptor is known to signal the presence of noxious stimuli to the CNS, it has been suggested that it may also be important for pain transmission (5). Neurogenic inflammation mediated by the NK_1 receptor in the dura mater has been proposed to be involved in the etiology of migraine headache (6), while similar processes within the joint spaces have been suggested to play a role in chronic inflammatory joint disorders, in particular rheumatoid arthritis (7). Considerable evidence has been amassed supporting an essential role for NK_1 receptors in emetic responses to diverse stimuli, including cisplatin, ipecac, morphine, whole body irradiation and provocative motion (8-10). The NK_1 receptors mediating the emetic response appear to be centrally located, since CNS-penetrant NK_1 antagonists are anti-emetic while poorly CNS-penetrant NK_1 antagonists are ineffective in this regard (11,12).

This chapter summarizes a number of recent developments in the medicinal chemistry, pharmacology and clinical sciences of neurokinin receptor antagonists, with an emphasis on results published since last year's review in this series (13). Besides those cited above, several other reviews of NK receptors (14, 15) and their antagonists (16-18) have appeared recently.

New NK Receptor Subtype - Pharmacological heterogeneity of the three neurokinin receptors across species is well-established by both molecular cloning and antagonist binding studies, but the possible existence of more than three neurokinin receptors within a single species has been controversial (19). However, it has recently been reported that an orphan receptor cloned from a human placental cDNA library bound the NKB analog senktide with a Kd of 39 nM and could be stimulated with SP, NKA, and NKB to hydrolyze inositol phospholipid with a rank order of potency similar to that of the NK_3 receptor, and maximal levels of efficacy about one-half that of the other neurokinin receptors (20). This protein displays 60-85% sequence homology with the other neurokinin receptors. Characterization of its interaction with NK receptor antagonists remains to be described.

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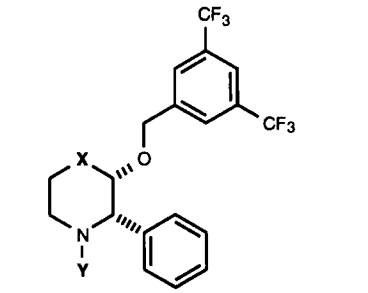
Genetic Disruption of the NK_1 Receptor - A strain of mice rendered deficient in the NK_1 receptor by gene-targeting techniques ($NK\text{-}1R^{-/-}$) was used to characterize the inflammatory cascade driven by immune complex-mediated injury (21). Such phenomena may play a significant role in arthritis and systemic lupus erythematosus. In this model, deposition of antibody-antigen complexes cause increased local vascular permeability to plasma proteins as well as a substantial inflammatory cell infiltrate, a process dependent on complement component C5. This protocol resulted in substantially increased number of polymorphonuclear leukocytes (PMNs) infiltrating into the lungs of the wild type mice but not the $NK\text{-}1R^{-/-}$ mice. Similarly, the wild type mice also displayed a significant enhancement of lung endothelial permeability relative to the $NK_1R^{-/-}$ animals. Thus, activation of the NK_1 receptor at a site of immune-complex mediated injury enhances microvascular permeability, allowing leakage of both antigen and antibody, whose complex stimulates additional release of SP, further enhancing permeability. The protein leakage also provides a local source of complement, which in the presence of the immune complexes generates C5a, the anaphylatoxin necessary for the inflammatory response in this context. Since mice deficient in the C5a receptor displayed the same phenotype as the NK_1 $receptor^{-/-}$ mice, both the NK_1 receptor and the C5aR appear to be necessary in this model to generate inflammatory injury.

NK_1 Receptors in Pain Models - Electrophysiological studies carried out in anesthetized or decerberate animals indicate that NK_1 receptor antagonists can play a role in the signalling of prolonged or intense noxious stimuli to the spinal cord (22). Conflicting results have been obtained in conscious animals using first generation NK_1 receptor antagonists, possibly because these compounds possess significant affinity for peripheral ion channels. To minimize this complication, the piperidine L-733,060 (**1**; NK_1 IC_{50} = 0.6 nM), which has a long central but short peripheral half-life, was identified (23). In the gerbil formalin paw assay, **1** inhibited the late-phase response with an i.v. ID_{50} of 0.17 mg/kg, while its enantiomer was at least 50-fold less potent. The effect of **1** on the early phase was considerably weaker, incomplete and displayed only modest enantioselectivity. These data suggest that the immediate response to formalin may be largely dependent on neurotransmitters other than SP. However, the sensitivity of the late phase to NK_1 receptor blockade is intriguing, since clinically useful non-steroidal antiinflammatory drugs (NSAIDs) and opiates also perform well in this regard.



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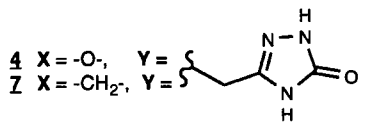
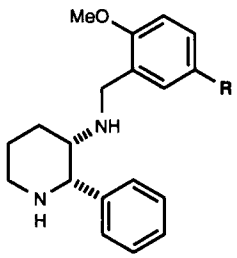
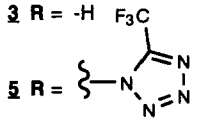


4 X = -O-, Y =
7 X = $-CH_2-$, Y =



2 R = $-OCF_3$

3 R = -H



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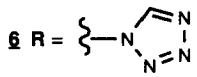


6 R =

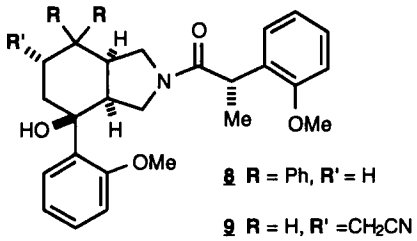
PRECLINICAL STUDIES OF NEUROKININ RECEPTOR ANTAGONISTS

NK1 Antagonists - The piperidine CP-122,721 (**2**), designed to improve on the oral activity of its parent CP-99,994 (**3**), proved to be 400-fold more potent orally than **3** in a study measuring inhibition of aerosolized capsaicin-induced lung plasma

extravasation in the guinea pig. However, it also displayed an increased affinity (IC_{50} = 390 nM) for the L-type calcium channel (24). In ferrets, emesis induced by cisplatin, loperamide, ipecac syrup or copper sulfate was inhibited by oral doses of **2**, with complete blockade of cisplatin-induced retching and vomiting seen with 1 mg/kg (25). Piperidine **2** failed to block the gagging and lip-licking that preceded emesis, raising the question of whether NK_1 receptor antagonists will inhibit prodromal events such as nausea in humans. Delayed emesis, defined as emesis occuring more than 24 hr after cisplatin dosing, has been observed in ferrets. This delayed response can be significantly inhibited over a 72 hr period by 10 mg/kg i.p. of compound **3** dosed every 8 hr, while dosing at this level every 4 hr completely blocked retching and vomiting over a 20 hr period (26).

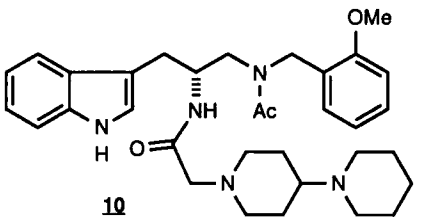
While the receptor affinity of compound **2** is only modestly improved over **3** (human NK_1 (hNK_1 receptor) IC_{50}'s of 0.15 nM and 0.5 nM, respectively), analysis of saturation binding studies with the hNK_1 receptor in IM-9 cells indicated that compound **2** did not interact with the receptor in a classically competitive manner (24). These results were supported by studies in a SP-induced hypotension assay in the dog, wherein increasing oral doses of **2** produced a progressive decrease in maximal response, as well as the expected dose-related shift to the right of the SP dose-response curve (>100-fold at 0.3 mg/kg p.o.). Using the high affinity morpholine hNK_1 receptor antagonist L-742,694 (**4**; NK1 Kd = 0.037 nM), it has recently been shown that such pseudo-irreversible behavior may result from a very slow off-rate from the receptor (27). The 5'-(5-trifluoromethyl)tetrazol-1-yl analog GR205171 (**5**; hNK_1 IC_{50} = 0.025 nM) was derived from the parent GR203040 (**6**) by a survey of the receptor binding and anti-emetic activities of a series of tetrazole substituents (28). Compound **5** displays antiemetic activity against a wide array of emetogens, including X-irradiation and motion, with efficacy demonstrated in the dog and house-musk shrew as well as the ferret (29). The human pharmacology of piperidine **5** is under study (30).

Inhibition of SP-induced dermal extravasation in guinea pigs after oral dosing of 1-substituted-3-benzyloxypiperidines was improved more than 20-fold by replacement of the 1-aminocarbonylmethyl group with 1,2-dihydro-3*H*-1,2,4-triazol-3-one-5-ylmethyl (31). The resulting compound, L-741,671 (**7**; NK_1 IC_{50} = 0.05 nM) displayed an ID_{50} of 0.037 mg/kg in the extravasation assay, and showed 24-46% oral bioavailability in rat and rhesus. Compound **7** also featured an ID_{90} of 1.0 mg/kg i.v. to inhibit cisplatin-induced emesis in ferrets. The related morpholine **4** featured an ID_{50} of 0.009 mg/kg p.o. in the guinea pig extravasation model. This high degree of oral activity was consistent with the chemical stability of the acetal **4** in simulated gastric fluid *in vitro*, wherein no degradation was noted over a 4 h period at 37° C (32).



8 R = Ph, R' = H

9 R = H, R' = CH_2CN



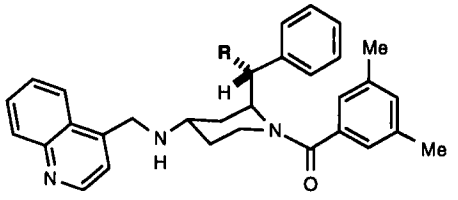
10

The first perhydroisoindole-based antagonist that was selective for the human rather than the rat isoform of the NK_1 receptor was erispant (RPR 100893, **8**; hNK_1 IC_{50} = 30 nM). Deletion of the gem-diphenyl group and addition of a 7-cyanomethyl substituent provided compound **9**, which features an IC_{50} of 0.3 nM on the hNK_1 receptor and displays an ED_{50} of 0.004 mg/kg p.o. for the inhibition of septide-induced plasma extravasation in guinea pig (33). The substantially lower activity observed for compound **9** in the formalin paw analgesia screen (ED_{50} = 17 mg/kg p.o.) may indicate modest or slow CNS penetration. Although the development status of **9** has not been

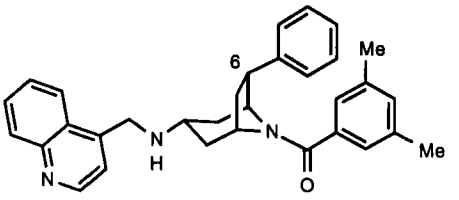
disclosed, a synthetic route suitable for its preparation on a multi-kilo scale has been reported (34).

Systematic evaluation of a series of tryptophan-derived amides bearing basic, lipophilic side chains led to the identification of laneipitant (LY303870, **10**) as a high affinity (hNK_1 IC_{50} = 0.2 nM), selective, orally active hNK_1 receptor antagonist with long duration of action in guinea pig (35). Compound **10** blocked the biting and scratching behavior induced by intrathecal (i.t.) injection of a selective NK_1 receptor agonist to mice, with ED_{50}'s of 0.21 nmol i.t. and 1.7 mg/kg after intraperitoneal administration. Electrical stimulation of the dura in guinea pig provokes neurogenic extravasation, which can be antagonized by **10** with an ED_{50} of 15 ng/kg i.v. Simultaneous administration of **10** and the anti-migraine 5-HT_{1D} agonist sumitriptan is reported to produce synergistic effects in this model, shifting the dose-response curve for **10** more than 3 orders of magnitude to the left in the presence of 1.2 ng/kg of sumitriptan (36). It was suggested that if this model is relevant to human migraine, an NK_1 receptor antagonist may be used to lower the efficacious dose of a serotonin agonist in the clinic.

The stress of an adverse environment induces spontaneous colitis and colon cancer in the cotton-top tamarin, and it has been suggested that the early stage has a neurogenic component. Compound **10** administered at 10 mg/kg twice daily for 12 months in this model produced a decrease in polymorphonuclear leukocyte levels measured from endoscopic mucosal biopsies, without affecting plasma cell and monocyte levels (37). These results suggest that blockade of the NK_1 receptor interferes with initiation of the acute inflammatory phase of spontaneous colitis without affecting the mucosal cell population increases associated with chronic colitis.

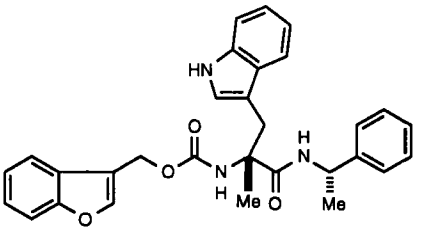


11 R = H **12** R = Me



13

Detailed chemical and SAR studies for a series of *N*-acyl piperidine NK_1 receptor antagonists related to CGP 49823 (**11**; hNK1 IC_{50} = 12 nM) highlighted the requirement for 3,5-disubstitution on the *N*-aroyl group, while the 4-amino group tolerated a broad array of acyl and alkyl functionality as long as one N-H group remained (38). Methyl substitution on the 2-benzyl methylene produced diastereomers with an 80-fold difference in hNK_1 receptor binding (39). The conformation of the more active isomer **12** (hNK_1 IC_{50} = 30 nM), established by X-ray crystallography, proved useful in predicting the 15-fold greater activity of constrained analog **13** (hNK_1 IC_{50} = 44 nM) over its C6 epimer.



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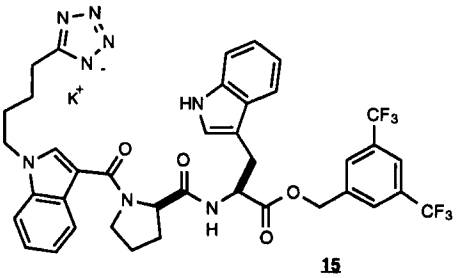

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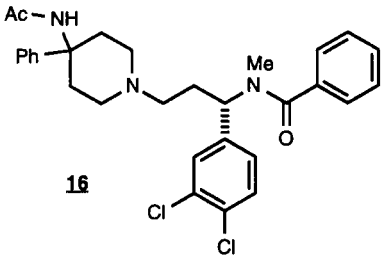
A drug-design strategy based on elaboration of the minimum active fragment of SP led to the identification of PD 154075 (14) as a high affinity NK_1 receptor ligand (hNK1 IC_{50} = 0.4 nM). Compound 14 displayed 49% absolute bioavailability in rats and was orally active in several assays measuring NK_1 receptor mediated neurogenic extravasation (40). The central activity of compound 14 was evaluated in a gerbil assay measuring [Sar^9,Met(O_2)SP-induced foot-tapping, where a minimally effective dose of 10 mg/kg s.c. was measured (41). A 30 mg/kg i.p. dose of compound 14 provided substantial inhibition of acute emesis induced by cisplatin in the ferret, and delayed emesis occurring over a 72 hr period was almost completely suppressed by 10 mg/kg i.p. of benzofuran 14 given every 8 hr. Another tryptophan-based antagonist, tetrazole 15, was designed with a 4-(2H-tetrazol-5-yl)butyl side chain to enhance water solubility and duration of action when dosed as an aerosol (42). Compound 15 inhibited capsaicin-induced plasma extravasation in the bronchi of guinea pigs with an IC_{50} of 26 μg/kg i.v., and as an aerosol blocked SP-induced bronchostriction with an ID_{50} of 50 nmol/kg.

NK_2 Antagonists - The best characterized non-peptide, high affinity NK_2 receptor antagonists to date are SR 48,968 (16) and GR 159,897 (17) (hNK_2 IC_{50}'s of 0.5 nM and 0.3 nM, respectively) (43, 44). More recently, conformational analysis of the cyclic peptide NK_2 antagonist L-659,877 was used to calibrate molecular dynamics and NMR studies, which led to the identification of the bicyclic peptide MEN 10627 (18) as a potent NK_2 receptor antagonist with good resistance to plasma peptidases (45). Compound 18 displayed a pK_B of 10.1 for the NK_2 receptor in hamster trachea and proved to be roughly equipotent to the non-peptide antagonist 16 in inhibiting urinary bladder contraction induced by the NK_2 selective agonist [βAla^8]NKA(4-10) in rats after i.v. and intraduodenal dosing.



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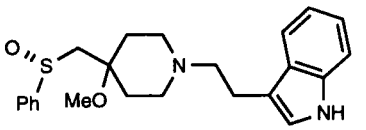

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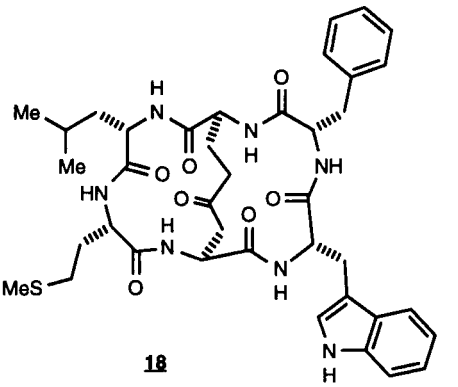
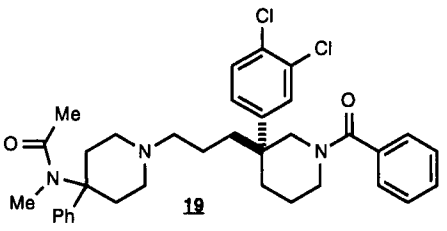


18

NK_3 Antagonists - Although NK_3 receptors have been characterized in several non-human mammals, their role in human disease has not been established. Recently, non-peptide NK_3 antagonists have become available to begin addressing these issues (46). The first such agent described was SR 142,801 (19; hNK_3 IC_{50} = 1 nM; note corrected stereochemistry (47)). Piperidine 19 displays a slow onset of action in blocking contraction of guinea pig ileum elicited by the selective NK_3 agonist [MePhe^7]NKB, and the antagonism that results is largely irreversible (48). In contrast, 2-phenyl-4-quinolinecarboxamide 20, which possesses the same hNK_3 affinity as 19, did not show time dependence in its onset of activity (49). In addition, the effects of 20 could readily be washed out, suggesting that it functioned as a reversible, competitive hNK_3 ligand. Interestingly, both 19 and 20 showed measurable affinity for the hNK_2 receptor (IC_{50}'s of 40 and 144 nM, respectively), in contrast to the usual 3-



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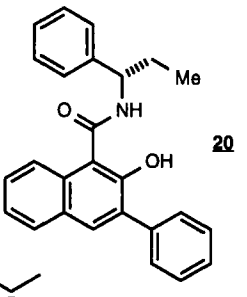

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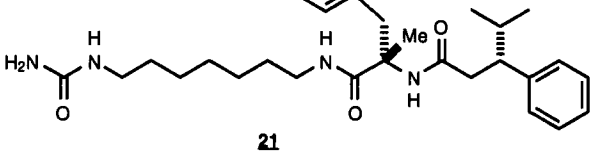
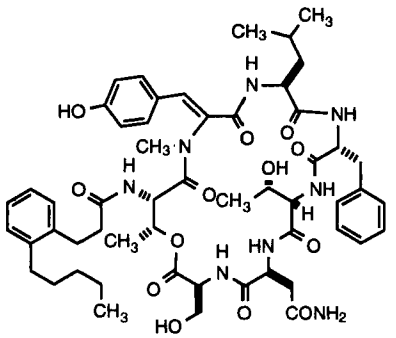


21

4 orders of magnitude of selectivity observed for other neurokinin antagonists. The recently reported peptide-derived antagonist **21** displays the same trend (50). This property may simply reflect residual activity of the leads from which these compounds were derived, or it may indicate that the hNK_2 and hNK_3 receptors contain similar binding domains, with hNK_3 having more restrictive requirements for high affinity binding.

NK1/NK2Antagonists - Speculation on the role of NK_1 and NK_2 receptors as mediators of inflammatory airway disease (3,4) led to clinical studies of the macrocyclic peptide FK224 (**22**) administered as an aerosol (51). Interest in non-peptidic structures that might be utilized orally led to the identification of MDL 105,212 (**23**), which was derived from the NK_2 selective compound **16** by modeling comparisons with NK_1selective compounds, followed by side chain optimization (52). At a dose of 50 mg/kg orally, pyrrolidine **23** reduced pulmonary insufflation pressure and microvascular leakage in sensitized guinea pigs and also significantly inhibited airway hyperresponsiveness (53).



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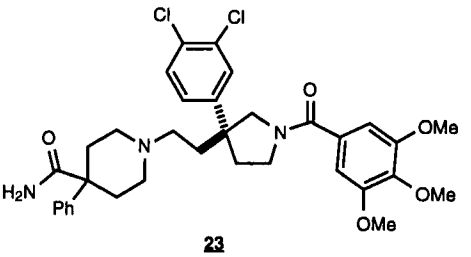
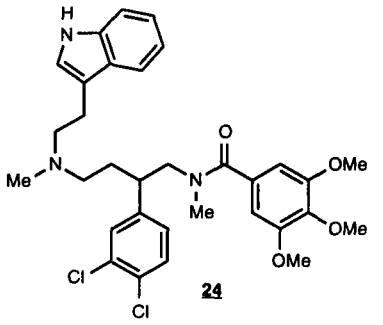


23

Another class of dual antagonists described in the patent literature is exemplified by compound **24** (hNK_1 K_i = 12 nM; hNK_2 K_i = 10 nM), which contains elements of the NK_2 antagonists **16** and **17**, and compound **23**'s trimethoxybenzamide subunit (54). The 3,5-bis(trifluoromethyl)benzyl ether drivative **25** (hNK_1 K_i = 4.3 nM; hNK_2 K_i = 12 nM) was also disclosed as one member of a series of non-selective neurokinin antagonists that feature a variety of highly polar side chains vicinal to the benzyl ether (55).



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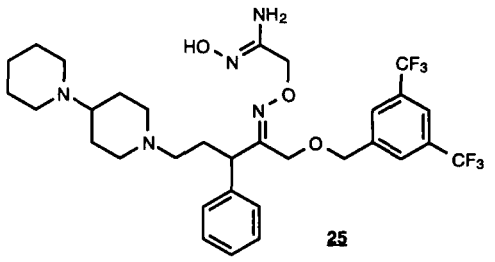
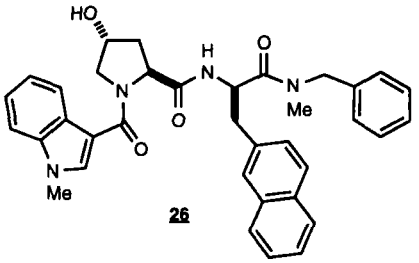


25

CLINICAL STUDIES OF NEUROKININ RECEPTOR ANTAGONISTS

Respiratory Studies - The peptide macrolactam **22** was reported to inhibit bradykinin-induced bronchoconstriction in stable asthmatics when a 4 mg dose was administered as an aerosol (51). When a similar study of **22** was carried out using a 2 mg aerosol dose over a several week period, its effect on key respiratory parameters was not significant and was similar to the variability in bradykinin responsiveness over the period of the study (56). In another study of similar design but employing inhaled NKA instead of bradykinin, no effect of 4 mg of aerosolized **22** could be detected on the evoked bronchoconstriction (57). In agreement with these later studies, **22** administered by aerosol (4 mg q.i.d.) in a double-blind, placebo-controlled trial involving 40 asthmatic patients over a 4 week period had no effect on asthma symptoms or on bronchial reactivity to methacholine (58). It was proposed that the relatively weak affinity of **22** for the neurokinin receptors may be responsible for the lack of efficacy in this study. In support of this analysis is a recent study of the high potency NK_2 selective antagonist **16** in male asthmatics, where a 100 mg p.o. dose significantly inhibited NKA-induced bronchoconstriction for up to 24 hr (59).

Clinical studies of the airways effects of selective NK_1 antagonists have provided mixed results. In a trial measuring bronchoconstriction and cough evoked by inhaled hypertonic saline in patients with mild asthma, 0.25 mg/kg i.v. of CP 99,994 (**3**) was ineffective (60). The 30-70 ng/ml plasma concentrations of the drug that were attained in this study would have been expected to efficiently block airway NK1 receptors, based on responses in guinea pig. These results are consistent with guinea pig studies indicating that neurogenic bronchoconstriction is driven primarily by NK_2 rather than NK_1 activation (3,4). The potent and selective NK_1 antagonist FK888 (**26**; hNK_1 IC_{50} = 0.7 nM), dosed as an aerosol, significantly shortened the recovery phase in a trial of exercise-induced airway narrowing, although it did not affect the maximal fall in specific airway conductance (61). The plasma levels of compound **26** in the subjects was between 6 and 18 ng/ml, which would be expected to effectively block airway NK_1 receptors. The authors interpreted these data in light of the above-mentioned preclinical results,suggesting that blockade of airway wall edema was responsible for these results, rather than a diminution of bronchial smooth muscle contraction.



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Migraine - In preclinical models in which dural extravasation is driven by local release of neuropeptides, modest peripheral levels of potent NK_1 antagonists are sufficient to block the evoked response (35). The supposition that this process plays a central role in the initiation or exacerbation of migraine headache has been tested in two clinical studies. Erispant (**8**) was reported to be inactive at oral doses up to 20 mg in a migraine trial (62). Since plasma levels of drug were not reported it is possible that the oral absorption of **8** was insufficient to provide adequate circulating levels. However, lanepitant (**10**), in a study testing its ability to abort moderate to severe migraine pain at p.o. doses up to 240 mg, also was not efficacious, and the low ng/ml plasma levels reported appeared adequate for peripheral receptor blockade (63). Although these studies suggest that low peripheral levels an NK_1 antagonist will not halt an ongoing migraine, it is possible that these agents may be useful for prophylactic therapy. Alternatively, if NK_1 antagonists block central nociceptive pathways activated during a migraine attack, larger doses of highly CNS-penetrant antagonists may be required to demonstrate efficacy.

Pain -The piperidine **3** proved to be ineffective in relieving pain in a trial of patients with peripheral neuropathy when dosed at 0.2 mg/kg i.v. (64). In contrast, in a study of dental pain induced by oral surgery, compound **3** was superior to placebo in suppressing pain when a 0.75 mg/kg dose was infused over 5 hr, beginning 2 hr before surgery (65). However, it was less effective than 600 mg of ibuprofen, and its effects had dissipated after 150 min. In a second trial, compound **3**, ibuprofen, and placebo were each dosed 30 min prior to surgery, and **3** was more effective than placebo up to 2 hr post-surgery, although it was not as effective as ibuprofen. Side effects were reported to be similar in the treated and placebo groups. This study constitutes the first published evidence that a selective NK_1 receptor antagonist possesses analgesic activity in humans. The duration of the surgery was not reported, nor were pharmacokinetic parameters for compound **3** in man, so it is not known what plasma levels of drug were required for analgesic activity. In a study of lanepitant (**10**) in osterarthritic patients with moderate joint pain, twice daily doses of up to 300 mg p.o. over a three week period proved similar to placebo and ineffective relative to 375 mg p.o. of naprosyn (63). The disparate results from these two trials may reflect differences in pharmacokinetic and tissue distribution properties between the two drugs, although the significant differences in the nociceptive stimuli may also be relevant.

Emesis - The orally bioavailable NK_1 antagonist **2** has been studied in a clinical trial of cisplatin-induced emesis at doses up to 200 mg p.o. in patients with advanced solid tumors (66). Five subjects received only compound **2**, while another arm of 12 patients received **2** as well as a $5HT_3$ antagonist and dexamethasone. The patients receiving only piperidine **2** experienced a median of one emetic event in the first day, while only 3 of the 17 patients in the study experienced delayed emesis, considerably fewer than would be expected from historical controls. Compound **2** was also reported to be well-tolerated. These results are consistent with the extensive preclinical studies demonstrating the anti-emetic activity of brain-penetrant NK_1 antagonists (8-10, 25).

Conclusion - The field of neurokinin receptor antagonists, which has experienced broad and sustained industrial interest since the first non-peptides were identified in the early 1990s, has now moved to the first stages of clinical evaluation. Given the numerous therapeutic targets that have been proposed for these agents, it is not surprising that initial studies in humans have had widely varying outcomes. The significant results seen for NK_1 antagonists in emesis, and the positive trends observed in clinical trials of dental pain and airway dysfunction should encourage continuing studies of these intriguing classes of receptor antagonists.

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SECTION II. CARDIOVASCULAR AND PULMONARY DISEASES

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Chapter 7. Endothelin Inhibitors

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Introduction - Endothelin-1 (ET-1), originally isolated from the culture supernatant of porcine aortic endothelial cells by Yanagisawa *et al.*, belongs to a family of peptides with potent biological actions including vasoconstriction and mitogenesis (1,2). Discovery and development of agents to block the actions of ET-1 with ET receptor antagonists, or the production of ET-1 with endothelin converting enzyme (ECE) inhibitors, have been the subject of much research (3-6). With several newly discovered ET inhibitors, investigators soon confirmed the possible involvement of ET-1 in number of disease states including systemic hypertension (HP), pulmonary hypertension (PH), renal and myocardial ischemia (RI and MI), stroke, subarachnoid hemorrhage (SAH), restenosis (RT), congestive heart failure (CHF) and chronic renal failure (CRF) (7-15). Currently, several ET receptor antagonists have been brought to early development stages with a few already having advanced into clinical evaluation (16). Although less advanced, persistent effort in ECE inhibitor area has led to the discovery of several novel inhibitors. The discovery of ET antagonists has been a subject of several reviews in this series for the past three years (17-19). Recent progress in the discovery and development of new ECE inhibitors and ET receptor antagonists will be reviewed in this article with a brief introduction of the endothelin system.

ENDOTHELIN CONVERTING ENZYMES

ET-1 and isopeptides ET-2, ET-3 are produced from peptide precursors of approximately 200-amino acid residues. These precursors are first processed by prohormone processing enzyme(s) into biologically inactive, 38-41-amino acid intermediates called big ET-1, ET-2, and ET-3 (20). Big endothelins are then cleaved between Trp^{21} and Val^{22}/Ile^{22} in the critical proteolytic step to yield 21-amino acid ETs by ECE (1). The physiologically relevant ECE is a membrane-bound metalloprotease which is inhibited by phosphoramidon (PA), a non-selective peptidic metalloprotease inhibitor, but is insensitive to inhibitors of other metalloproteases such as thiorphan (neutral endopeptidase 24.11, NEP) and captopril (angiotensin converting enzyme) (21-27). Enzyme purification from rat lung or from porcine aortic endothelium rapidly led to cloning of the gene for one of the isoenzymes, ECE-1 (22-25). Subsequently, cDNA for the human enzyme was also obtained (28,29). Since then, another ECE family member was cloned and termed ECE-2 (30). ECE-2 has an overall 59% amino acid identity to ECE-1 and has an acidic pH optimum in contrast to the neutral pH optimum for ECE-1. Additionally, two isoforms of ECE-1 with distinct N-terminal tails have been identified and termed ECE-1a and ECE-1b (31,32). ECE-1a and ECE-1b are encoded by the same gene by way of two distinct promoters. The amino acid sequences of the ECE-1 enzymes were shown to be highly homologous among mammalian species (24,25,28,29,31,32). Both ECE-1 and ECE-2 are type II integral membrane proteins with a short N-terminal cytoplasmic tail, a transmembrane hydrophobic domain, and a large extracellular domain containing the catalytic site and a Zn-binding motif, HEXXH. They show significant sequence similarities, particularly in the C-terminal region, to NEP and the human Kell group protein. Both ECE-1 and ECE-2 have 10 sites predicted to be N-glycosylated and the high glycosylation level is evident from molecular weight estimation (24,25,28). ECE-1 is highly specific for the conversion of big ET-1 to ET-1

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shown by no further degradation of the products (22,25). Targeted disruption of ET-1, ET-3, and ET_B receptor genes in mice has demonstrated an important role for endothelins in the development of neural crest-derived tissues (33-35). Interestingly, ECE-1 knockout mice develop in a similar way to ET-1 and ET_A receptor knockout mice, but they also lack myenteric neurons, a phenotype that occurs in ET-3 or ET_B knockout mice. This indicates that ECE-1 is mainly responsible for the production of ET-1 and ET-3 (36). ECE-1 mRNA is expressed in most tissues tested, with the highest expression in the ovary and testis, followed by the adrenal gland (25). ECE-2 mRNA is expressed at much lower levels with the highest expression in the neural tissues, *i.e.* cerebral cortex, cerebellum, and adrenal medulla, where its signals are still several fold lower than those for ECE-1 mRNA. In cultured endothelial cells (ECs), ECE-2 mRNA is expressed at levels only 1-2% of those of ECE-1 mRNA (30). The relative abundance of ECE-2 mRNA in neural tissues suggests that ECE-2 may be the major ECE in neurons, glia, and certain neuroendocrine cells. PA at 100 μM has been shown to increase ECE-1 expression in ECs by up to 10-fold, but no corresponding change in mRNA levels for ECE-1 was observed (28,37). Northern analysis also indicated that ECE-1b is expressed more abundantly than ECE-1a in pancreas, peripheral blood leukocytes, prostate, testis, and colon, whereas lung, spleen, placenta, and small intestine had higher expression of ECE-1a (31). Northern analysis showed that ECs express primarily ECE-1a while vascular smooth muscle (VSM) cells express ECE-1b. Further localization studies by immunohistochemical staining indicated that ECE-1a in ECs is predominantly intracellular, particularly in the Golgi, while ECE-1b in VSM cells is predominantly extracellular (36). ECE-2 is mainly localized intracellularly (30). The acidic pH optimum of ECE-2 precisely matches the luminal pH of the trans-Golgi network, which was measured directly at 5.5-5.7. Since both ECE-1 (ECE-1a and ECE-1b) and ECE-2 were shown to cleave big ET-1 more efficiently than big ET-2 or ET-3 (22-25,28-30,32), there may be additional ECE(s) which would preferentially convert big ET-2 and/or big ET-3.

ENDOTHELIN RECEPTORS

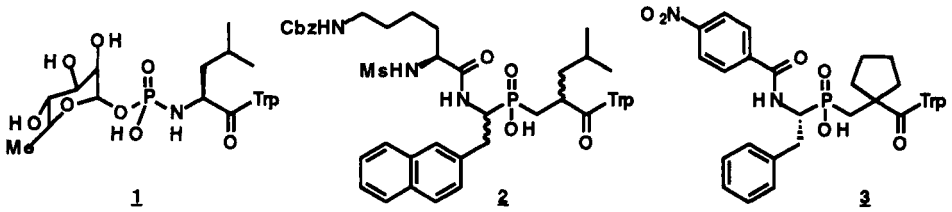
Endothelin-A (ET_A) and endothelin-B (ET_B) receptor subtypes are defined based on their gene regulation, structural features and the relative affinity for ET-1, ET-2 and ET-3. Human ET_A and ET_B receptors have been cloned and belong to the seven-transmembrane, G protein-coupled superfamily (38-41). The cloned human ET_A subtype has subnanomolar affinity for ET-1 and ET-2 but about 33-fold lower affinity for ET-3, whereas the cloned human ET_B subtype has subnanomolar affinity for all three isopeptides (42). In addition, subtypes of the ET_A (ET_{A1} and ET_{A2}) and ET_B (ET_{B1} and ET_{B2}) receptors have been proposed based on the relative selectivity of ET receptor agonists and antagonists (43-45). These subtypes may be products of alternative RNA splicing which has been reported for both human ET_A and ET_B receptors (46,47).

VSM ET_A receptors mediate vasoconstriction through the release of intracellular stores of Ca^{++} from the sarcoplasmic reticulum and extracellular Ca^{++} influx through non-voltage dependent Ca^{++} channels (48,49). In addition, ET_A receptors mediate VSM cell proliferation (50-52). ET_B receptors are present on vascular endothelium as well VSM of certain vascular beds (53-55). Activation of endothelial ET_B receptors releases nitric oxide and PGI_2 causing vasodilation, whereas activation of smooth muscle ET_B receptors produces vasoconstriction (56).

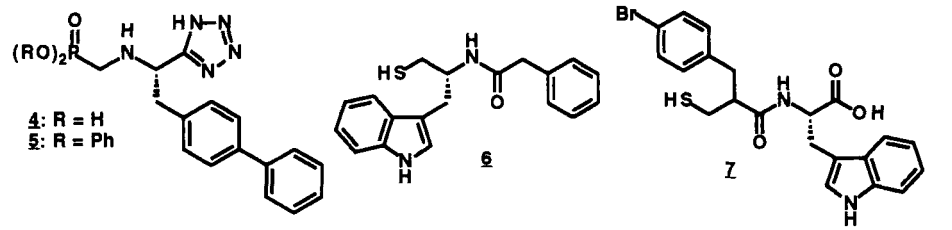
ECE INHIBITORS

Phosphoramidon (**1**), the first ECE inhibitor reported, inhibits both ECE-1 and ECE-2 albeit with a 250-fold difference in potency (IC_{50} = 1 μM and 4 nM for ECE-1 and ECE-2, respectively) (30). It also potently inhibits other metalloproteases such as NEP with IC_{50} values in the low nanomolar range (57). The chemical and metabolic instability, low selectivity, and low potency of **1** against ECE-1 make it of limited therapeutic interest. The early efforts of several groups in the development of ECE inhibitors were

focused on synthesis of PA analogs with improved properties. Modifications included replacement of the rhamnose ring by alkyl groups, replacement of the phosphoramide group by phosphate, phosphonate, or metal-chelating groups such as thiol and hydroxamate, and introduction of a P_1 lipophilic group either adjacent to the phosphoryl group or between the phosphoryl group and the leucinyl residue (58-60). However, these efforts met with limited success. More recently, chemically more stable phosphinic acid inhibitors bearing structural similarity to **1** at the P_1' and P_2' sites such as **2** and **3** have been reported. Compound **2** inhibited ECE-1 with an IC_{50} of 25 nM and showed at least >10-fold selectivity to ECE-1 over NEP (61). Compound **3**, designed by incorporating structural features of **1** and a reported dual ACE/NEP inhibitor, exhibited inhibition against ECE-1, ACE, and NEP with IC_{50} values of 50, 1.5 and 55 nM, respectively (62). Whether inhibition of ECE-1 with concomitant inhibition of ACE and/or NEP may constitute an effective therapeutic strategy for the treatment of vascular diseases remains to be demonstrated.



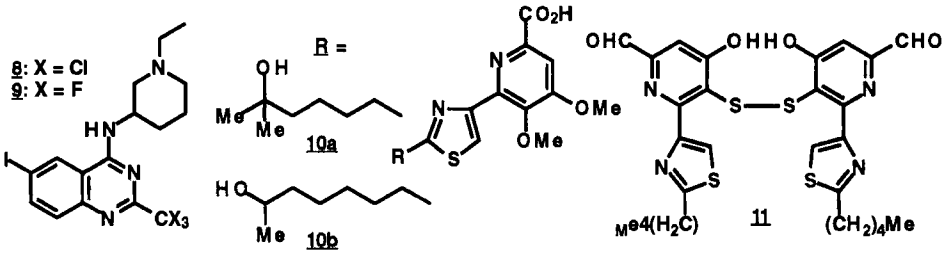
The non-peptidic, phosphonic acid tetrazole inhibitor, CGS 26303 (**4**), inhibits ECE-1 and ECE-2 with similar potency (IC_{50} of 0.3-1.1 μM) but is >1000-fold more potent against NEP (IC_{50} of 0.9 nM) (63,64). Compound **4** blocks the pressor action of exogenously administered big ET-1 *in vivo*. CGS 26393 (**5**), a prodrug of **4**, is orally active showing a long-lasting antihypertensive effect in the deoxycorticosterone acetate (DOCA) salt hypertensive rat (30 mg/kg p.o.) (65). It also inhibits big ET-1 induced vasoconstriction of basilar cerebral arterial in rabbit with either intravenous (i.v.) or intraperitoneal (i.p.) administration. I.p. administration of **4** significantly attenuated the delayed spastic response of the basilar artery to an intracisternal injection of autologous blood and therefore may have therapeutic efficacy in preventing cerebral vasospasm after SAH (66). In addition, compound **4** efficiently protected exogenously administered atrial natriuretic peptide (ANP) from degradation by NEP *in vivo* (3 mg/kg i.a.) and reduced the mean arterial pressure in spontaneously hypertensive rats (SHR) during chronic administration (5 mg/kg/day for 13 days), once again raising the hypothesis that an ECE/NEP inhibitor might offer a therapeutic benefit for the treatment of cardiovascular and renal diseases (63).



Thiorphan is a potent NEP inhibitor but is inactive against ECE-1 and ECE-2. Selective mass screening of zinc protease inhibitors revealed an analog of retrothiorphan to be moderately active to ECE-1 (67). Structure-activity relationship studies have resulted in **6** inhibiting ECE-1 with an IC_{50} of 1.7 μM. The selectivity of compound **6** against other metalloproteases is not known. Another thiol-based inhibitor, **7**, was also developed through structural optimization of a lead obtained from screening of a NEP inhibitor library (68). ECE-1 activity requires the natural

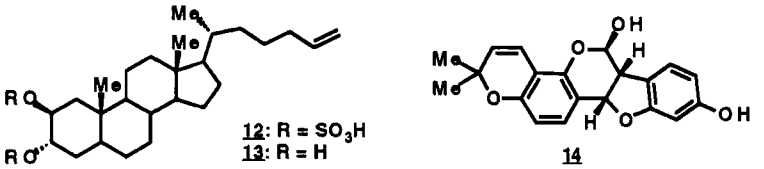
S-stereochemistry of *L*-Trp at the P_2' position but is much less discriminative at the other center. Although **7** was reported to have an IC_{50} of 20 nM, **1** also had a similar potency (IC_{50} = 10 nM) under the assay conditions.

A series of quinazolines represented by PD 069185 (**8**) and PD 159790 (**9**), were developed through compound library screening and structure-activity studies. They were reported to have IC_{50} values of 0.9 and 2.6 μM, respectively, for ECE-1 inhibition. Compound **8** is a competitive inhibitor with a K_i of 1.1 μM and shows high ECE-1 specificity. ECE-2 is not inhibited by **8** at up to 100 μM. In addition, compound **8** has little effect on inhibiting other metalloproteases, such as NEP, stromelysin, gelatinase A, and collagenase at up to 200-300 μM, the first ECE inhibitor reported to have such selectivity profile. Compound **9** at 10-30 μM was found to be effective in attenuating the increase in perfusion pressure induced by big ET-1 in isolated rat mesentery (69).



Thiazolyl pyridine carboxylic acids, WS75624 A (**10a**) and WS75624 B (**10b**), isolated from *Saccharothrix* sp. were originally reported to inhibit ECE-1, NEP, and collagenase with IC_{50} values of 0.03, 1.0, and 1.25 μg/ml (~80, 2600, and 3300 nM), respectively (70). Total synthesis of **10b** was reported recently (71). However, both synthetic sample and the sample from the natural source which are identical spectroscopically were shown to have a same IC_{50} of 1.5 μM against ECE-1 in this report, considerably higher than the original report of ~80 nM (72). The reason for the discrepancy is unknown. SAR studies from the same report indicated that the removal of the long alkyl chain did not affect the potency for ECE-1 inhibition. A dimeric oxazolyl pyridine compound **11**, obtained from *Blastobacter* sp. SANK71894, is reported to selectively inhibit ECE-1 versus NEP with potency similar to PA for ECE-1 and an IC_{50} of 75 μM for NEP (73).

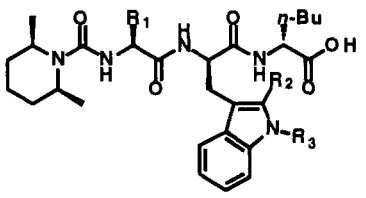
Halistanol disulfate B (**12**), a novel sulfated sterol isolated from the sponge *Pachastrella* sp., is reported to inhibit ECE-1 with an IC_{50} of 2.1 μM (74). Interestingly, the diol **13** was completely inactive towards inhibition of ECE-1. A new phytoalexin, daleformis (**14**), was isolated from the roots of *Dalea filiciformis* and was reported to inhibit ECE-1 with an IC_{50} of 9 μM (75).



Mainly due to the difficulties previously encountered with the purification and characterization of the enzyme responsible for the *in vivo* cleavage of big ET-1, the development of ECE inhibitors had been slow. In recent years considerable progress has been made due to the cloning of human ECE isoezymes and thus development of potent ECE inhibitors may now be an achievable target.

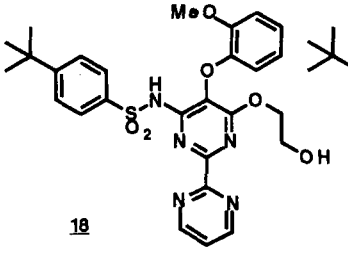
ET RECEPTOR ANTAGONISTS

Many new compounds reported last year were identified using an SAR approach around previous lead compounds and feature different receptor subtype selectivity, improved pharmacokinetics and/or improved solubility. For example, BQ-788 (**15**), a linear tripeptide, was the first ET_B selective antagonist reported with IC_{50} values of 1300 and 1.2 nM to ET_A and ET_B receptors, respectively (76). Modification with additional 2-substituents on the *D*-Trp residue resulted in BQ-017 (**16**), an analog with similar activity and selectivity as BQ-788 but with much improved solubility in saline. A mixed ET_A/ET_B antagonist, BQ-928 (**17**), was also derived from this approach and exhibited binding affinities of 13 and 1.1 nM to ET_A and ET_B receptors, respectively (77).



15: R_1= *t*-BuCH_2, R_2=H, R_3=CO_2CH_3
16: R_1= *i*-Pr, R_2=CN, R_3=H
17: R_1= *c*-Pr, R_2=Br, R_3=H

Replacement of the second pyrimidine ring found in a leading non-selective ET antagonist, Bosentan (**18**), with a *p*-methoxyphenyl ring and the modification at the side chain yielded the first non-peptide ET_B selective antagonist, Ro 46-8443, with IC_{50} values of 6800 and 34 nM to the ET_A and ET_B receptor, respectively (**19**) (78). IRL 2500 (**20**) is a dipeptide ET_B selective antagonist reported to have K_i values of 1 and 440 nM to the ET_B and ET_A receptors, respectively (79). It exhibits antagonism to the ET_B mediated contraction induced by ET-3 in the guinea pig tracheal but had no effect on the ET-1 induced contraction of the aorta up to 30 μM. The pathophysiological role of ET_B receptor activation in various disease states has been a subject of debate, and the discovery of new ET_B selective antagonists has been helpful in addressing this important issue. For example, compound **19** was shown to decrease blood pressure in normotensive rats suggesting the predominant effect of activating vascular ET_B receptors under normal conditions is vasoconstriction. In contrast, **19** produced a pressor effect in spontaneously hypertensive rats (SHR) and DOCA salt hypertensive rats, suggesting the predominant effect of activating vascular ET_B receptors under hypertensive conditions is vasodilation (80).



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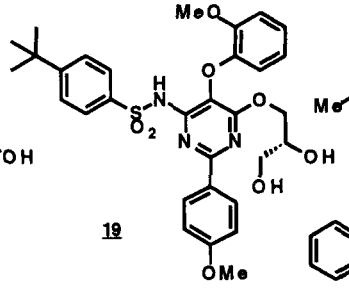

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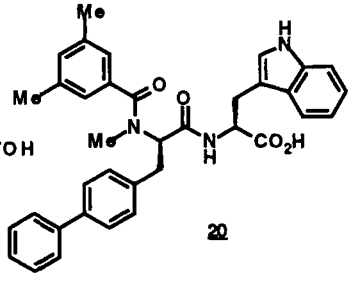
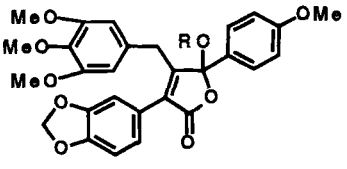
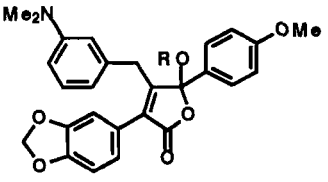


20

Continued SAR studies in the butenolide series of potent ET_A selective antagonists represented by PD 156707 (**21**), have yielded several series of analogs with comparable potency and selectivity. PD 163140 (**22**) and PD 163610 (**23**) are the γ-carbamate and ether analogs of **21** in the ring closed form (81, 82). Their affinities to the ET_A receptor were reported to be 0.3 and 50 nM, respectively.



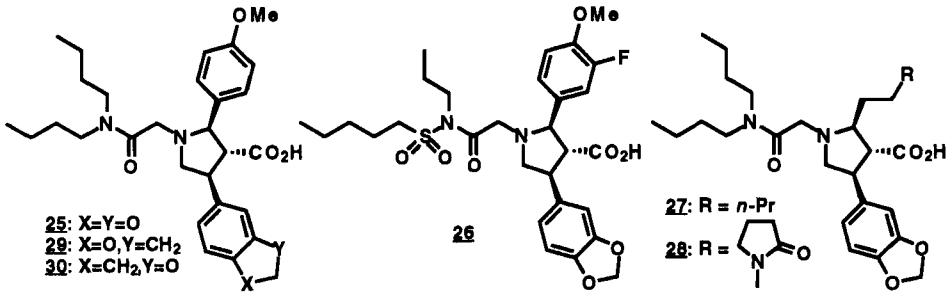
21: R = H
22: R = $CONHCH_2CO_2Et$
23: R = $(CH_2)_3CO_2H$



24

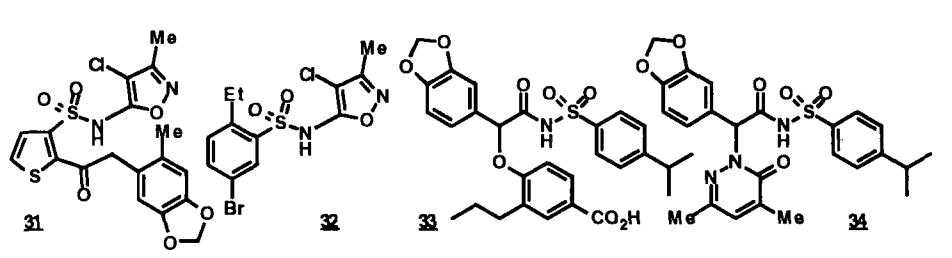
Compound **22** is readily converted to the parent **21** in rat intestinal perfusate acting as a prodrug of **21**. Modification at the R_2 ring led to PD 163070 (**24**), an orally active ET_A selective antagonist (ET_A = 3 nM, ET_B = 2500 nM) with an 83% bioavailability and pharmacokinetic half life of 5.4h through an active demethylated metabolite (83).

Subtle structural modifications of A-127722 (**25**) series produced a dramatic effect on receptor binding selectivity. Thus, changing one of the amide side chains to a *S*-alkyl sulfonamide led to a series of mixed ET_A/ET_B antagonists highlighted by **26** which



was reported to have ET_A binding affinity of 0.1 nM with ET_A /ET_B ratio of 3 (84). Compound **26** is reported to have a pharmacokinetic half life of 8.1h in rats with 54% oral bioavailability (84). Replacement of the anisyl moiety with an alkyl or a heterocyclic substituted alkyl afforded compound **27** (ET_A = 2.5 nM, ET_A /ET_B = 19000) and **28** (ET_A = 0.17 nM, ET_A /ET_B = 106000) , respectively, that are potent and more ET_A selective than **25** (85). The methylenedioxy benzene moiety, a common group in several reported leading ET antagonists, is known to inhibit cytochrome P_{450} and efforts have been reported in the A-127722 series to explore other groups to replace the methylenedioxy ring. Simple deletion of either oxygen atom from the parent structure led to compounds **29** and **30**. Both compounds showed similar ET_A affinity and selectivity to the parent compound without inhibiting P_{450} (86).

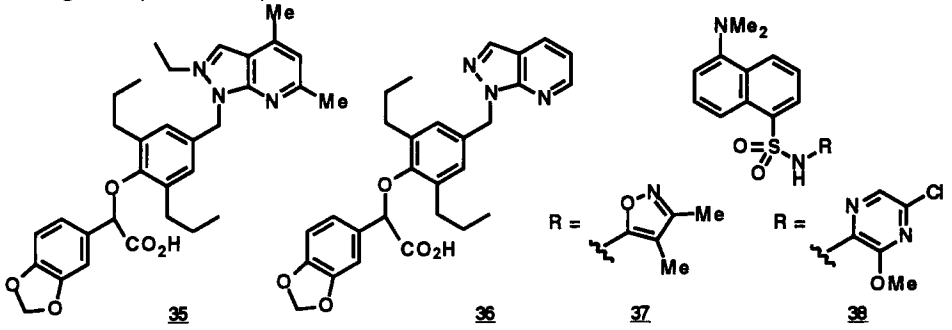
Continued SAR in a series of isoxazolyl sulfonamide series, represented by TBC-11251 (**31**), has led to the identification of several benzene sulfonamides, such as TBC-10662 (**32**), which are potent ET_A selective antagonists (87). Using molecular modeling techniques to replace the aryloxy in the non-selective antagonist L-749,329 (**33**) with other heterocycles resulted in a pyridazinone derivative **34** with a binding affinity of $2.9x10^{-8}$ M to the ET_A receptor, comparable to the parent compound **33** ($4.1x10^{-8}$ M) (88).



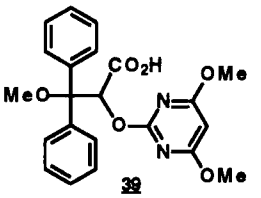
A series of α-phenoxyphenyl acetic acid derivatives was previously reported as angiotensin II antagonists. Structural modifications produced a dual AT_1/ET antagonist **35** [IC_{50} (ET_A) = 20 nM, IC_{50} (AT_1) = low nM]. Fine tuning of the substituents on the imidazopyridine moiety resulted in a further increase in ET_A potency and a loss of AT_1 activity to lead to the potent ET_A selective antagonist, L-744,453 (**36**) with IC_{50} values of 4.3 nM to ET_A and >1 μM to AT_1 (89). Incorporation of the methylenedioxy benzene moiety was one of the key contributing factors for increasing the ET_A affinity. Compound

36 is active *in vivo*, blocking the acute cardiovascular responses to exogenously administered ET-1 or big ET-1 and is reported to have an oral bioavailability of 48%.

The power of automated synthesis to optimize a lead compound was demonstrated by the discovery of **38** (90). Inspired by BMS 182874 (**37**), an earlier reported ET_A selective antagonist ($5.0x10^{-8}$ M), replacement of the isoxazole ring with different heterocycles followed by evaluation of different substituents led to **38** which has an ET_A receptor binding affinity of 7.9 nM and is orally active at 10 mg/kg in inhibiting the big ET-1 pressor response in rats with a duration of action ≥ 5h.



LU 127043 (**39**) represents a newly reported novel class of orally active ET_A selective antagonists. This class of compounds was developed through structural optimization of the earlier leads which were initially identified through compound library screening. LU 127043 is potent in binding to the ET_A receptor (K_i values of 6 nM to ET_A and 370 nM to ET_B) and is reported to be orally active with long duration of action (91). Orally administrated LU 135252, the active enantiomer of LU 127043, was shown to inhibit neointima formation dose-dependently in response to endothelial injury in rats (92).



THERAPEUTIC INDICATIONS AND DEVELOPMENTAL STATUS OF ET INHIBITORS

Table 1 summarizes possible therapeutic indications and developmental status of the leading ET inhibitors. Possible indications are based on positive preclinical and clinical efficacy studies (3-16). Phosphoramidon has been widely used in defining the physiology and pathophysiology of ET-1, and although it is unlikely to be developed it has provided important evidence that ECE inhibitors may be efficacious in treating HP, PH, CHF, and RT. CGS 26303 and its prodrug CGS 26393 are the only ECE inhibitors reported to be in preclinical development which have demonstrated *in vivo* efficacy in pathological models such as SAH and PH.

Most of the ET_A selective antagonists in Table 1 have shown efficacy in preclinical models of CHF and PH. In addition, PD 156707 has shown efficacy in stroke, and BQ-123 is efficacious in RF and SAH. TBC-11251 is reported to be in phase I clinical trials for CHF and LU 135252 is effective in preventing RT. The ET_B selective antagonist BQ-788 has been widely used to define the physiological role of ET_B receptor activation. To date, however, *in vivo* efficacy in a pathological model has not been reported with BQ-788 or any other ET_B selective antagonists.

The non-selective antagonist Bosentan appears to be the most advanced in development having shown efficacy in phase II clinical trials in CHF patients. Preclinical efficacy has been demonstrated with Bosentan in several diseases including SAH, PH and MI. TAK-044 is the most potent of ET_A peptide antagonist but is only about 500-fold selective and is therefore often referred to as a non-selective agent (93). It is reported to be in phase II clinical trials most likely for short term parenteral treatment in MI and RF

due to its peptidic structure. SB 209670 and SB 217242 are structurally related non-selective antagonists which differ mainly in their oral bioavailability (19). SB 209670, having poor oral bioavailability (<10%), prompted the development of SB 217242 which is 66% orally bioavailable with a plasma $t_{1/2}$ of 3.3 hours in rats. Consequently SB 209670 is reportedly being developed for acute i.v. indications with preclinical efficacy shown in PH, ARF and stroke whereas SB 217242 is reported to be in development for chronic PH and chronic obstructive pulmonary disease (COPD).

Table 1. Therapeutic indications and developmental status of ET inhibitors[16]

Compound	Inhibitor	Efficacy	Status
Phosphoramidon	ECE	CHF, RT, HP	Not Being Developed
CGS 26303	ECE	SAH	Preclinical[94,95]
CGS 26393	ECE	HP	Preclinical[94,95]
TBC-11251	ET_A	PH, CHF	Phase I[94]
BQ-123	ET_A	CHF, PH, SAH, RF	Phase I (Discontinued)[94,95]
PD 156707	ET_A	Stroke, PH, CHF	Preclinical[94,95]
A-127722	ET_A	PH, CHF	Preclinical[94,95]
LU 135252	ET_A	RT	Preclinical[94,95]
BQ-788	ET_B	--	Preclinical[94,95]
Bosentan	ET_A/ET_B	CHF,SAH ,PH, MI	Phase II[94,95]
TAK-044	ET_A/ET_B	CHF, MI, RF	Phase II[94,95]
SB 209670	ET_A/ET_B	PH, RF, Stroke	Phase I[94]
SB 217242	ET_A/ET_B	Stroke, PH, COPD	Phase I[94,95]

Summary - Discovery and development of endothelin inhibitors have been one of the fastest growing research areas in the pharmaceutical industry. ECE inhibitors and ET antagonists of diverse structural types have been identified and optimized with different profiles in terms of potency, selectivity, efficacy and pharmacokinetics. With several compounds currently in clinical trials and additional candidates under preclinical evaluation, the role of ET in a variety of important human diseases will be elucidated.

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Chapter 8. Antithrombotics/Serine Proteases

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Introduction-The use of anticoagulants in the treatment and prevention of both acute and chronic thrombosis-related disorders is growing at a rapid pace in part due to an increasing geriatric population and the recognition of intravascular clot formation as a causative factor in a number of important cardiovascular diseases such as myocardial infarction, unstable angina, deep vein thrombosis, pulmonary embolism and ischemic stroke. The mechanism of all current anticoagulant strategies is the inhibition of one or more of the trypsin-like serine proteases that catalyze blood coagulation through the sequential amplified activation of the corresponding zymogens for these enzymes (1). To date there are only two anticoagulant strategies that have been used clinically for over 50 years in the treatment of acute and chronic thrombosis; heparin and related derivatives (low molecular weight heparins (LMWH)) and vitamin K antagonists (warfarin), respectively. The anticoagulant mechanism of heparin and its derivatives results from its ability to serve as an activating co-factor primarily for a circulating serpin antithrombin III (AT-III) which forms an immediate stable inhibitory complex with several of the serine proteases in the coagulation cascade (2). In contrast, the vitamin K antagonists like warfarin act to inhibit the post-translational vitamin K-dependent γ-carboxylation of several key serine protease zymogens which results in the down regulation of thrombus formation that takes several days to have its maximal effect (3). Both the heparins and vitamin K antagonists have been used effectively as parenteral and oral anticoagulants, respectively, despite significant limitations in maintaining a therapeutic dose and efficacy in certain clinical indications as well as unwanted side-effects, the most prominent of which is the risk for prolonged bleeding. The side effect profiles of both the heparins (2) and vitamin K antagonists (3,4) is thought to be principally due to their lack of specificity toward numerous serine proteases involved in thrombus development and in the case of the vitamin K antagonists this includes a significant impairment of the natural anticoagulant pathway mediated through the serine protease activated protein C (aPC).

The limitations of both the heparins and warfarin have stimulated the search for new therapeutic alternatives based on the selective inhibition of the principle serine proteases catalyzing thrombus formation. This has been aided by the determination of the three dimensional crystal structure of virtually all of the key enzymes in the blood coagulation cascade which has helped in the design of synthetic compounds that specifically block enzymatic activity (5-8). Additionally, the use of exquisitely selective natural inhibitors derived from blood feeding organisms has also provided key insight into the pharmacological effects of selective inhibition of the coagulation serine proteases at different levels in this cascade (9,10). While there has been much progress toward the goal of introducing new therapeutic anticoagulants into the clinic over the past 5 years, there remain significant obstacles in achieving the appropriate selectivity, potency, pharmacokinetics and oral bioavailability. This chapter will focus on the recent developments in the search for a new generation of synthetic anticoagulants with some discussion on the use of natural anticoagulants to gain further insight into inhibitor design and pharmacological efficacy.

THROMBIN INHIBITION

Thrombin-Introduction - The initiating event triggering the blood coagulation response occurs following the rupture of an established atherosclerotic plaque as in the case of occlusive thrombus development in the coronary arterial vasculature (11) or damage to

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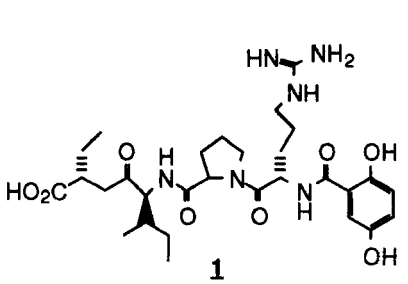
the intimal endothelial barrier induced by physical manipulation or inflammation as is often observed in the venous vasculature (12). This results in the triggering of an amplified cascade of proteolytic events leading to the formation of the serine protease thrombin (1). As the terminal enzyme in the coagulation cascade, thrombin primarily acts to form insoluble fibrin from circulating fibrinogen and activate circulating platelets leading to aggregation (13). The action of thrombin coupled with the particular rheological environment found in diseased or damaged vascular beds, result in thrombi with compositions ranging from platelet-rich found primarily in the arterial vasculature, to fibrin-rich, platelet-poor clots in the venous vasculature. As opposed to the heparins, thrombin inhibitors which directly act to neutralize the procoagulant effects of this enzyme without the requirement of AT-III, have been shown in some cases to be more effective antithrombotic agents in the prevention of arterial and venous thrombosis compared to heparin anticoagulants (13,14). However, the issue has not been resolved on whether direct thrombin inhibitors will be more beneficial both from an efficacy and safety viewpoint compared to the heparin anticoagulants especially in the clinical setting of acute arterial thrombosis (15-17).

Naturally Occurring Thrombin Inhibitors - Two examples of this class have been isolated which are potent inhibitors of the fibrinogen exosite in thrombin without interacting with the active site. Triabin, a small protein (142 amino acids) isolated from the saliva of the triatome, *Triatoma pallidipenis*, inhibits the thrombin catalyzed hydrolysis of fibrinogen (Ki=3 pM) but does not inhibit thrombin's amidolytic activity in chromogenic peptide substrate assays (18). Bothrojaracin, isolated from the venom of *Bothrops jararaca*, also utilizes primarily the fibrinogen exosite along with some interactions at the heparin exosite to give effective inhibition (Kd = 0.6 nM)(19). As determined by competition with the hirudin 54-65 peptide it's binding to the fibrinogen exosite alone is somewhat weaker (Ki = 50 nM). Rhodniim, isolated from the assassin bug, *Rhodnius prolixus*, is a highly specific inhibitor of thrombin (Ki = 0.2 pM)(20). A 2.6Å crystal structure shows two Kazal-type domains in which the N-terminal domain binds in a substrate-like manner to thrombin's active site and the C-terminal domain binds via electrostatic interactions to the fibrinogen exosite. Rhodniim is unusual in that it has a histidine at the P1 position that makes a unique interaction with both the Asp 189 at the bottom of the S1 pocket and to Glu 192. Nazumanide A, **1**, isolated from the marine sponge *Theonella sp.*, is a weak inhibitor of thrombin (IC_{50} 4.6 μM) but has the unusual feature, shown by x-ray crystallography of complexes with thrombin, of binding in a retro mode with the C-terminal α-aminobutyrate in the S4 pocket, the isoleucine in the S3, the proline in S2, and the arginine in S1 with the 2,5-dihydroxybenzene in S1' (21). A partial structure has been determined by 2D NMR for A90720A **2**, a cyclic depsipeptide thrombin inhibitor (Ki=274 nM) isolated from the blue-green alga *michochaete* (22). The presence of several NOE's indicates a rigid, constrained structure with all L-amino acids in the ring and a D-leucine and R-glycerate in the sidechains. An x-ray structure of the complex of cyclotheonamide A (CtA) **3** with thrombin has been reported along with a comparative study of its complex with



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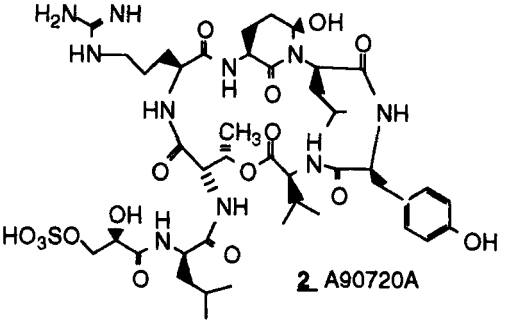
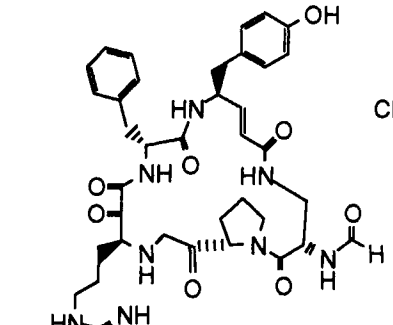


2 A90720A

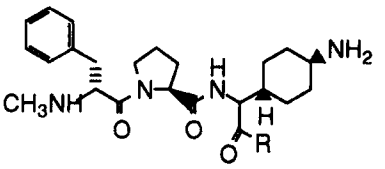
trypsin (23,24). Specific interactions of the (D)-Phe side chain of CtA with different residues in thrombin and trypsin is believed to be responsible for its relative trypsin selectivity.

Transition-State Inhibitors - Recent reviews have raised several issues related to inhibitors based on transition state mimetics (25-28). These include concerns about selectivity, particularly against the fibrinolytic enzymes as well as trypsin; the slow-tight binding kinetics often observed with this class and whether these inhibitors can complete successfully with the rapid production of thrombin during vascular injury; side effects such as hypotension possibly associated with highly basic guanidine functionality; short plasma half-lives; and oral bioavailability. A number of synthetic strategies to deal with these issues are described below and include reducing the basicity of the P1 group, rigidifying the P1 group and making it more hydrophobic, changing the nature of the transition states, altering the nature of the P2 groups to take advantage of thrombin's unique "60-insertion loop", and constraining and introducing non-peptide functionality into the P3-P4 regions.

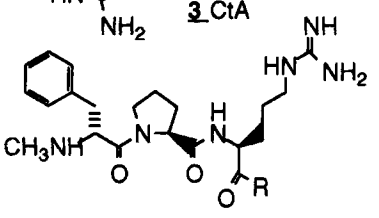
Transition -State P1 Variations - One approach for quickly evaluating structure-activity relationships in the P1 position was to synthesize several carboxamides, H-(D)-Phe-Pro-X-NH2 as a guide for later conversion to the more difficulty synthesized transition state inhibitors (29). Using this strategy **4** was the most potent carboxamide found (Ki = 5 mM, 160 fold selective) and conversion to the α-ketoamide resulted in **5** L-370,518



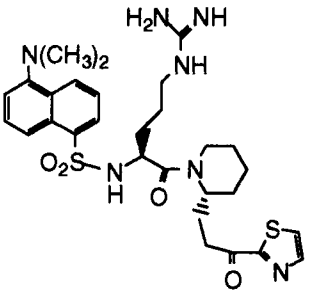
3 CtA



4 R = NH_2
5 R = $C(=O)NHCH_3$



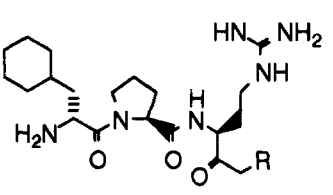
6 R = benzthiazole (RWJ-50353)
7 R = 2-thiazole
8 R = 2-benzothiaphene
9 R = 2-pyridine



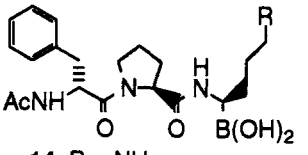
10 RWJ-50215

(Ki = 90 pM, trypsin 12,800x). The α-ketobenzthiazole transition state, which provides both an electrophilic carbonyl and a template to extend functionality into the S' regions, has been coupled with the (D)-Phe-Pro-Arg motif to give **6** RWJ50353 (Ki = 0.18 nM, trypsin 16x) (30,31). The x-ray structure of **6** and thrombin shows the nitrogen of the benzthiazole forms an H-bond with His-57 and the benzthiazole ring stacks in a face-to-edge interaction with the indole ring of Trp 60D. Compound **7** (Ki = 3.6 nM, trypsin 0.4x) illustrates the importance of aromatic stacking with Trp 60D and **8** (Ki = 2400nM) and **9** (Ki = 85 nM) show that two proximal hetero atoms, which activate the

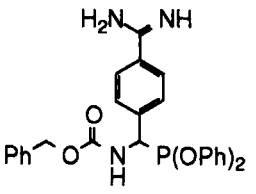
electrophilic carbonyl, are necessary for potent activity (30). Compound **6** inhibited thrombin-induced platelet aggregation (IC_{50} = 23 nM) comparing favorably with efegatran **50** (IC_{50} = 23 nM) and argatroban (IC_{50} = 52 nM). RWJ-50215 **10** (Ki = 1.2 mM) has an α-ketothiazole group attached to the dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide motif of DAPA and argatroban (32). The crystal structure of **6** and **10** in complex with thrombin show they bind in the active site of thrombin in the same conformations as the templates (32). Other transition state inhibitors **11** (Ki = 0.19 nM) utilize the pyridinium methyl group to activate the electrophilic carbonyl (33). Removing the charged nitrogen (**12**)(Ki = 1.25 nM) or the charge and the aromatic ring (**13**) (Ki = 60 nM) substantially reduced activity.



11 R = $N^+C_5H_5$ (P598)
12 R = C_6H_5 (P601)
13 R = CH_3 (P607)



14 R = NH_2
15 R = CH_2NH_2
16 R = $CH_2CH_2NH_2$
17 R = $CH_2C(=NH)NH_2$
18 R = $NHC(=NH)NH_2$



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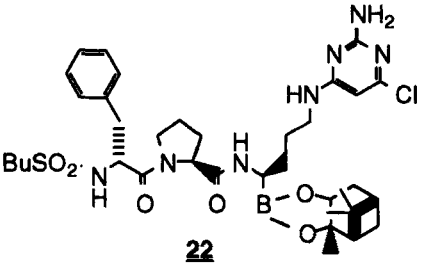

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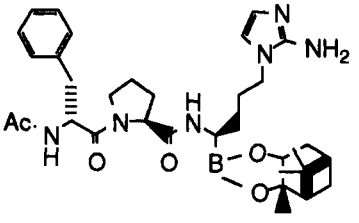

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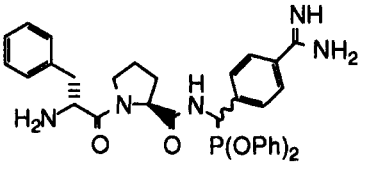

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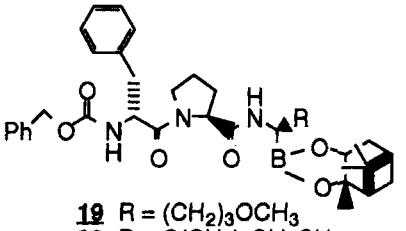


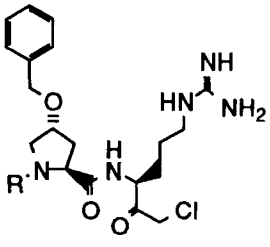
19 R = $(CH_2)_3OCH_3$
20 R = $C(CH_3)_2CH_2CH_3$
21 R = $(CH_2)_4CH_3$

Contributions from the P1 group in the boronic acid transition states were studied by replacing the arginine in Ac-(D)-Phe-Pro-boroArg-OH **18** (DuP714, Ki= 0.04 nM)

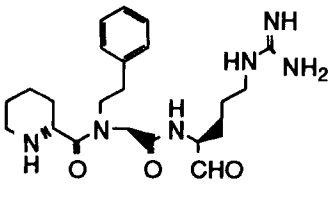
with ornithine **14** (Ki = 79 nM), lysine **15** (Ki = 0.24 nM), homolysine **16** (8.1 nM), and amidine **17** (0.29 nM) (17). The resulting range of affinities (3 orders of magnitude) reflect differences in the specific interactions that were detailed in x-ray crystallographic studies of their respective complexes with thrombin (34). While **18** is a slow, tight-binding inhibitor of thrombin the other analogs did not show time dependent binding, with no greater than a 3 fold difference in binding affinities between initial and steady state. The P1 group in the boronic acids has also been replaced with neutral sidechains **19-21** (35). Linear alkyl chains, **21** (Ki = 19 nM, trypsin 17x, plasmin 153x, fXa 31x), branched alkyl chains **20** (Ki=7 nM, trypsin 39x, plasmin 786x, fXa 371x), or methoxy substituted alkyls **19** (Ki= 7 nM, trypsin 1114x, plasmin 1114x, fXa 443x) have little effect on potency but do influence selectivity. A series of boronic acids with a variety of amino-substituted pyrimidine and imidazole heterocycles ranging in size and basicity replacing the guanidine in the arginine side chain of DuP714 gave as the most potent pyrimidine **22** (Ki = 2.1 nM, trypsin 102x, fXa>2860x) and the most potent imidazole **23** (Ki = 1.7 nM, trypsin 11.7x, fXa >3529x)(36).

Phosphonates have also been used as transition state mimetics for inhibitors of trypsin-like proteases. Phosphonate **24** reacts with trypsin to form a trigonal bipyramidal, pentacoordinated phosphorus atom with the donor and acceptor ligands in the apical positions. Incorporation of the phosphonate into the (D)-Phe-Pro motif led to **25** which, however, had a surprisingly low activity ($k_{obsd}/[I]$ = 700 M^{-1} s^{-1}) compared to Phe-Pro-Arg-chloromethylketone (PPACK) (kobsd/[I] = $9.6x10^6$ M^{-1} s^{-1}) (37).

Transition-State P2 Variations - Molecular models suggested that benzyloxy groups in the 4-position of a P2 proline could occupy the same conformational space as the (D)-Phe in the (D)-Phe-Pro-Arg motif (38). The trans-4-benzyloxy **26** (IC_{50} = 0.9 nM, fXa 1088x, plasmin 244x) was 5000 times more potent than the cis isomer. Compound **26** was active in the $FeCl_3$ model of induced thrombosis in a rat carotid artery with an iv bolus (3 mg/kg) and a constant infustion (0.3 mg/kg/min) (38). Arterial patency was maintained and the thrombin mass reduced. Elimination of the carbonyl that forms the critical H-bond to Gly 216 of thrombin illustrated by **27** (IC_{50} = 8600 nM), gave a compound 10,000 times less active. N-substituted glycines in the P2 position are expected to induce the same type II beta turn as proline. Replacing the P2 proline in a series of aldehyde transition state inhibitors with N-substituted glycines resulted in a number of potent inhibitors, e.g. **28** (Kd = 11.5 nM, trypsin 32x, plasmin 200x) (39). Increasing the length of the N-substituent from methyl to propyl, as well as α, β or γ branching, had little effect on potency. A crystal structure of **28** in complex with thrombin shows the S2 subsite is unoccupied in contrast to the proline cases (39).



26 R = CH_3CO-
27 R = CH_3CH_2-



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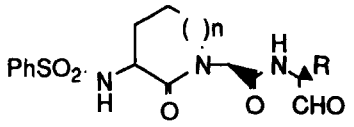
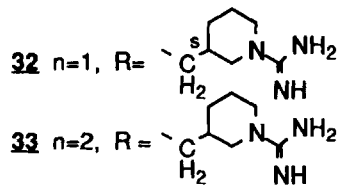
Transition-State P3-P4 Variations - Selectivity, particularly against fibrinolytic enzymes as well as trypsin, has been a particular concern with transition state inhibitors which utilize the interaction between an electrophilic atom and the Ser 195 Oγ to achieve potency (25,26). Early examples of such inhibitors, e.g. DuP714 and efegatran, lacked

the necessary selectivity. Incorporating a 6- or 7-membered lactam at the P3 positions of argininal inhibitors, however, gives **29** (Ki = 1.01 nM, trypsin 128x, fXa 403x) and **30**

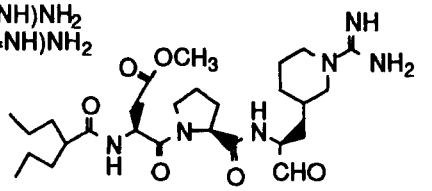


29 (CVS1578) n=1, R=-$(CH_2)NHC(=NH)NH_2$
30 (CVS1778) n=2, R=-$(CH_2)_3NHC(=NH)NH_2$



32 n=1, R=

33 n=2, R =



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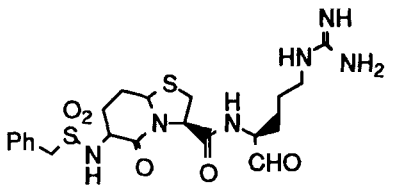

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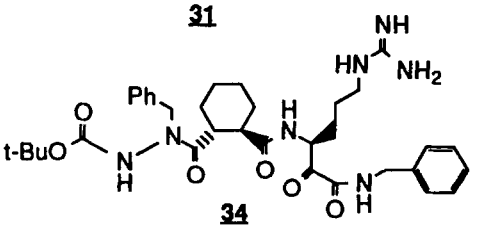

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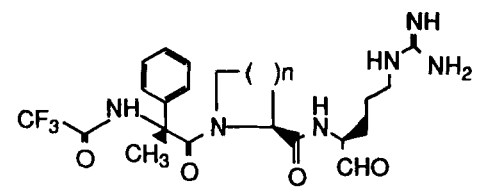
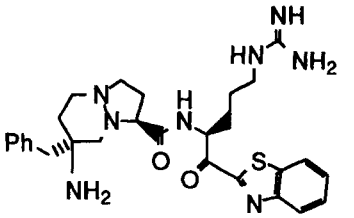
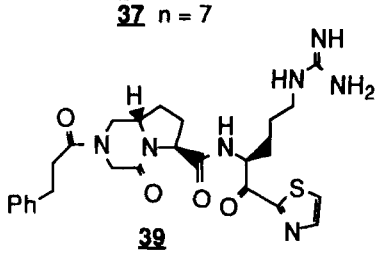


36 n = 1
37 n = 7



38 MOL-144



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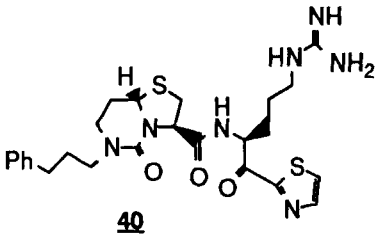

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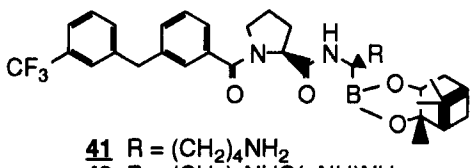
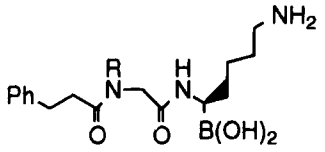
(IC_{50} = 0.71 nM, tryspin 463x, fXa 29x), respectively, with substantially increased selectivity including against trypsin (40). An x-ray structure of **29** with thrombin confirms the substrate-like binding with the lactam ring and P2-methylene occupying the S2 site as expected (40). Similar improvements in selectivity are achieved by substituting the rigid 3-piperidyl(N-guanidino)alaninal **31** (IC50 1.5 nM, trypsin 47x, fXa 1667x) as an arginine mimetic in the P1 position (41). Combining these structural features, a lactam at P3 and rigid arginine surrogate at P1, gave potent and very selective inhibitors **32** (IC_{50} 0.67 nM, trypsin $1x10^5$x, fXa $3.7x10^4$x) and **33** (IC_{50} = 0.57 nM, trypsin $8x10^4$x, fXa $4.3x10^4$x) (41). X-ray structures of their complexes with thrombin are described (41). Since both D- and L- configurations at the P3 site have been shown to be accomodated in thrombin's active site, a series of azapeptides, e.g. **34** (IC_{50} = 0.88 mM) was made where the trigonal N-alkylated nitrogen can adopt an orientation intermediate between the D- and L- forms (42). This strategy also

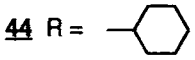
eliminates a chiral center in the molecule. Variations of the N-terminal group, the N-alkyl of the azaamino acid, and the transition state (aldehydes, α-ketoamides) failed to improve the potency. A fused bicyclic lactam **35** (IC_{50} 16.4 nM, trypsin 0.7x, plasmin 56x, fXa inact) introduced into the P2-P3 position lacked the selectivity seen with simple lactams (43). Modification of the P3 groups in the argininal series by α-methylation gave **36** (IC_{50} 12 nM, trypsin 0.8x, plasmin 508x, fXa 8166x) and **37** (IC_{50} 64 nM, trypsin 2.8x, plasmin 703x, fXa 2968x) (44). When P3 was an α-alkylated phenylglycine, P2 was optimally azetidine (**37** vs **36**). Mol-144 **38** is an example of a rigid P3 modification with the α-ketobenzthiazole transition state (45). This compound shows inhibition of platelet deposition in vivo in a baboon vascular graft model after iv administration and is claimed to give extended thrombin times in rats after oral dosing. Compound **39** (Ki = 7nM) also shows good in vitro potency and is active in the $FeCl_3$ model at an iv dose of 0.75 μg/kg (46). A further variation is shown with **40** (Ki = 40 nM) which incorporates a bicyclic urea at the P2-P3 position (47). A series of peptidyl boronic acid inhibitors have been prepared in which the P3 residues of Ac-(D)-Phe-Pro-boroLys-OH and 3-phenylpropionyl-Pro-boroLys-OH were replaced by conformationally restricted benzoic acid groups, e.g. **41** (Ki = 0.07 nM) (48). X-ray crystallographic results show that the borolysine side chain is extended more deeply into the S1 pocket than with the Ac-(D)-Pro- analog apparently as a consequence of the steric bulk of the bridged biaryl P3 residue (48). Interestingly, the corresponding boroarginine **42** (Ki = 0.4 nM) shows *weaker* activity than the lysine analog in contrast to the earlier findings where arginines were generally ten times more potent than lysines. As expected the length of the P1 chain and the precise postioning of the basic group in the S1 pocket have dramatic effects on potency. More non-peptide like inhibitors have been synthesized by replacing the usual P3 (D)-Phe with 3-phenylpropanoyl and substituting N-alkyl glycines for the P2 proline, **43** (Ki 0.69 nM, trypsin 1.6x, plasmin 230x), **44** (Ki 4.9 nM, trypsin 1.7x, plasmin 50x), **45** (Ki 1.4 nM), **46** (Ki 4.68 nM) and **47** (Ki 0.42 nM, trypsin 6x, plasmin 400x, tPA 1620x) (49). Most of the active site interactions in the original (D)-Phe-Pro were retained, specifically the edge-to-face aromatic interaction with Trp 215 and one of the H-bonds to Gly 216 (carbonyl of Gly 216 to amide NH). One of these analogs (**47**) was reported to be orally active in dogs elevating the aPTT >2 fold for 2 hrs at a dose of 5 mg/kg. The binding mode of these analogs was confirmed by X-ray analysis of their complexes with thrombin (49). The series X-Pro-boroLys-OH **48** (Ki = 5.6 nM) and **49** (Ki=0.5 nM) was designed to introduce aromatic functionality to optimize interactions with Trp 215 (50).



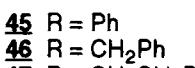
41 R = $(CH_2)_4NH_2$
42 R = $(CH_2)_3NHC(=NH)NH_2$



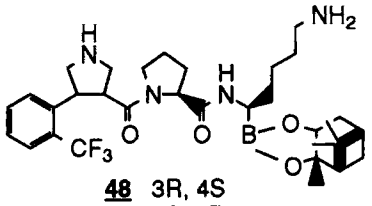
43 R =



44 R =



45 R = Ph
46 R = CH_2Ph
47 R = CH_2CH_2Ph

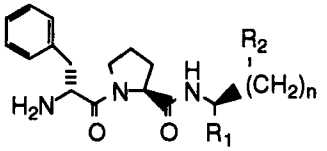


48 3R, 4S
49 3S, 4R

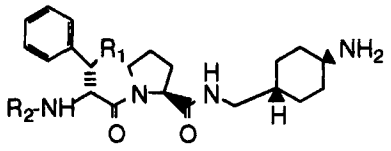
Non-Covalent Inhibitors - Because of the non-peptide nature of the non-covalent inhibitors such as NAPAP and argatroban as well as their rapid kinetics of inhibition,

the last few years has seen a substantial effort to modify these templates to generate inhibitors with improved properties, particularly oral biovailability and longer plasma half-lives (see ref 25). X-ray crystallography of their complexes with thrombin has played an increasingly important role in the design of more optimized structures. This has been essential since, particularly in the case of non-transition state compounds, the modes of binding may not always predictable (e.g. 51). Small structural changes have sometimes led to substantial alterations in the fit to the active site (52,66). A second successful approach has been to simply remove the transition state moiety from optimized inhibitors of this class. In conjunction with that deletion it has also proven necessary to replace the P1 group with a rigidified arginine mimetic to restore potency. Additional effort has been focussed on reducing the basicity of the arginine in the belief this will improve oral bioavailability and reduce side effects thought to be associated with the guanidine functionality.

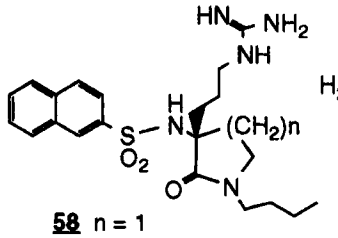
Modified P1 Arginine Surrogates - Starting from the known, potent aldehyde inhibitors (D)-Phe-Pro-Arg-H **50** (efegatran, Kd = 2.17 nM, trypsin 5.2x, plasmin 260x) , the aldehyde functionality was removed to produce agmatine **51** (Kd = 181 nM, trypsin 33x, plasmin 4600x) (53). Replacing the propylguanidine with a rigid mimetic gave **52**



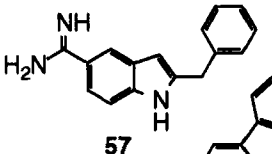
50 R_1 = CHO , R_2 = NHC(=NH)NH_2, n=3
51 R_1 = H, R_2 = NHC(=NH)NH_2, n=3
52 R_1 = H, R_2 = 4-C_6H_4-C(=NH)NH_2, n=1



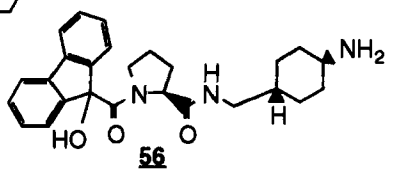
53 R_1 = H, R_2 = CH_3
54 R_1 = H, R_2 = $PhCH_2SO_2$
55 R_1 = Ph, R_2 = $PhCH_2SO_2$



58 n = 1
59 n = 2
60 n = 3



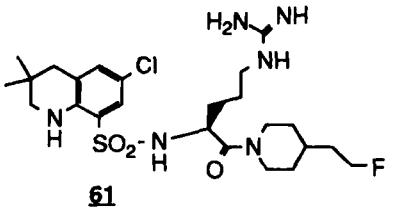
57



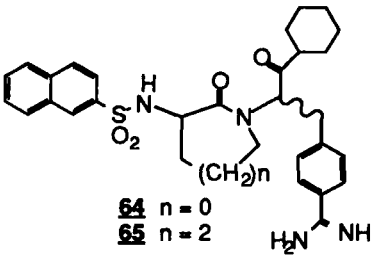
56

(Kd = 1.47 nM, trypsin 130x, plasmin 26000x), equipotent with the original argininal (54). These results confirm the idea that the binding energy provided by the covalent transition states can, in some cases, be achieved with non-covalent interactions. The x-ray structure of **52** with thrombin shows the fit to the active site is essentially identical to the covalently bound analog (54). The two order of magnitude difference in the binding constants of **51** vs **50** indicates the C-terminal aldehyde contributes approximately 3 Kcal/mol of binding energy to the complex. Interestingly, this binding energy is recovered by the rigid P1, presumably by favorable entropic and hydrophobic effects. A number of less potent analogs suggests that selectivity can be manipulated by varying the tether length of the P1 functionality. Removing the α-ketoamide transition state in **5** gave **53** (Ki = 5 nM, trypsin 2200x)(56) and substituting a benzylsulfonyl at the N-terminus of **53** gave **54** (Ki = 0.4 nM) (55), enhancing the potency by ten fold. The crystal structure of **54** in complex with thrombin is described (55). Incorporation of a second phenyl ring in the P3 position (diphenylalanine) gave an extremely potent inhibitor **55** (Ki = 2.5 pM, trypsin [4 nM] 1600x, plasmin $2x10^5$x) (56). An important point should be made about trypsin selectivity with respect to compounds such as **55**. Because the inhibitor is not simultaneously exposed to trypsin

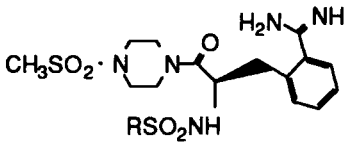
and thrombin (trypsin in gut, thrombin in plasma), for very potent inhibitors such as **55**, the absolute magnitude of the trypsin inhibition may be more important than relative



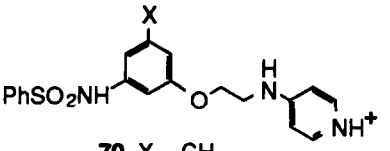
61



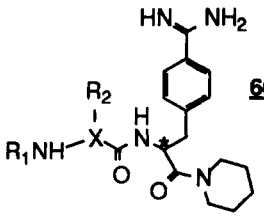
64 n = 0
65 n = 2

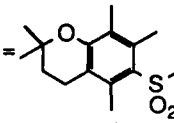


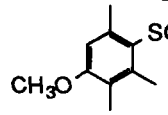
62 R = 2-Naphthyl-
63 R = 2,2,5,7,8-pentamethylchroman-

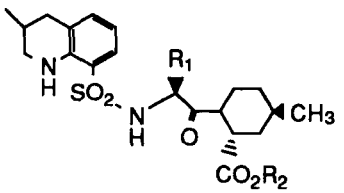


70 X = CH_3
71 X = H
72 X = OCH_3

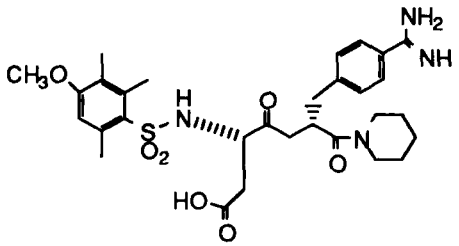


66 R_1 =  , R_2= H, X= N, * = (D)

67 R_1  , R_2 = CO_2H, X= (L)CH, * = d,l



68 R_1 = $CH_2ONC(=NH)NH_2$, R_2 = H
69 R_1 = $CH_2CH_2ONC(=NH)NH_2$, R_2 = C_2H_5



73 CRC 220

selectivity. Compound **56** is an interesting example of an inhibitor which retains good potency when an OH replaces the NH which forms the H-bond to the Gly 216 carbonyl (57). A small arginine mimetic **57** (Ki = 260 nM) was shown to have surprising potency even though it lacked the important H-bonds to Gly 216 and did not occupy the P4-P3 hydrophobic pocket (58). It could serve as a template for further design.

Inhibitors based on NAPAP or Argatroban motifs - β-Naphthylsulphonylglycyl-D,L-4-amidino-phenylalanylpiperidide (NAPAP) (Ki = 11 nM, trypsin 499x, fXa 63,400x, plasmin 39,000x), while a potent and relatively selective thrombin inhibitor, has a short plasma half-life and is not orally bioavailable (59,60). Both NAPAP and the related argatroban have been targeted by several groups as useful templates for the design of novel inhibitors with improved pharmacological profiles. Efforts to rigidify the P1 group of the NAPAP framework led to **58** (Ki = 270 nM), **59** (IC_{50}= 920 nM) and **60** (IC_{50} = 3.2 mM) (61). Although weak inhibitors **58** and **59** show good selectivity (>100 fold) against trypsin, plasmin, and factor Xa. Modifying the substitution pattern in

against trypsin, plasmin, and factor Xa. Modifying the substitution pattern in argatroban to improve its properties gave as an optimal compound **61** (Ki= 7 nM) (62), although oral biovailability and selectivity were not reported. Other variations leading to nonpeptidic inhibitors resulted in **62** (Ki = 2.8 nM, trypsin 68x, fXa 7857x) and **63** (Ki = 5.3 nM, trypsin 60x, fXa 6981x) (63). Rigidifying the P2 position of the NAPAP motif by formation of lactams gave potent inhibitors **64** (IC_{50} 11 nM) and **65** (IC_{50} 1.6 nM) (64). The mode of binding of these analogs was confirmed by x-ray crystallography of their complexes with thrombin (64). Modifications of the N-terminal groups have improved potency and selectivity, **66** (Ki = 2.4 nM, trypsin 2035x, fXa 31400x, plasmin 7500x) and **67** (Ki = 3.1 nM, trypsin 670x, fXa $1.7x10^5$x, plasmin 21000x) (60). In screening compound libraries, **70** BM14.1248 (Ki = 23 nM) was discovered (65). Meta-substitution of the aryl ring was shown to be important as was the size of the aryl substituent (**71** (Ki = 79 nM), **72** (Ki = 170 nM)). X-ray crystallography of **70** confirmed that the 4-aminopyridine is an arginine surrogate fitting into the S1 pocket although the compound showed an unusual binding motif with an unexpected main chain reconformation of two critical thrombin residues, Ser 214 and Trp 215 (65).

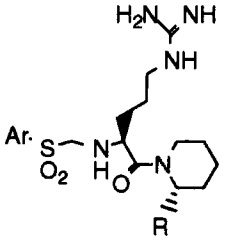
Caco-2 cell studies - Since for chronic therapy the most desirable mode of drug administration is the oral route, to date major disadvantages of most non-covalent inhibitors has been both their low oral bioavailability and short plasma half-life. It is known that small di- and tri-peptide-like molecules can be absorbed by passive diffusion as well as specific carrier-mediated transport systems(67). Caco-2 cells are known to express carrier mediated transport systems including those for dipeptides (78). As a model for intestinal absorption, permeability in these cells can be used as a rapid measure of potential oral biovailability of drug candidates. The permeability of several argatroban analogs with reduced basicity in the P1 functionality were studied in the model using Caco-2 cell monolayers(69). Reducing the basicity of the P1 group also substantially reduced potency as shown with **68** (IC_{50} 410nM, pKa ~7) and 66 (IC_{50} 1.3 mM, pKa~7). The inhibitor, **69**, the compound with the best permeability coefficient (~70 nM/sec) in the Caco-2 cell studies, did not show any oral activity at 50 mg/kg in mice. An in-depth study has been carried out on one reversible potent dipeptide inhibitor **73** (CRC220, Ki= 2.5 nM) in an effort to gain some understanding for its lack of systemic bioavailability in the face of proteolytic stability and proven enteral absorption (70). The results suggest that almost complete first pass elimination of the compound is due to an active carrier-mediated transport process in hepatocytes and this transport occurs via a bile acid transport system. The transport rate of **73** has been shown to increase, however, with different permeation enhancers in the Caco-2 cell culture medium with the conclusion that enhancement is possible, without cell damage, with sodium taurocholate (NaTC) and with NaTC mixed micelles containing oleic acid (71). The permeability of five structurally related thrombin inhibitors was evaluated in the Caco-2 cell cultures and compared to oral bioavailability in the rat (72). Although a quantitative correlation was poor, a similar rank order was found.

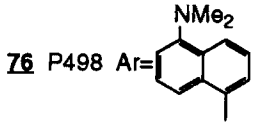
Bivalent Inhibitors - Hirudin (65 residue protein,Ki = 20 fM), and particularly the C-terminal anion exosite binding region (residues 45-65), has been used as a starting point for the design of bifunctional inhibitors (73). Hirudin binds to the active site of thrombin in a conformation opposite to that of substrates and does not occupy the S1 pocket. Interestingly, however, it is still characterized as a slow, tight binding inhibitor although the k_{on} approaches diffusion controlled kinetics under physiological conditions (74). Small synthetic versions of hirudin, Hirunorm IV **74** (Ki = 0.134 nM) and V **75** (Ki = 0.245 nM), bind to the active site in a similar manner to hirudin and are reversible, tight-binding inhibitors (75,76). They have been evaluated and found to be effective in a number of animal models of thrombosis (77). In an alternate strategy to link known effective active site motifs with an exosite-binding peptide, P498 **76** (Ki = 0.13 nM), P500 **77** (Ki = 20 pM) , and P552 **78** (Ki = 5 pM) were synthesized as bifunctional inhibitors. They incorporate known, non-mechanism based active site directed inhibitor

74 Hirunorm IV Chg-Arg-2-Nal-Thr-Asp-dAla-Gly-βAla-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr- Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-dGlu

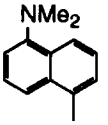
75 Hirunorm V Chg-Val-2-Nal-Thr-Asp-dAla-Gly-βAla-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr-Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-dGlu

Chg=cyclohexylglycine, 2-Nal= 2-=Naphthylalanine



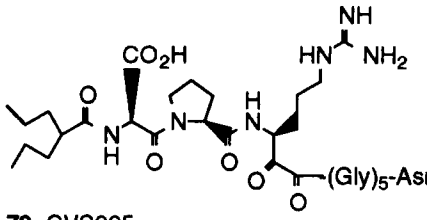


76 P498 Ar= ,R= βAla-Gly-Gly-(5-aminopentanoic acid)-hirudin$^{55-65}$



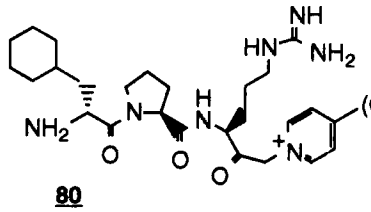
77 P500 Ar= , R= (12-aminododecanoic acid)-Gly-hirudin$^{55-65}$

78 P552 Ar= t-BuC6H5 , R= (12-aminododecanoic acid)-(g-aminobutyricacid)-hirudin$^{55-65}$



(Gly)$_5$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu

79 CVS995



(Gly)$_5$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala

80

motifs (DAPA-like) with novel linkers to the C-terminal hirudin sequence (79,80). High resolution crystal structures of complexes with thrombin have been solved. Transition state mimetics have also been incorporated at the P1-P1' site to yield compounds such as CVS995 **79** (2 pM), utilizing an α-ketoamide, and **80** (Ki = .045 pM) which incorporates a pyridinium methyl ketone (81,82). An analysis of analogs related to **79** has demonstrated that the interaction energies of the active-site component, the transition state, and the exosite regions of these inhibitors are not additive (81). The crystal structures of both **79** and **80** have been reported (81,82). Electrostatic contributions of the fibrinogen exosite binding of hirudin have been estimated by comparison of the calculated and experimental binding energies in a number of

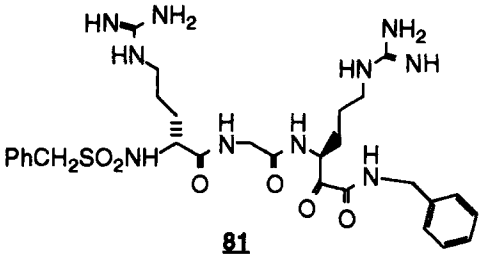
mutants (83). Contributions of the nonpolar interactions in this exosite binding was assessed by using five analogs of **78** by systematic replacement of Phe56, Ile 59, Pro60, Tyr63 and Leu64 in hirudin(55-65) with Gly (84).

Computational Approaches - Computer-aided design of thrombin inhibitors by combinatorial selection of fragments that may bind favorably to the known 3D structure of thrombin has been reported (85). This approach reproduces interaction patterns of known inhibitors and generates sets of closely related compounds. A 3D pharmacophore search of existing data bases has been used to identify lead compounds for further optimization (86). The suggestion is made that these hits could serve as novel templates for further optimization using medicinal chemistry or combinatorial chemistry approaches. The multiple copy simultaneous search (MCSS) method has been used to construct functionality maps for thrombin (87). Many of the features of known thrombin inhibitors are reproduced by this method and the results could be useful in the design of improved binding interactions as well as unused interaction sites. A mathematical model inclusive of the several paths in the coagulation cascade has been developed to predict thrombin generation under a variety of inhibitory conditions (88). Conclusions of the model were 1) that predicted thrombin generation times are relatively insensitive to changes in the Ki values until these values are < 1 nM; 2) most effective inhibitors are those that inhibit all three enzymes, fIIa, fXa, and fVIIa; and 3) inhibition of thrombin generation decreases as compounds that have high affinities for fVIIa and fIXa assume some thrombin inhibitory activity from 1000 to 5 nM. This latter situation is especially dramatic for compounds that also have high affinity for fXa.

FACTOR Xa INHIBITION

Factor Xa-Introduction - The pathway leading from the initiation of the coagulation response to thrombin proceeds through the serine protease factor Xa (fXa). This enzyme directly activates the circulating zymogen of thrombin, prothrombin, to the active enzyme. This occurs following the formation of a macromolecular catalytic complex (prothrombinase) composed of fXa and the non-enzymatic co-factor factor Va (fVa) and/or the fXa receptor, Effector Cell Protease Receptor-1 (EPR-1) (89,90). The assembly of prothrombinase and binding of the substrate prothrombin, requires an appropriate phospholipid surface which is typically provided by activated platelets or inflammatory cells adhered to the site of vascular damage (1). Attempts to target the formation of thrombin rather than directly inhibit its catalytic activity have relied on direct inhibitors of the prothrombinase complex via the inhibition of fXa. Both standard heparin and more specifically the family of LMWH, have been shown to effectively and in some cases selectively, inhibit uncomplexed fXa via antithrombin III (AT III). However, the contribution of this inhibition to the overall antithrombotic effect of these agents *in vivo*,
remains speculative since it has been shown that fXa is restricted from inhibition by AT-III when assembled in the prothrombinase complex (91,92). In contrast, several studies have compared selective direct and potent natural inhibitors of fXa in the prothrombinase complex with other anticoagulant strategies such a direct and indirect thrombin inhibition which have suggested a potential advantage of intervening at this point in the cascade particularly in situations such as arterial thrombosis where thrombin generation is prominent due to an accumulation of a significant prothrombotic platelet surface (93).

Factor Xa Inhibitors - The presence of an S4 pocket formed by the aromatic residues Trp215, Tyr99, and Phe174 has been proposed as the basis for a cation-π interaction site which is unique to Factor Xa and may form the basis for selectivity at least among



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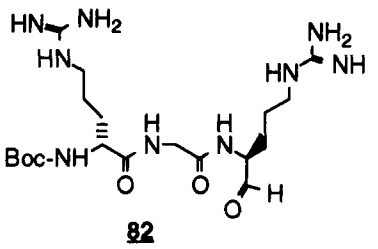

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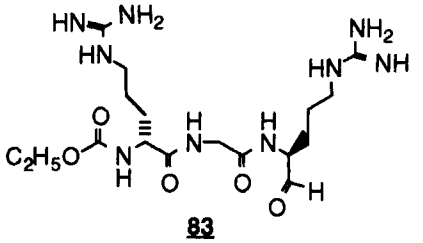

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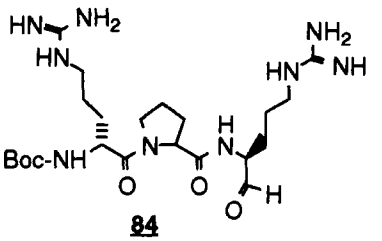

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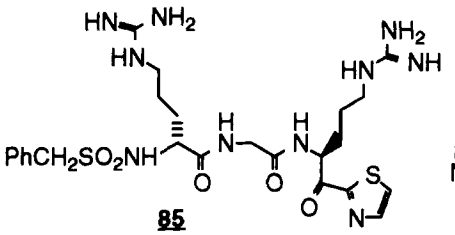

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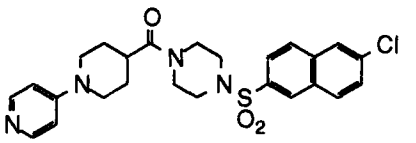

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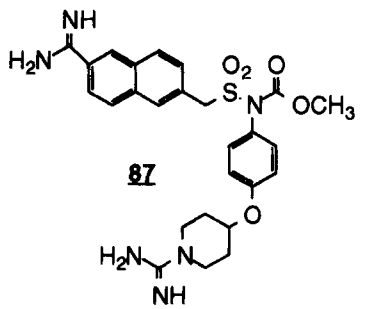

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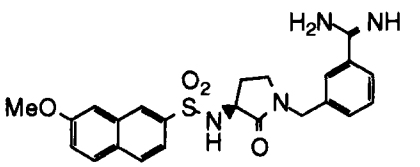

88

the coagulation factors (94). The corresponding residues in thrombin are Trp215, Leu99 and Ile174. The apparent preference for known fXa inhibitors to have cations in the P3,P4 position may be directly related to the ability of the fXa S4 site to stabilize these cations. Consistent with this hypothesis a series of α-ketoamides, e.g. **81** (IC_{50} = 3 nM, thrombin 330x) , have been shown to be potent and selective fXa inhibitors (95). This class shows comparable inhibition for both free fXa as well as fXa in the prothrombinase complex. A number of variations at the P4 postion as well as substitution in the aryl ring of the phenethylamide did not improve potency. Analogous compounds with the aldehyde transition state, e.g. **82** (IC_{50} = 50 nM, prothrombinase 4 μM, thrombin 3600x), were also good and selective inhibitors (96). The P3 position

appeared to require the D-configuration and preferred a guanidino group. Gly was optimal at P2 and the optimal P4's were the benzylsulfonamides. Interestingly, and in contrast to ketoamides, **82** inhibited fXa in the prothrombinase complex 80 fold less than free fXa. This differential activity does not appear to be due to the aldehyde functionality since alternate substitution at P4 (**83**)(IC_{50} = 30 nM, prothrombinase 135 nM, thrombin >3300x)) reduces this to <5 fold and replacing the P2 Gly with Pro, **84** (IC_{50} = 46 nM, prothrombinase 21 nM, thrombin 4x), results in essentially no difference (96). Utilizing the carbonylthiazoles as the transition state gave **85** (IC_{50} 0.65 nM, prothrombinase 0.45 nM, thrombin 15385x) (97). A number of non-transition state fXa inhibitors have been patented. These include **86** (IC_{50} = 3 nM, thrombin 11333x), **87** (conc to double coagulation times 40 nM), and **88** (Ki = 35 nM) (98-100).

The molecular details of how the potent and selective fXa inhibitor, tick anticoagulant peptide (TAP), binds to fXa continues to be an enigma. X-ray structures showing the binding of a two domain Kunitz-like inhibitor, ornithodorin, to the active site of thrombin have suggested a similar mode of binding for TAP to fXa (68). In this conformation TAP's Tyr 1 would point towards the hydrophobic pocket formed by fXa's Tyr 99, Phe174, and Trp 215 while Arg 3 would fit the hydrophobic pocket of fXa shaped by Phe 174 and reaching towards the electronegative pocket of carbonyls formed by Ile 175, Thr 98, Glu 97, and the acidic side chain of Glu 97. The Asn 2 would make H-bonds to both the enzyme and core structure. This is suggestive of similar 'parallel binding' seen for hirudin in thrombin.

Factor Xa-Implications for Drug Development From Natural Inhibitors - While there has been rapid progress in the design and synthesis of synthetic fXa inhibitors, the majority of the experimental pharmacological evidence that has validated this enzyme as a target for antithrombotic drug development has come from a variety of natural inhibitors derived from hematophagous or blood feeding organisms (93). In contrast to the majority of synthetic inhibitors, the potency and exquisite selectivity of these small protein inhibitors has allowed an unambiguous discrimination between the role of thrombin generation mediated by fXa in prothrombinase and pre-formed or existing thrombin trapped within the growing clot (92,93). Natural fXa inhibitors have also provided valuable insights into the most efficient way to inhibit this enzyme and how this may differ from the approaches taken with thrombin. The requirement for several macromolecular interactions between fXa, fVa and the phospholipid surface for maximal prothrombin activation, differs from that observed with thrombin in the proteolytic cleavage of fibrinogen to form fibrin. In addition, the 100,000-fold rate enhancement of prothrombin activation observed with prothrombinase versus uncomplexed fXa is not observed in the cleavage of synthetic peptidyl substrates or the inhibition by small molecule inhibitors (see the description of compound **82** above). This is thought to be due to the fact that small substrates and inhibitors are restricted to binding at the active site of fXa and therefore lack the extended macromolecular interactions expected for the natural substrate prothrombin (101). In contrast, the efficiency of fibrinogen and synthetic substrate hydrolysis by thrombin is comparable as is the inhibitory profile of active-site directed inhibitors for both substrates. Therefore, the strategies employed in the design of synthetic, active-site inhibitors of fXa in the prothrombinase complex must take into account the effects of the co-factor(s) on the macromolecular recognition of the natural substrate and how this will be reflected in the kinetic mechanism of inhibition which will be dependent on the type of substrate used. The interaction of natural inhibitor TAP with fXa has provided valuable insight into the functional identification of macromolecular recognition sites distant from the active site of fXa (exo-sites) influenced by fVa (102,103). These studies have been extended recently to directly demonstrate that the affinity of prothrombin for prothrombinase is principally governed through the interaction at fVa-induced exo-site(s) which precedes cleavage of the R^{323}-I^{324} bond within the active site of fXa leading to thrombin formation (104). Therefore, the expectation that small molecule, active site-directed inhibitors will effectively compete with the natural substrate prothrombin for fXa in prothrombinase may not be realized since the binding

of prothrombin to exo-sites on fXa may be the principal contributing factor leading to the maximal catalytic efficiency of prothrombinase and not the interactions of the primary recognition residues of the substrate within the active site of the enzyme. This hypothesis is supported by observation that several small molecule, active site-directed inhibitors of fXa in the prothrombinase complex that have been shown to be competitive with small synthetic substrates are classical non-competitive inhibitors of prothrombin activation (104). Thus, there may be limitations in the overall effectiveness of active site-directed fXa inhibitors as anticoagulants *in vivo* due to the lack of competitive competition with the natural substrate prothrombin. This implies that strategies aimed at developing inhibitors that can directly compete with prothrombin for binding to fXa at exo-sites with and without active site occupation, may be a more amenable to achieving the type of potent antithrombotic effects observed with many of the natural fXa inhibitors like TAP (9,93).

FACTOR VIIA INHIBITION

Factor VIIa and Factor IXa-Introduction - Initiation of the blood coagulation response to vascular injury follows the formation of a catalytic complex composed of serine protease factor VIIa (fVIIa) and the non-enzymatic co-factor, tissue factor (TF) (1). This response appears to be exclusively regulated by the exposure of subendothelial TF to trace circulating levels of fVIIa and its zymogen factor VII (fVII), following a focal breakdown in vascular integrity or the recruitment of activated inflammatory cells such as monocytes that have surface exposed TF. It is believed that the small amount of fXa that is initially formed catalyzes further activation of fVII and primes the coagulation response through the proteolytic modification of factor IX to factor IX_{α} which in turn is converted to the active serine protease factor IXa_{β} by the factor VIIa/TF complex (105). This leads to the propagation of the coagulation response through increased fXa (prothrombinase) generation following the assembly of $fIXa_{\beta}$ and the non-enzymatic co-factor factor VIIIa on an appropriate phospholipid surface (106).

Factor VIIa Inhibitors- In contrast to thrombin and fXa, there has been limited work on the synthesis of potent and selective inhibitors of fVIIa/TF. In addition, the requirements for such an inhibitor to be an effective *in vivo* antithrombotic agent may be difficult to achieve due to the fact that picomolar quantities of this enzymatic complex can initiate the highly amplified series of reactions resulting in the stoichiometric conversion of prothrombin to thrombin in seconds (107). Therefore, definition of the potency and selectivity requirements for an effective fVIIa/TF inhibitor are being addressed using a variety natural and genetically engineered inhibitors. These include tissue factor pathway inhibitor (TFPI)(108) and nematode anticoagulant peptide c2 (NAPc2)(109) both of which bind fXa prior to the formation of a quaternary inhibitory complex with the fVIIa/TF complex as well as small protein direct inhibitors (110) and inactivated forms of fVIIa (111,112). Additionally, there have been several approaches aimed at targeting the macromolecular interaction of fVIIa with TF. These include the use synthetic peptides and soluble forms of mutant TF which retains binding affinity for fVIIa but reduced co-factor activity (113, 114). A mutant TF with reduced co-factor activity was also used to target a small protein fVIIa inhibitor to enhance the specificity of the chimeric protein (115). The pre-clinical pharmacological evaluation of several of these inhibitors has led to the conclusion that the fVIIa/TF may be an attractive target for the development of inhibitors that have a unique therapeutic profile based on the dissociation between surgical bleeding and prevention of intravascular thrombosis (116). However, it is also clear from these studies that synthetic inhibitors of fVIIa/TF may be a very challenging objective due to the strict requirements for effective intervention at this point in the coagulation cascade.

Conclusions - Anticoagulants, particularly bifunctional inhibitors of thrombin and more recently small, naturally occurring protein inhibitors of factor VIIa and Xa,

continue to be investigated as possible acute agents. In recent years, however, substantially more effort has been directed at the discovery of synthetic small molecule inhibitors of thrombin and factor Xa that are potent, selective, and orally bioavailable. Two general structural classes have been investigated, the non-covalent inhibitors as exemplified by NAPAP and argatroban, and the mechanism-based inhibitors represented by the tripeptide argininals, boronic acids, and ketoheterocycles. While the design and synthesis of optimized inhibitors in each class has resulted in potent (subnanomolar) direct inhibitors of thrombin with selectivity against both the fibrinolytic enzymes as well as trypsin, there remain unresolved issues with each class. Despite considerable effort by several groups, examples in the non-covalent class that are orally bioavailable with appropriate plasma half-lives are rare. Additionally, their fast kinetics coupled with short plasma half lives can substantially compromise the duration of their antithrombotic effects. On the other hand, a major question regarding the intrinsic likelihood of mechanism-based inhibitors to show significant selectivity has been answered with examples of argininals which, upon appropriate substitution, can show orders of magnitude selectivity, including against trypsin. Possibly a more problematic issue with this class, however, is the nature of their kinetics which is often characterized as slow and tight binding. The concern here is that this general class of inhibitors will not effectively suppress the high levels of local thrombin generated at the site of a vascular lesion particularly in the presence of significant platelet recruitment as seen in small vessel arterial thrombosis. Recent work on the mechanism-based inhibitors, however, has shown that time-dependent kinetics are not necessarily associated with the transition state functionality but result instead from an incompletely understood combination of structural features. There has also been substantially greater success, to date, in generating orally active inhibitors in the mechanism-based class. The final word, however, with regard to the optimal combination of properties in either class that will lead to useful therapeutics will have to await more detailed biological evaluation of these compounds and ultimately clinical trials.

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Chapter 9. Leukotriene Modulators as Therapeutic Agents in Asthma and Other Inflammatory Diseases

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Introduction - The search for new therapeutic agents for the treatment of asthma has been an arduous and predominantly unsuccessful endeavor. Asthma incidence is increasing and standard therapies show significant flaws (1-3). In the last 3-4 years however a new class of agents, the leukotriene modulators, has shown promise in treating the disease. A number of agents have reached the clinic and have been examined in challenge protocols in asthmatics. Four agents have progressed to large outpatient studies and thus far continue to show promise. Clearly however there is a need for more precise definition of the utility of these new agents in asthma and other inflammatory diseases and possibly for newer more potent entities. The exploration of new target enzymes and receptors offers potential new uses and perhaps greater selectivity. This class of agents was last reviewed in this forum several years ago, however a number of reviews on the clinical utility of the agents have recently appeared (1, 4-8).

THE LEUKOTRIENE PATHWAY AS A THERAPEUTIC TARGET FOR ASTHMA

Asthma is characterized by reversible airway obstruction, airway hyperreactivity and airway inflammation (1). Events leading to airway obstruction include edema of airway walls, infiltration of inflammatory cells into the lung, production of various inflammatory mediators and increased mucous production. There is a large body of literature indicating that leukotrienes could be involved in many of the components of the disease (9-11). Although leukotriene involvement in airway hyperreactivity is controversial these mediators are clearly capable of causing bronchial smooth muscle contraction and inflammatory cell influx into pulmonary tissues (12). Since classical therapy effects either smooth muscle contraction (β agonists) or airway inflammation (steroids), agents which modulate leukotriene action or synthesis should be unique and distinct additions to the treatment of the disease because they effect both major aspects of the disease with mechanisms predominantly different from either bronchodilators or steroids.

The two classes of leukotrienes are derived from the sequential action of 5-lipoxygenase (5-LO) on arachidonic acid followed by the action of either LTA_4 hydrolase to produce LTB_4 or LTC_4 synthase to produce the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. There is apparently only one type of LTB_4 receptor designated BLT_1 and two types of cysteinyl leukotriene receptors designated Cys-LT_1 and Cys-LT_2. Thus there are three biosynthetic enzymes and three receptor types which could be targets for drug design. In addition, a protein designated as FLAP for 5-LO activating protein has been shown to be required for leukotriene formation (13). There has been either preclinical activity and/or clinical activity in searching for agents affecting all seven potential targets.

Mechanistic Studies with Leukotriene Modulators - 5-LO is the first committed enzyme in the biosynthetic pathway leading to leukotrienes. Thus complete inhibition of the enzyme results in eliminating both LTB_4 and the cysteinyl leukotrienes. Appropriately potent and effective inhibitors should have effects on both smooth muscle contraction (cysteinyl leukotrienes) and inflammatory cell influx (LTB_4). Effects on antigen-induced pulmonary smooth muscle contraction have been shown both *in vitro* and *in vivo* in animal systems, however selective agents are not general bronchodilators (14, 15). These agents are thus broad in scope. Concerns about the toxicology of inhibiting the entire pathway have recently been examined by engineering mice with the 5-LO gene

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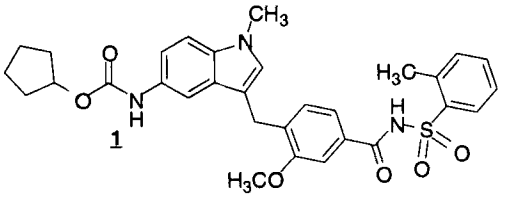
deleted. These mice appear to be grossly normal (16, 17). They do have decreased inflammatory responses to some but not all stimuli compared to littermate controls (16, 17). Recently a decreased ability to fight parasites was reported (18).

Antagonists of FLAP, like 5-LO inhibitors, would be expected to inhibit both branches of the leukotriene pathway. Two possible differences between the two types of inhibitors have been observed. First, in systems where there is a high arachidonic acid concentration, leukotriene effects tend to be more weakly inhibited by the FLAP class of inhibitor than by direct 5-LO inhibitors (19). However this phenomena has not been observed thus far in animal models where physiological stimuli cause leukotriene production. Secondly, the FLAP protein has been shown to be structurally related to LTC_4 synthase. However to date although a FLAP inhibitor has been shown to inhibit LTC_4 synthase, (20) no detailed study of the relative potency of a class of FLAP inhibitors against the two activities has been published. Finally as with the 5-LO, a strain of mice has been engineered with the FLAP gene deleted and has been shown to be phenotypically normal with modestly diminished responses to some inflammatory stimuli (21).

The remaining targets for leukotriene modulators are selective for either LTB_4 or the cysteinyl leukotrienes. *BLT_1* antagonists and LTA_4 hydrolase inhibitors have been identified (see below). These agents are envisioned to be effective in blocking the anti-inflammatory component of leukotriene formation. Some effects in asthma on neutrophil and eosinophil influx are also possible given the anti-inflammatory effects of 5-LO inhibitors. In addition a few of these agents have been tested in other anti-inflammatory diseases, and more clinical research is in progress. Cys-LT_1 receptors appear to be the primary receptor for the cysteinyl leukotrienes in human airways. Cys-LT_2 receptors have only been described in selected tissues thus far (22). Until recently, cysteinyl leukotrienes were thought to be associated exclusively with the bronchoconstrictive aspects of airway disease with perhaps some effect on mucous production. However, recent reports indicate that LTD_4 and LTE_4 may be chemotactic for eosinophils *in vitro* and possibly *in vivo* (23, 24).

Leukotriene Modulators Undergoing Clinical Evaluation - The first leukotriene modulators tested in the clinic for asthma were Cys-LT_1 antagonists (25). Thus far a number of these agents have been tested in phase II challenge studies in asthmatics and three have progressed to outpatient trials for varying lengths of time. ZD-204219, zafirlukast **1**; MK-0476, montelukast **2**; and Ro 24-5913 cinalukast **3**, were effective against exercise-induced asthma in small studies (26-28). Zafirlukast, and montelukast were also effective in antigen-induced bronchospasm (29,30) Zafirlukast (as an aerosol) and ONO-1078, pranlukast **4**, also gave some protection in cold-air challenge studies (31, 32). In addition, MK-679 **5**, (now discontinued) was effective against aspirin-induced bronchoconstriction (33).



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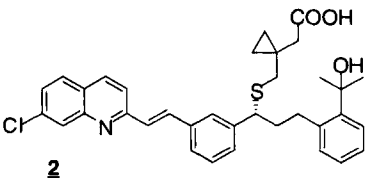

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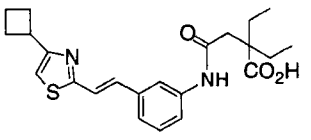

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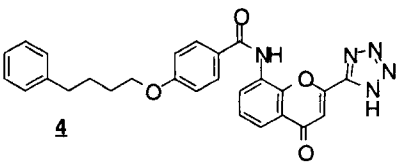

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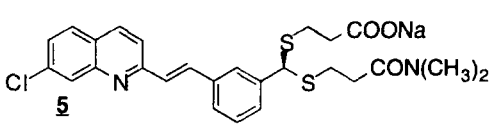

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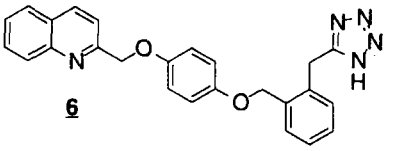

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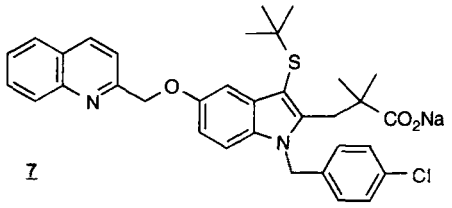
Four Cys-LT$_1$ antagonists have progressed to Phase 3 clinical trials in outpatients. RG 12525 **6**, gave modest improvement in FEV1 (forced expiratory volume in one minute, an objective measure of lung function) in a small study (34). Zafirlukast (20 mg bid) was shown to statistically increase FEV1 in one 3 month trial but not in another (35, 36). However in both studies, symptoms and peak flow measurements were positively effected. Pranlukast has been studies in larger trials and showed positive effects on FEV1 at a 450 mg BID dose (37). However, the effect tended to diminish at the end of the study. Finally a daily dose of 10 mg of montelukast was recently reported to increase FEV1 in an outpatient trial (38).



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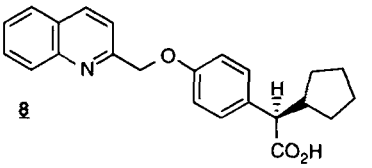

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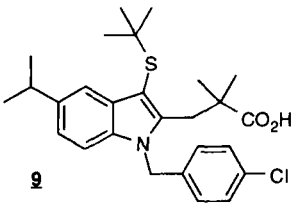

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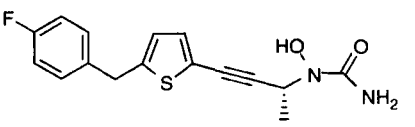

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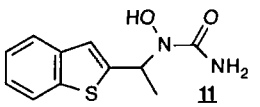

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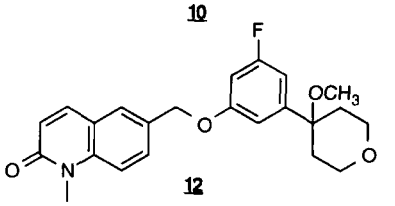


12

FLAP antagonists and 5-LO inhibitors have also been tested in asthma challenge studies. The FLAP antagonists MK-0591, **7** , Bay X 1005 **8**, and MK-0886 **9** and the 5-LO inhibitor ABT-761 **10**, were effective against antigen-induced bronchospasm in asthmatics (39-42). A-64077, zileuton **11** and ZD-2138 **12** were not effective against this type of challenge (43, 44), but were both effective against aspirin-induced bronchospasm (41, 45). Zileuton and **9** were also shown to be effective against cold air-induced asthma and zileuton partially inhibited bronchospasm caused by exercise challenges (46). Three of these agents have been examined in Phase 3 outpatient trials, **7** and **8** in small dose-ranging studies (47-49), and zileuton in a dose ranging and in two large pivotal studies (50-52). In these studies, zileuton (600 mg qid) was effective in causing FEV1 improvements as well as positive changes in peak flow measurements and symptom scores. Perhaps more significantly the compound also decreased the number of acute asthma episodes.

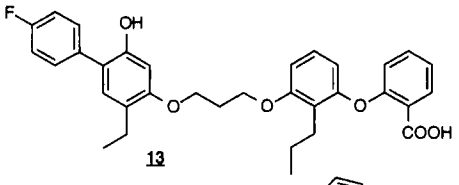
The data with the 5-LO inhibitor zileuton and the Cys-LT$_1$ antagonists zafirlukast (one of two studies), pranlukast and montelukast (preliminary data) indicate improvements in pulmonary function such as FEV1 and peak flow as well as in symptom scores. In these parameters, although quantitative differences in these

parameters are seen between compounds, no dramatic qualitative differences have been observed between the four agents on these three measurements. To date no comparative studies have been performed to definitively rank the efficacy of the four agents.

Two studies have been reported which may differentiate 5-LO inhibitors from Cys-LT_1 antagonists. In nocturnal asthma zileuton caused inhibition of airway hyperresponsiveness (53, 54). In addition, zileuton was shown to decrease eosinophil influx in a segmental antigen-challenge study indicating a clear anti-inflammatory effect in these patients (55). Thus far, similar studies have not been reported for Cys-LT_1 antagonists.

One unanticipated observation with both biosynthesis inhibitors and antagonists was the bronchodilatory effect seen after the first dose. This response was seen in moderate to severe asthmatics but not in normals (29, 56, 57). These data have been interpreted to mean that continual leukotriene production is responsible for some of the loss in lung function in moderate to severe asthmatics (58).

A single clinical study has been reported with the BLT antagonist LY293111 **13** (59). In that study, the compound failed to inhibit antigen-induced bronchospasm but significantly reduced the number of neutrophils in lung lavage fluids following challenge. These data are consistent with effects on the chemotactic effects of LTB_4 and thus the compound could be anti-inflammatory in the disease.



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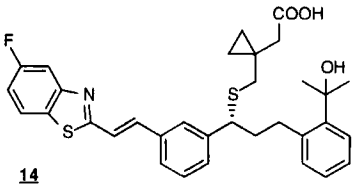

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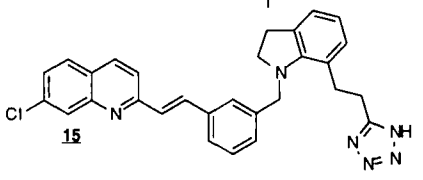

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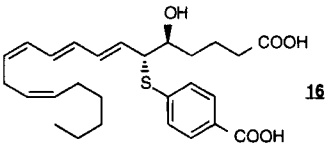

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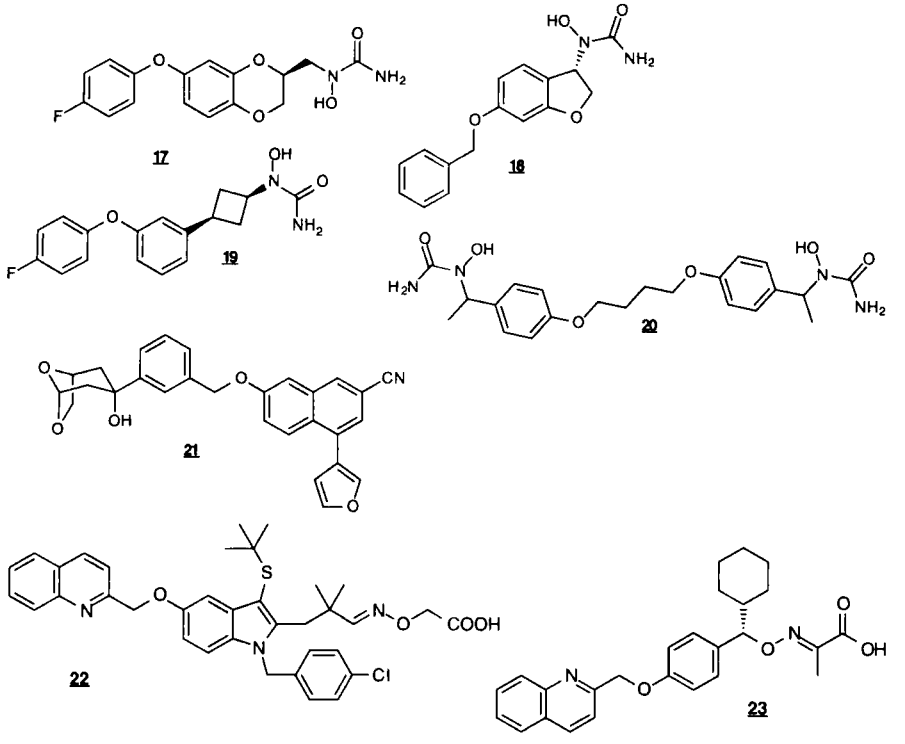
NEW LEUKOTRIENE MODULATORS

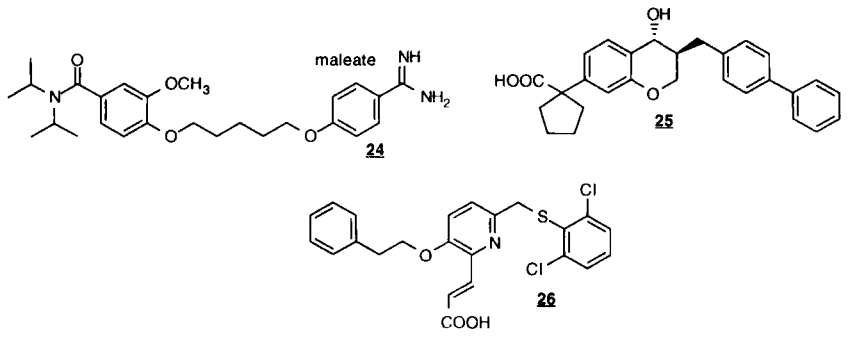
Cys-$LT_{1/2}$ Antagonists - Three new antagonists described recently are montelukast, the fluoro-quinoline OT 4003,**14**, and the quinolone substituted dihydroindole LY 302905 **15** (60-62). All three compounds are potent orally active antagonists of the Cys-LT_1 type. In addition to these compounds, a novel antagonist with Cys-LT_2 activity has also been described. This compound, Bay u9773 **16** was shown to be selective for Cys-LT_2 receptors in guinea pig ileum preparations which contain both types of receptors (63).

Biosynthetic Inhibitors - Several structural classes of new leukotriene biosynthesis inhibitors have been described. A number of reports described new N-hydroxyurea containing 5-LO inhibitors. These include CGS 25997 **17**), SB 202235 **18** orally active in guinea pig bronchospasm and pulmonary inflammation; BW 862c **19** an achiral inhibitor; **20**, a bishydroxamic acid and ABT-761 **10**, a propynyl containing compound which has progressed to phase II challenge models in asthmatics (42, 64-69). In addition, L-708780 **21**, a potent 5-LO inhibitor structurally related to **12**, was described. Close congeners of L-708780 were subsequently found to be toxic (70).

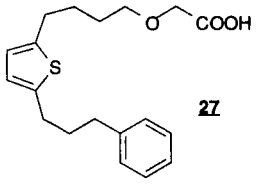
The new FLAP antagonist Bay X1005 **9** was reported to be a FLAP antagonist with good *in vivo* activity (48, 71). Two other leukotriene biosynthesis inhibitors have also been reported (A-81834 **22**, A-93178 **23**) which structurally resemble **8** and **9** respectively but which have not yet been shown to be FLAP antagonists in binding assays (72, 73).



BLT Antagonists - Several series of BLT antagonists have been described. Compounds with *in vivo* activity include CGS 25019c **24**, an aryl amidine antagonist with activity in an arachidonic acid ear edema model (74); CP-105696 **25** which was active in collagen-induced arthritis models in mice; SB 209247 **26** orally active in mouse models of inflammation; (75) **13** active in LTB_4 challenged cynomolgus monkeys (76).



LTA$_4$ Hydrolase Inhibitors - LTA$_4$ hydrolase although purified in 1985 has only recently become a target for inhibitor development (77). Relatively few inhibitors have been described thus far. RP 64966 **27** was identified as the best inhibitor in a series of ω-arylalkyl aryl alkanoic acid inhibitors. **28** is a potent inhibitor and the mercapto amine **29** (78, 79). The only inhibitor active in vivo thus far is SC-57461 **30**, which is active orally in rodents (21). Unfortunately it also has a long-lived metabolite with potential toxicity (80).



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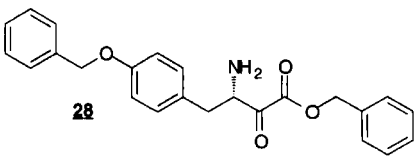

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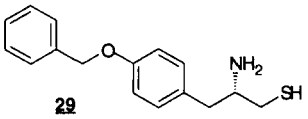

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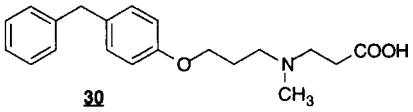

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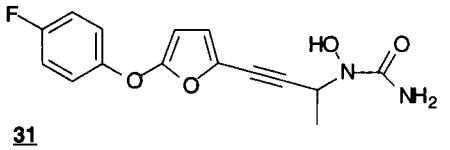
LTC$_4$ Synthase Inhibitors - In 1994 two laboratories cloned and expressed LTC$_4$ synthase (81, 82). The protein is a 17 kd protein with no sequence homology to GSH S transferases, but unexpected homology to FLAP. It is possible that both FLAP and LTC$_4$ synthase act as arachidonic acid binding proteins. No detailed inhibition studies have been published, but **9** does have some inhibitory effect on LTC$_4$ synthase (81).

OTHER THERAPEUTIC APPLICATIONS FOR LEUKOTRIENE MODULATORS

Although asthma has been the primary focus of clinical research with leukotriene modulators to date, a number of other diseases have been examined. Thus far more clinical data for rhinitis treatment is available than for other possible indications. Rhinitis has been of particular interest because cysteinyl leukotrienes have been shown to cause congestion when applied to nasal passages (83). Several leukotriene modulators have shown some efficacy in alleviating rhinitic symptoms. In the earliest study, the 5-LO inhibitor zileuton reduced nasal congestion after antigen challenge (84). In another study, the compound also attenuated symptoms in aspirin challenged asthmatics with rhinitic symptoms (85). A-78773 **31**, an N-hydroxyurea 5-LO inhibitor was also effective in increasing nasal airflow and inhibiting rhinorhea but not itch or sneeze in rhinitis patients (86). Cys-LT$_1$ antagonists have also been effective in phase II studies in rhinitis. The Cys-LT$_1$ antagonist zafirlukast relieved ragweed induced symptoms in rhinitis patients as did pranlukast (87, 88).

There has also been considerable clinical activity in treating ulcerative colitis. Early studies indicated greatly elevated levels of LTB$_4$ in colonic tissue of these patients (89). The 5-LO inhibitors zileuton and FPL 6410XX **32** both decreased LTB$_4$ production in colitis patients (90,91). Zileuton also showed some efficacy in the treatment of the disease but MK-591 did not (92-94). High LTB$_4$ levels are also seen in psoriatic lesions (95). A number of agents have been examined in the disease, but several 5-LO and FLAP inhibitors have had little effect thus far. In a single study zileuton (600 mg qid) was effective in alleviating symptoms in an 8 week study in lupus patients (96). Clearly in SLE as well as in the other conditions listed, the number of studies as well as the number and type of agents needs to be expanded to more clearly define the role of leukotriene modulators in treatment of these diseases.

Conclusions - Leukotriene modulation is clearly effective in asthma as evidenced by the recent approval of pranlukast (Ultair™, Japan), zafirlukast (Accolate™,US) and zileuton (Zyflo™ US) for this indication. It is unclear yet whether Cys-LT$_1$ antagonists or biosynthesis inhibitors will prove to be superior therapy or whether new targets such as LTC_4 synthase will prove more effective and selective. In anti-inflammatory diseases other than asthma the clinical experience is still very preliminary. It is possible that new studies and/or new agents will expand the utility of leukotriene modulators beyond the treatment of asthma.



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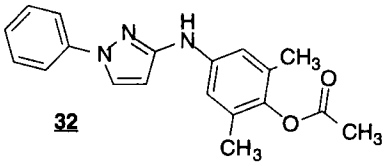

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Chapter 10. Emerging Opportunities in the Treatment of Atherosclerosis

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Introduction - Cardiovascular disease is the leading cause of death in industrialized nations. Atherosclerosis, characterized by the deposition of lipids and fibrotic material in the arterial wall, is recognized as the leading contributor to cardiovascular disease, accounting for over half of all cardiovascular deaths (500,000 in the US alone in 1994). The total healthcare cost for atherosclerotic coronary heart disease (CHD) in 1997 is projected at $91 billion (1).

High levels of low density lipoprotein cholesterol (LDL-C) is a well established risk factor for CHD. Clinical trials with HMG-CoA reductase inhibitors (statins), the most commonly prescribed LDL-C lowering therapy in the US, have demonstrated a decrease in cardiovascular morbidity and mortality in patients with (2) and without (3) preexisting CHD. It is generally accepted that low levels of high density lipoprotein cholesterol (HDL-C) and high levels of triglycerides are also important risk factors, resulting in a growing emphasis to treat low HDL-C and high triglycerides (4). In addition to conventional lipid therapies, modification of the underlying mechanisms of plaque formation and stabilization within the arterial wall presents an alternative potential therapy for the treatment of atherosclerosis. The following article will highlight some of the recent developments in LDL-C lowering and HDL-C elevation approaches, as well as approaches targeting the atherosclerotic plaque, focusing primarily on programs in preclinical or early clinical development.

LDL-C LOWERING APPROACHES

The statins (lovastatin, pravastatin, simvastatin, fluvastatin) have proven to be safe and efficacious agents. However, some patients taking the statins do not achieve cholesterol levels recommended by the National Cholesterol Education Program (NCEP) guidelines (4). This fact, coupled with the recent data demonstrating a positive correlation between LDL-C lowering and mortality, has resulted in significant effort being extended in other LDL-C lowering approaches in search of a more effective therapy. Standards by which potential new LDL-C lowering agents will be judged are likely to have increased with the entry into the marketplace of the newest statin, atorvastatin, which shows an unprecedented 50-60% LDL-C lowering in addition to significant reductions (up to 45%) in triglycerides (5). Ongoing clinical trials with higher doses of simvastatin have shown comparable efficacy with a corresponding increase in adverse effects (6). The following section will highlight some of the advancements in LDL lowering approaches. A more detailed description can be found in several recent reviews (7-9).

Cholesterol Biosynthesis Inhibitors - The statins inhibit the rate-limiting step in the cholesterol biosynthesis pathway. Recent effort has focused attention on other steps in the pathway with an emphasis on the three enzymes involved solely in sterol synthesis - squalene synthetase, squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase - as they do not interfere with the formation of other isoprenoids (i.e., dolichol and ubiquinone) required for cell growth. Extensive reviews on inhibitors of these enzymes have been published (10, 11). While many cholesterol biosynthesis inhibitors appear more efficacious than the statins in animal models, no clinical data on these inhibitors has been reported, and so it remains to be seen if they offer distinct advantages over the statins.

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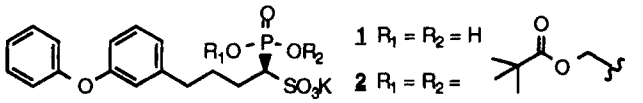
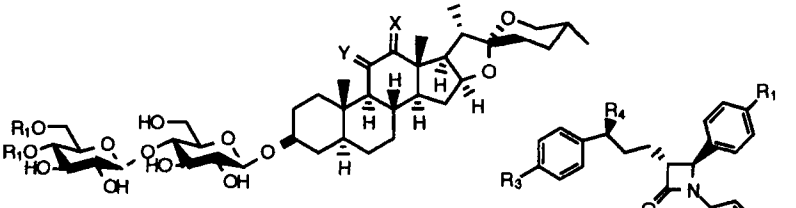


The majority of efforts have focused on inhibition of squalene synthetase, the first committed step to cholesterol. The substrate for the enzyme, farnesyl pyrophosphate, is water soluble and easily metabolized, thereby potentially avoiding harmful effects due to accumulation of the substrate during inhibition. A number of substrate mimetics have been described including the α-phosphonosulfonic acid 1. A potent inhibitor of squalene synthetase *in vitro*, 1 lacked oral efficacy *in vivo* due to poor absorption. BMS-188,494 (2), a prodrug of 1, exhibited a tenfold increase in oral potency (12). Of particular note is the ability of 2 to lower cholesterol in the rat after oral administration, a model insensitive to lipid lowering with the statins (13). In addition to the substrate mimetics, many carboxylate-based inhibitors exist including the fermentation products known as both the zaragozic acids and the squalestatins. While 2 showed lipid lowering in the rat, the zaragozic acids were reported to cause a lethal toxicity, speculated to be mechanism-based, with minimal cholesterol lowering in both rats and dogs at high doses. These picomolar inhibitors did lower plasma cholesterol in primates without any toxic effects (14,15).

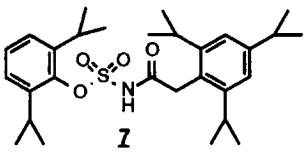
Cholesterol Absorption Inhibitors - In addition to inhibiting cholesterol biosynthesis, reducing dietary cholesterol intake by inhibiting absorption at the intestinal wall exists as an alternative method of reducing LDL-C. Several agents shown to inhibit cholesterol absorption and to reduce LDL-C in animal models through an unknown mechanism of action have recently been reported to possess modest clinical efficacy. The synthetic plant saponin derivative pamaqueside (CP-148,623; 3) (16) inhibited cholesterol absorption by 35-40% in normolipidemic individuals with a resulting 10-12% decrease in LDL-C at 300 mg BID (17). SAR studies indicated that modifications at the 4" and 6" positions of the sugar moiety of 3 resulted in analogs, exemplified by CP-242,184 (4, ED_{50} = 0.07 mg/kg), 50-100 times more potent in the cholesterol-fed hamster model (18).



Azetidinone SCH 48461 (5) was also reported to reduce plasma cholesterol in man (19). Originally designed as acyl-coenzymeA:cholesterol O-acyltransferase (ACAT) inhibitors, *in vivo* activities of analogs from this series showed no correlation to ACAT inhibition and appear to be acting at the intestinal wall through an unknown mechanism (20). In an effort to identify a more potent compound, the metabolic lability of 5 was examined (21, 22). Based on these findings and SAR studies, SCH 58235 (6) was identified and showed 50-fold greater potency than 5 in the cholesterol-fed hamster model, greater metabolic stability and an improved pharmacokinetic profile (23).

Acyl-CoenzymeA:Cholesterol O-Acyltransferase (ACAT) Inhibitors - ACAT is the key enzyme responsible for intracellular cholesterol esterification. Initial ACAT inhibitors were structurally diverse but generally lipophilic amides, ureas and imidazoles that were nonsystemic agents targeting inhibition of cholesterol absorption in the intestine. More recently agents of this class have also targeted ACAT in the liver and at the arterial wall through increased water solubility with a subsequent increase in absorption and bioavailability. Several inhibitors have been reported to reduce LDL-C in animal models without affecting cholesterol absorption. Evidence exists supporting the involvement of hepatic ACAT in the secretion of cholesterol ester containing lipoproteins (24). In addition, inhibitors without lipid lowering properties have been shown to retard the progression of atherosclerosis in hypercholesterolemic animal models by reducing the amount of cholesterol ester in macrophages, supporting the role of ACAT at the arterial wall. Thus, ACAT inhibitors have the potential to function as antiatherogenic as well as hypocholesterolemic agents (25, 26).

Despite the considerable effort extended in this area, these agents have offered limited success clinically. Adrenal toxicity, believed to be mechanism-based, has plagued many agents of this class. CI-1011 (7) has distinguished itself from all other agents by demonstrating cholesterol lowering in non-cholesterol fed animal models, a finding that may hold significance clinically as no other ACAT inhibitor has shown efficacy in this model (27).



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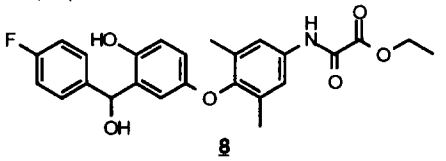
Bile Acid Sequestrants - Bile acid sequestrants are high molecular weight cationic ion exchange resins which bind the anionic bile acids in the intestine, thereby preventing their reabsorption to the liver. To accommodate this loss in bile acid recirculation, the LDL receptor (LDL-R) is upregulated, resulting in the removal of LDL-C from the plasma which is utilized in synthesizing new bile acids. The bile acid sequestrants on the market display modest efficacy (10-20% LDL-C lowering) but suffer from poor palatability (28). Efforts have focused on improving the palatability of these agents. Attempts to decrease the amount of resin administered by increasing the binding capacity and affinity for bile acids have led to several agents, DMP 504 (29), GT31-104 (30) and cholebine (MCI-196) (31), which have been reported to be more potent than cholestyramine in animal models.

Thyromimetics - The thyroid receptor is a nuclear protein located in multiple tissues in varying amounts (32). Thyroid hormone (L-T_3) enhances hepatic LDL-R gene transcription resulting in a concomitant decrease in LDL-C (33). Unfortunately, the lipid lowering action of L-T_3 is also associated with unfavorable cardiovascular events. It is unknown whether these events are a direct consequence of thyroid hormone on the heart or are secondary effects due to thermogenesis (34). CGS 26214 (8) has been reported to display lipid lowering properties without significant cardiovascular or thermogenic effects when compared to L-T_3. In hyperlipidemic rats and normolipidemic dogs, 8 lowered LDL-C by 35% and 59%, respectively, at 1 μg/kg. While displaying picomolar affinity for both the rat liver (IC_{50} = 0.1 nM) and cardiac (IC_{50} = 0.2 nM) thyroid receptors, 8's favorable *in vivo* profile was attributed to its inability to penetrate the nucleus of myocytes and not to its affinity for the different thyroid receptor isoforms (35).



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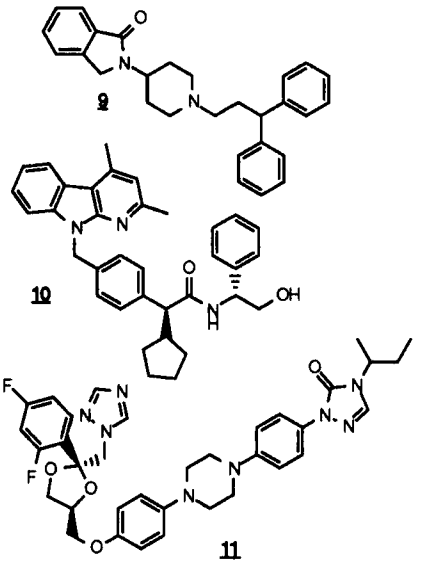
Very Low Density Lipoprotein (VLDL) Assembly/Secretion - Microsomal triglyceride transfer protein (MTP) is an intracellular lipid transfer protein that catalyzes the transport of triglycerides, cholesteryl ester and phospholipid. A deficiency of MTP has been shown to be the proximal defect in abetalipoproteinemia, a disease characterized by low levels of plasma cholesterol and triglycerides due to a defect in the assembly and secretion of apolipoprotein B (apo-B) containing lipoproteins such as chylomicrons and VLDL, the precursor to LDL (36). This finding has suggested MTP inhibition as a novel mechanism for lowering plasma lipids. Studies with the MTP inhibitor BMS-200150 (**9**) support the role of MTP-mediated transport of lipid in the assembly of apo-B containing particles (37). BMS-197636 (structure not disclosed, IC_{50} = 12 nM), a 50-fold more potent analog of **9**, has been reported to inhibit VLDL secretion in hypertriglyceridemic rats (ED_{50} = 2.9 mg/kg p.o.) and to lower plasma cholesterol and triglycerides in the hamster (38).



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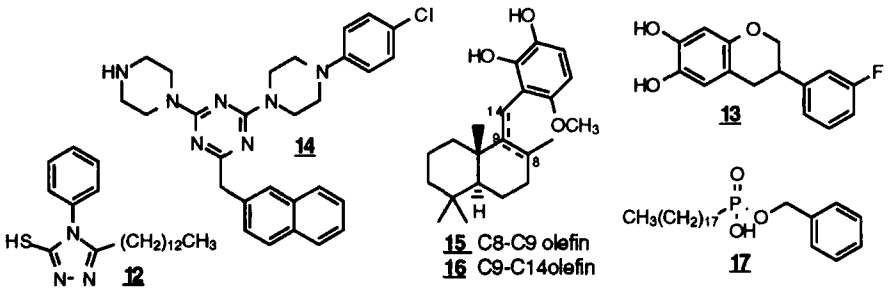
The cycloalkanoindol derivative **10** has also been reported to inhibit VLDL secretion *in vitro* (IC_{50} = 1.1 nM) as well as in the hamster (ED_{50} = 3-6 mg/kg p.o.) but through an undisclosed mechanism of action. In the rat, **10** was also shown to inhibit triglyceride absorption (39). A series of triazolones, exemplified by **11**, were reported to inhibit the synthesis of apo-B, the structural protein of VLDL and LDL, in HepG2 cells (IC_{50} = 170 nM) (40).

AGENTS TARGETING HDL-C

The mechanism by which HDL provides protection against CHD is unknown. HDL may stimulate the movement of cholesterol from peripheral tissues, such as the vessel wall, to the liver for excretion, a process referred to as reverse cholesterol transport (41). HDL may also act directly on the vessel wall, possibly reducing oxidation and aggregation of LDL or preventing cytokine-mediated injury (42). Understanding the role of HDL in atherogenesis is complicated by the heterogeneity of HDL which is composed of subpopulations of particles varying widely in size, lipid and apoprotein composition. The subfraction(s) responsible for protection against CHD is controversial. Several factors, including synthesis of apo-AI (the major apoprotein of HDL) and the activities of several plasma proteins (including lecithin:cholesterol acyltransferase (LCAT), lipoprotein lipase, hepatic lipase, and cholesteryl ester transfer protein) determine plasma HDL levels and subpopulation profiles (43). Also, triglyceride levels are inversely correlated with HDL-C, although the mechanism and range of triglyceride concentrations over which this relationship exists is not clear (44). Manipulation of any of these factors could potentially raise HDL, with each potentially producing a different profile of HDL particles and thus varying degrees of efficacy against CHD. Current therapies to elevate HDL have remained unchanged for years and include fibrates, mainly effective against hypertriglyceridemia but modestly raises HDL-C (10-20%), and niacin, which can elevate HDL-C up to 30% but is poorly tolerated (45).

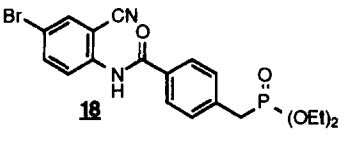
Cholesterol Ester Transfer Protein (CETP) Inhibitors - CETP is a plasma protein which catalyzes the transfer of HDL cholesteryl ester for VLDL and LDL triglyceride (46). The current understanding of the role of CETP in CHD is incomplete. Initial studies on a limited number of CETP-deficient subjects reported substantial elevations (2.5-3 fold) in HDL-C with no indication of premature atherosclerosis (47). Also, overexpression of CETP in mice, a species which normally lacks CETP, results in decreased HDL-C levels and an increase in atherosclerosis when fed a high cholesterol diet (48). In certain hyperlipidemias, plasma CETP levels are elevated (49). These observations support a proatherogenic role for CETP and suggest CETP inhibition as a therapeutic target. However, several recent observations have challenged this view. Expression of CETP in transgenic mice made hypertriglyceridemic by overexpression of apo-CIII inhibited the development of early atherosclerotic lesions (50). Perhaps more relevant is the finding that partial CETP deficiency in a population of Japanese-American men led to increased CHD when compared to normals with similar intermediate HDL levels (40-60 mg/dl) (51).

PD 140195 (**12**) was reported to inhibit CETP in a plasma free *in vitro* system, but showed poor CETP inhibitory activity in the rabbit after intravenous administration. *In vitro* studies in whole plasma, the site of action, resulted in diminished inhibitory activity, presumably due to its affinity for other plasma proteins. This protein binding was attributed to the poor CETP inhibitory activity of **12** *in vivo* (52). The synthetic isoflavan CGS 25159 (**13**) retains significant activity in whole plasma (IC_{50} = 22 μM versus 10 μM) and is the first inhibitor to show CETP inhibition *in vivo* (normolipidemic hamsters) with an increase in HDL-C (53). Several additional classes of CETP inhibitors have recently been reported with SAR studies resulting in the identification of only low micromolar inhibitors in a plasma free assay. Representative examples include triazine **14** (54) and fermentation products wiedendiols A (**15**) and B (**16**) (55). In addition, SAR studies identified a series of phosphonate-containing analogs of cholesterol ester demonstrating a preference for smaller lipophilic groups as replacements for the steroid moiety exemplified by the benzyl derivative **17** (56). The effectiveness of these lipophilic agents *in vivo* remains to be seen.



Lipoprotein Lipase (LPL) Activators - Like CETP, the role of LPL in atherogenesis is controversial. LPL hydrolyzes chylomicron and VLDL triglyceride. In addition to decreasing plasma triglycerides, activation of LPL is expected to enhance the formation of HDL particles via the release of phospholipid-rich surface components during catabolism of triglyceride-rich lipoproteins as well as through a decreased CETP-mediated loss of cholesteryl ester from HDL as a result of fewer triglyceride-rich lipoproteins (57). Clinical studies have demonstrated a positive correlation between LPL activity and HDL-C levels and a negative correlation with triglyceride levels in man (58). Also, transgenic mice overexpressing human LPL demonstrate a resistance to diet-induced hypercholesterolemia and hypertriglyceridemia (59). However, recent data indicates that LPL can enhance the binding and catabolism of LDL and triglyceride-rich lipoproteins in the artery wall, suggesting that increased LPL activity, and not mass, may be more desirable (60).

NO-1886 (18) was shown to increase the synthesis and activity of LPL and to lower plasma triglycerides and elevated HDL-C in rats. These changes were not observed when protamine sulfate, an LPL inhibitor, was coadministered, suggesting a correlation between the increased LPL activity and the observed lipoprotein profile. Formation of atherosclerotic lesions was inhibited in cholesterol-fed rats upon repeated administration of 18 (61). Cyclized analogs of 18, exemplified by quinazolinone 19, reportedly reduce serum triglycerides and total cholesterol in hypertriglycemic rats. However, the effects on LPL activity or HDL were not described (62).



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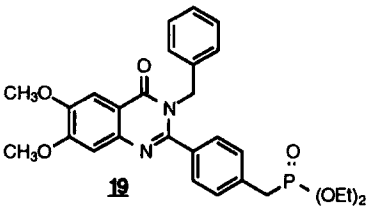

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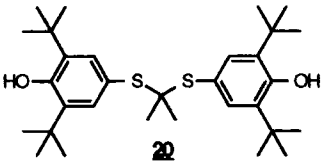
HDL-C: future therapeutic approaches - Future studies using transgenic and gene-targeted animals may better define the role of LCAT, hepatic lipase, phospholipid transfer protein (PLTP), and the recently identified scavenger receptor SR-BI, the putative HDL receptor, in lipoprotein metabolism and atherosclerosis (63, 64). Increasing apo-AI expression is an alternative and less controversial means of raising HDL. A variety of data exists linking apo-AI expression with an increase in HDL and protection from CHD in animals (65, 66). This data supports increasing apo-AI expression as a therapeutic target. However, there is currently no *in vitro* model predictive of an *in vivo* response. While the understanding of apo-AI regulation is incomplete, apo-AI expression and production appear to be regulated by a complex interplay of multiple transcription factors. These include several nuclear hormone receptors for all-*trans*-retinoic acid (RAR), 9-*cis*-retinoic acid (retinoid X receptor, RXR), HNF-4, and ARP-1 (67, 68). Members of the peroxisome proliferator-activated receptors (PPAR) may also be involved. Recent evidence has implicated PPARs in the hypolipidemic effect of fibrates, through suppression of apo-CIII gene expression, which may play a role in their ability to increase apo-AI transcription. Apo-CIII retards the clearance of triglyceride-rich remnant lipoproteins and has been shown to inhibit LPL *in vitro* (69). A greater understanding of how these factors interact to regulate apoprotein gene expression should result in more effective screening models for the identification and characterization of both HDL-elevating and triglyceride-lowering agents.

VESSEL WALL

Effort has recently centered on understanding the underlying mechanism(s) of plaque formation and rupture within the arterial wall as an alternative to traditional lipid approaches. Plaque composition appears to be important in the susceptibility of the lesion to rupture and to potentially result in myocardial infarction (MI). Evidence suggests that atherosclerosis is a chronic inflammatory and wound healing response to injury at the vessel wall. This process results in the expression of various chemotactic factors (e.g. MCP-1), adhesion molecules (e.g. VCAM), and colony stimulating factors (e.g. M-CSF) which recruit monocytes to the sub-endothelial space and promote their transformation to macrophages. The infiltration by macrophages is believed to cause accumulation of lipid within the lesion which contributes to erosion of the fibrous cap and eventual rupture (70, 71). One theory suggests that lipoprotein retention is the necessary and casual event in atherogenesis (72). Whatever the initiating element, modulating any of the above factors may provide a potential therapeutic intervention. However, the complexity of the inflammatory process in plaque formation suggests such intervention may not be straightforward.

Antioxidants - A large body of evidence exists suggesting that oxidized LDL (oxLDL) may play a role in the genesis of atherosclerosis. OxLDL has been found in atherosclerotic lesions of animal models and of humans and has been shown to be taken up by macrophages via scavenger receptors leading to the formation of foam cells and fatty streaks, the precursors to advanced lesions (73, 74). Dietary antioxidants such as vitamin E have been shown to inhibit the oxidative process and to reduce lesion formation in animal models. Recent clinical data with high doses of vitamin E demonstrated a beneficial effect on reducing non-fatal MI but not cardiovascular mortality (75). However, there is growing evidence questioning the efficacy of antioxidants in atherogenesis. Probucol (**20**), an antioxidant with modest cholesterol lowering properties, was recently withdrawn from the market based on the findings of the Probucol Quantitative Regression Trial (PQRST). While probucol provided protection against LDL oxidation, it showed no effect on the development of femoral atherosclerosis and lowered HDL-C by 35% (76). Whether oxLDL plays a casual role in plaque formation remains unclear and additional studies with other antioxidants are clearly needed to substantiate their use as a treatment for atherosclerosis (77).



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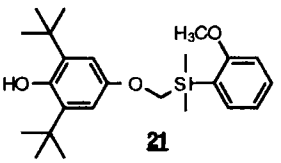

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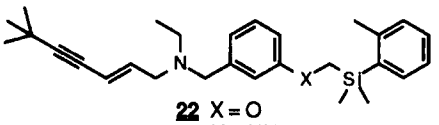

22 X = O
23 X = NH

Several probucol-like analogs have recently been described as antioxidants with cholesterol lowering properties of unknown mechanism in the cholesterol-fed rabbit. There was no correlation between antioxidant and cholesterol lowering properties. Members of this class displayed variable effects on HDL-C with the 2-methoxyphenylsilyl derivative (**21**) showing a two-fold increase in HDL-C (78). The structurally similar squalene epoxidase inhibitor **22** was modified to the (arylamino)methylsilane derivative **23** in an attempt to incorporate antioxidant activity. Compound **23** bound squalene epoxidase (IC_{50} = 100 nM), inhibited cholesterol biosynthesis in the rat and displayed more potent antioxidant activity than vitamin E *in vitro*. Removal of the benzylamine sidechain resulted in a loss of squalene epoxidase activity with retention of antioxidant properties (79).

Recent effort has focused on identifying pathways that could potentially generate oxidized LDL, including the lipoxygenase 15-LO. 15-LO has been located and shown to be active in developing lesions of animals and humans (80). However, conflicting data concerning the role of 15-LO in the development of atherosclerosis from transgenic animal studies exists (81, 82). In addition, paraoxonase and PAF acetylhydrolase, two enzymes transported on HDL, may inhibit LDL modification, suggesting other opportunities for intervention (83).

Conclusion - Significant effort and progress have been made in recent years in the development of agents which lower LDL-C. Future initiatives will undoubtedly be multifaceted, including HDL-C elevation, reducing the atherogenicity of various lipoproteins, and direct intervention at the arterial wall. In addition, given the complexity of atherosclerotic disease, advances made in other arenas, such as

inflammation, immunology or diabetes, may significantly add to potential therapeutic opportunities.

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SECTION III. CANCER AND INFECTIOUS DISEASES

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Chapter 11. New Approaches and Agents to Overcome Bacterial Resistance

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Introduction - Antibacterial resistance (AR) is a worldwide problem (1) with catastrophic potential (2). In this review, the challenges posed by AR are presented in four sections covering: 1) the magnitude and scope of AR and the problem organisms; 2) the mechanistic aspects of AR; 3) new bacterial targets and methods for screening and synthesis; 4) a review of selected antibacterials reported to offer some solution or promise toward overcoming AR.

SCOPE OF AR AND PROBLEM ORGANISMS

The recognition of antibacterial resistance as a serious health problem was reviewed earlier in the decade (1). In 1995, the American Society of Microbiology's task force issued a report (3) which called for national surveillance, education, basic research, and new prescribing patterns. The severe potential consequences of AR have been noted at the highest levels of government (4). The numbers associated with AR are staggering. In the US, deaths from infectious diseases have increased 58% from 1980-1992 (5). The mortality from pneumonia and bacteremia (28% of hospital infections) has increased significantly (6) as resistance has increased (3,7). The direct and indirect cost of AR to the US economy is $3 billion (3) and $30 billion (8), respectively. Many organisms such as *Staphylococci*, *Enterococci*, *Tuberculosis* and the pneumococci are resistant to six or more drugs (9) while *E. faecium* and *P. cepacia* are not sensitive to any currently approved drugs (10). *Tuberculosis* alone kills 3 million persons annually (11). We are on the edge of the post antibiotic era (1,10,12). Several reasons for this predicament have been offered: 1) Selective pressure on bacteria is enormous especially in hospitals and day care facilities (10). Over 110 million courses of office-based therapy were prescribed in 1992, 24 million for ear infections alone (3). 2) Bacteria are exceedingly "clever" at evading antibacterials (5,10,13,14). 3) Empiric therapy is the norm and a significant cause of AR (2,6,12). 4) As lifespans increase, so do immunodeficiency (5,15) and invasive procedures (6,7). 5) Antibacterials are often prescribed or used inappropriately and efforts to cut costs have impeded the proper management of some infections (3). 6) Drug companies reduced research and development of new agents in the belief that bacterial diseases had been conquered (3,5). The new awareness of AR has sparked debate regarding the use of antibacterials for OTC (16), and in animals (17).

Problem Organisms - *Staphylococci* are the most common hospital pathogens (6,18). Mortality in bacteremia is 25-63% (18). Penicillin resistance has reached 70-90% (3,7) (2.4% in 1975) (6). Methicillin resistance has reached 40% in *S. aureus* (MRSA) (14)

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and 50% in the coagulase negative strains (CNS), *S. epidermidis* (MRSE) and *S. heamolyticus* (6). Quinolone resistance in MRSA reached 78% in 18 months (7). Vanocomycin has become the drug of last resort for MRSA. While no resistance to vancomycin has yet been reported for MRSA, resistance has emerged in the CNS (7). Vancomycin resistant MRSA is health care's greatest nightmare (2,9,18). Strict control measures have already been proposed (19).

The *Enterococci* make up 12% of all hospital pathogens occurring often in urinary tract infections and endocarditis (6). Penicillin resistance is 12% (60% in *E. faecium*) and vancomycin resistance (VRE) was 14% in 1993 (0.4% in 1989) (6,20). Quinolone resistance is 15% (21). Mortality in VRE infections of any kind is 42-81% (22). VRE have been found in animals and food stuffs (17,23,24).

S. pneumoniae is a community and hospital pathogen accounting for 25% of community pneumonias, 50% of ear infections, and 18% of bacterial meningitis (18). It is associated with one million nosocomial pneumonias with 7% morbidity (25). *S. pyogenes* is less common, but substantially more virulent (18). The *Streptococci* are relatively susceptible to ß-lactams with only 6.6% high level resistance (7,26); such resistance in ear infections however, is 30 - 41% (18,27). The use of marginally active quinolones fostered resistance to these drugs (27).

Among the Gram negative pathogens, *Pseudomonas* is most problematical (12% in ICUs) (28) due to its intrinsically lower susceptibility to antibiotics (2,6). Resistance to imipenem is 14% overall (29) and up to 40% in some hospitals (0% in 1987) (6). Quinolone resistance is over 18%. *E. coli, Klebsiella, Proteus, Enterobacter, Serratia,* and *Citrocobacter* are all important hospital organisms (6,28) due to genes encoding multiple resistance (2,28). Multiple drug resistant TB is 3.5% in the US, but estimated to be much higher worldwide (11).

MODES OF RESISTANCE

Several overviews on the general mechanisms by which bacteria acquire resistance have appeared (2,10,13,28), and the modes of quinolone resistance in particular have been reviewed (30,31). Gyrase and Topoisomerase IV are the quinolone targets in bacteria (14). All high level quinolone resistance in Gram negatives (31,32), MRSA (33), *S. pneumoniae* (25,34), and *Enterococci* (21) have been shown to be associated with double mutations in gyrase at the Ser 83 and the AA at 87 (*E. coli* numbering). Porin and efflux mutations play minor roles in high level resistance (31,35). Mechanisms of tetracycline resistance involve efflux and ribosomal protection (36,37). The glycylglycines were the first tetracylines to overcome this resistance. These agents were shown to be poor substrates for the efflux pump and to bind five times more tightly to the ribosome (38). The mechanism of vancomycin resistance was reviewed (39). *Enterococci* with the vanB phenotype, are resistant to vancomycin but not teicoplanin (40). This resistant phenotype has been shown to originate with the two component signalling proteins vanS and vanR, which activate the gene cascade leading to VRE. In the vanB phenotype, the histidine kinase fails to recognize teicoplanin (41,42). Aminoglycoside (AG) resistance arises from acetylation, adenylation, or phosphorylation by AG modifying enzymes (2,43). Successful deactivation depends on the electrostatic interaction of the AG nitrogens in the enzymes' binding sites. Systematic removal of each nitrogen of neomycin and kanamycin A produced analogs which were poor substrates for the enzymes and which retained wild type activity vs. the resistant strains (44).

DISCOVERY OF NEW ANTIBACTERIALS

Every discussion of AR cites the need to discover new agents (3,4,5,14). Scientists have turned their attention to new bacterial targets, new assay methodology, and new methods of synthesis (5,14,45).

New Targets - To defeat MRSA, MecA (the modified penicillin binding protein) and several essential auxiliary gene products for cell wall synthesis were suggested as targets (14). Such genes have been identified by a novel selection technique (46). FemA and FemB are peptidases that add the third and fifth glycines to the pentaglycine crosslinking chain (14,47). FemX was shown to add the first glycine (47) and FemF adds an essential lysine to the alanylisoglutamine dipeptide (46). The proper function of MecA and all of the auxiliary gene products are essential for the MRSA phenotype (46). Lysine is synthesized from meso-diaminopimelic acid (DAP). The key enzyme in the biosynthesis of DAP and lysine is an amino acid transferase (DAP-AT) which was isolated and purified. Hydrazino-substrate analogs were synthesized with Ki's of 24-50 nM (48). VanX was suggested as a target for VRE (14), based partly on the successful inhibition of VanX by phosphinate transition state based compounds (49). Various disulfides were also shown to inhibit VanX by interfering with the catalytic Zn in the active site (50). Lipid A is the protein anchor in the lipopolysaccharide outer membrane of Gram negative bacteria and was described as a target in 1992 (51). Using an uptake assay, certain hydroxamic acid oxazolidines were identified as perturbing LPS integrity. The inhibition was traced directly to a key deacetylase in the biosynthesis of Lipid A. Analogs with Ki's of 50 nM displayed antibacterial activity *in vitro* and *in vivo* (52,53). Bacterial septation promoted by FtsZ is another target (45). FtsZ was shown to form an essential interaction with PBP3 and an assay to identify inhibitors has been described (54).

Aside from cell wall biosynthesis, protein (14) and DNA synthesis (45) are considered prime antibacterial pathways. One novel target out of many (55) in protein synthesis involves the amino acid *t*-RNA synthetases (56). These enzymes join a particular amino acid (AA) and ATP for attachment to their *t*-RNA. Transition state ATP-sulfamate mimics were designed with low nM inhibition of isoleucine-*t*-RNA transferase and antibacterial activity (56). Inhibitors of elongation factor Tu, which positions the AA-t-RNA onto the ribosome were described (57). For DNA synthesis inhibition, gyrase and Top IV remain important targets (58). Competitive inhibitors of the essential DnaA-ATP binding have been described (a series of bis-indoles) (59). DnaA is one of >30 proteins involved in DNA synthesis and initiates DNA replication in bacteria (59).

Protein secretion is another important bacterial process which includes several chaperone and export proteins, and a leader peptidase that frees the new protein from the secretory complex (45,60,61). Several penems were described as uM inhibitors of leader peptidase and demonstrated the accumulation of unprocessed protein in whole bacteria (62,63). Other possible antibacterial targets suggested include the histidine kinase-2-component signalling pathway (5,45) and iron uptake (64).

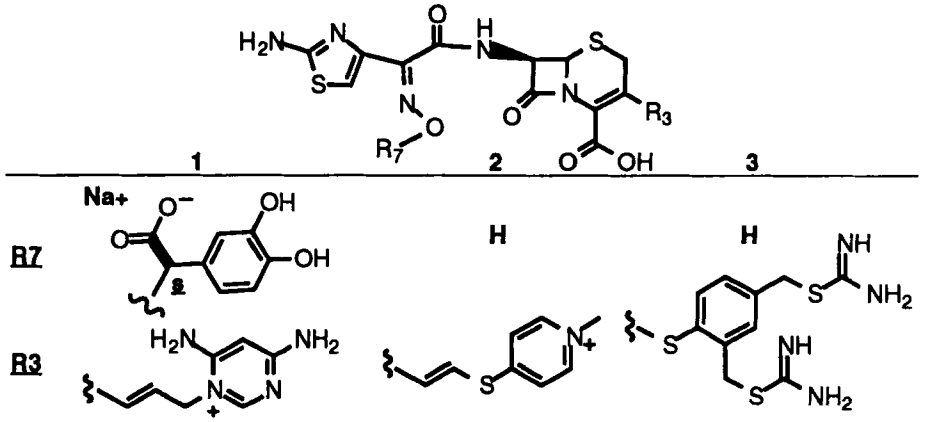
New Concepts in Screening - To speed the identification of potential targets and agents, new assay and mass screening methodologies have been described (5,45). To study bacterial adherence, a major aspect in infections near foreign devices, fibronectin coated membranes were employed. Agents that inhibit adhesion *in vitro* or *in vivo* (implanted in mice) may be identified (45). A multi-channel screening technology has been reported where a single compound is tested against a multitude

of uniquely constructed bacteria (or mammalian cells) to profile the mechanism of action, transport, efflux selectivity, and toxicity all in one assay (65).

New Methods of Synthesis - Combinatorial chemistry has been utilized to prepare libraries for screening vs. bacteria or single targets (45,66,67). Small peptides with MICs 2-10 μg/ml were identified (66). Both D- and modified amino acids have been employed improving biological properties while maintaining potency (66,68). Combinatorial approaches to more difficult molecules such as glycopeptides have been attempted (69,70). One unique twist has been the development of designer biosynthesis. Microbes that produce antibiotics often use clusters of genes ("cassettes") which produce all the enzymes to perform a complete biosynthesis. These cassettes have been cloned and manipulated to produce distinct desired variations in the natural product structures (71). Finally a reemphasis of the value of natural product diversity as a source of new drugs was described (72).

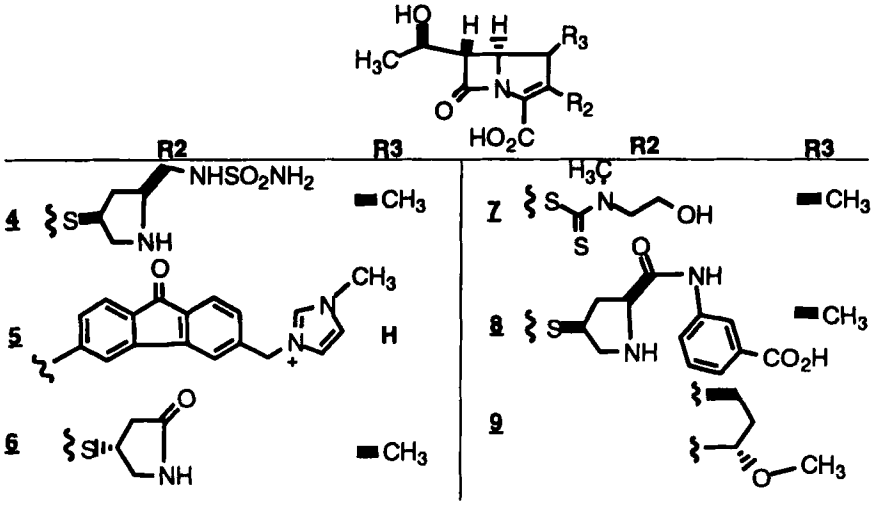
SELECTED AGENTS REPORTED AS ACTIVE VS. RESISTANT ORGANISMS

ß-Lactams - LB-10517 (1), a new catechol-cephalosporin using the iron-uptake pathway, was shown to have good activity against *P. aeruginosa* (MIC_{90} of 2.0 μg/ml) (73) and was potent against imipenem and ofloxacin resistant strains (74). TOC50 (2), a vinyl-thiopyridine derivative (75,76) has MIC_{90}s (μg/ml) of 3.13 against MRSA and 1.56 against MRSE. It is the first cephalosporin derivative to show potent activity against vancomycin-resistant *E. faecalis* (VREF) with an MIC_{90} of 1.56 μg/ml (77). MC-02,331 (3) shows good activity (MIC_{90}s, μg/ml) against MRSA of 4.0, MRCNS 8.0, ampicillin-(R) *E. faecium* 4.0, and penicillin-(R) *S. pneumoniae* (PRSP) 0.5 (78). In time-kill studies in MRSA, 3 was bactericidal at 1xMIC and more rapid than vancomycin.



A new carbapenem with a sulfonylurea substituted pyrrolidine side chain, S-4461 (4), exhibited better antibacterial activity than meropenem against Gram positive bacteria including MRSA, (MIC 0.2 vs. 0.4 μg/ml), and was comparable against *P. aerugenosa* (79,80). A 2-fluorenonylcarbapenem, L-695,256 (5), had MICs against methicillin sensitive *S. aureus* (MSSA) and MRSA in the range of 0.016-2.0 μg/ml. The activity against penicillin-(R) pneumococci was even better at 0.016-0.064 μg/ml (81,82). CS-834 is an orally absorbed prodrug ester of R-95867 (6). The latter possessed antibacterial activity against ofloxacin-(R) *S. aureus*, PRSP and ß-lactamase-producing *Enterobacteriaceae* with MICs of 0.78, 0.39 and 1.56-6.25 μg/ml respectively (83). CS-834 showed good efficacy in mice (84) against Gram positive

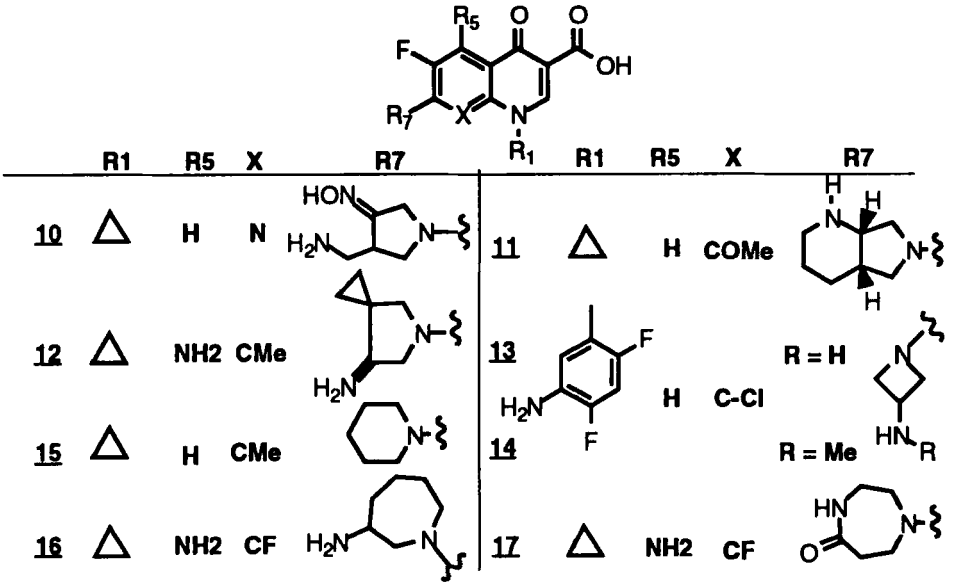
and Gram negative pathogens including PRSP (ED_{50}, 2.88 mg/kg/dose). BO-3482 (7) is a carbapenen with a dithiocarbamate side-chain specifically designed to have activity against resistant strains (85). It showed *in vitro* activity against high-level MRSA, low-level MRSA and MR-coagulase negative staphylococci with geometric



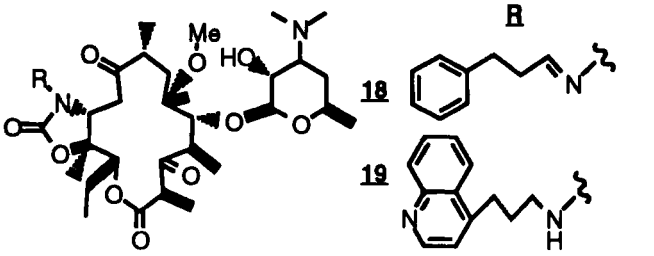
means of 4.36, 2.99 and 5.44 µg/ml respectively. The ED_{50} of 7 against high level MRSA was 19.5 mg/kg, (with cilastatin 4.80 mg/kg). Against low-MRSA, the combination had an ED_{50} of 0.46 mg/kg. The *in vitro* activity of the thioprolinecarbapenem, L-749,345 (8), was 1.5-16 times better than imipenem against multiresistant *E. cloacae, E. coli, K. pneumoniae, S. aureus* and *S. marcescens*, (MICs 0.125-2.0, 0.03, 0.03-0.5, 0.06-0.125 and 0.03 µg/ml respectively) (86). A tricyclic-carbapenem, GV-104326 (safetrinem 9), had antipneumococcal activity (MIC_{90}) of 1.0 µg/ml against 211 clinical isolates. Of 74 penicillin-resistant strains tested, all but 11 were inhibited at 0.5 µg/ml (87). The MIC_{90}s for MRSA, MRSE, PRSP and erythromycin-(R) *S. pyogenes* were 32, 4.0, 1.0 and 0.03 µg/ml, respectively (88).

Quinolones - Expanded studies (89) of a previously reported fluoronaphthyridone (90), LB-20304 (10), showed MIC_{90}s against oxacillin-(R) *S. aureus* (ORSA), ORSE, ORS *haemolyticus*, and *P. aeruginosa* of 2.0, 0.25, 4.0 and 2.0 µg/ml. Additional studies (91,92) confirmed the *in vitro* activity against MRSA, MRSE and PRSP as well as *in vivo* efficacy superior to ciprofloxacin and sparfloxacin against *S. aureus*, *S. pyogenes* and *S. pneumoniae*. BAY12-8039 (11), a new 8-methoxyquinolone, was superior to sparfloxacin vs. resistant Gram positive strains (MIC_{90}: cipro R MRSA 4.0 µg/ml) (93). It was equivalent to trovafloxacin vs. PRSP (MIC_{90}: 0.25 µg/ml) (94). A 5-amino-8-methylquinolone, HSR-903 (12), demonstrated MIC_{90}s of 1.56 and 0.05 against MRSA and PRSP. Compound 12 was four times more active than ciprofloxacin and ofloxacin in general (95). WQ -2756 (13) and WQ-2765 (14), two azetidinylquinolones, had MIC_{90}s (µg/ml) against MRSA (1.56 and 0.78), QRSA (1.56 and 0.78), *E. faecalis* (0.7 and 0.39) and *P. aeruginosa* (0.39 and 0.78). Both 13 and 14 were more efficacious than levofloxacin in systemic infections in mice (96). An 8-methyl-6-desfluoroquinolone (15), had potent activity against MRSA, cipro-(R) *S. aureus* and *S. pneumoniae* (MICs: 0.016 µg/ml for each) (97). FD501, a 5-

aminoquinolone with a 3-aminoazepine side chain (16), had activity against MRSA (MICs 0.024-0.1 μg/ml) and *P. aeruginosa* (1.56 μg/ml). The racemate and both stereoisomers were equipotent (98). A 5-aminoquinolone with a perhydro-diazepinone side chain, FA103 (17), was 2-8 times more active against Gram positive bacteria (including MRSA) than sparfloxacin (99). MICs for MRSA (μg/ml) were 0.024-0.05 for FA103 and 0.05-0.2 for sparfloxacin.

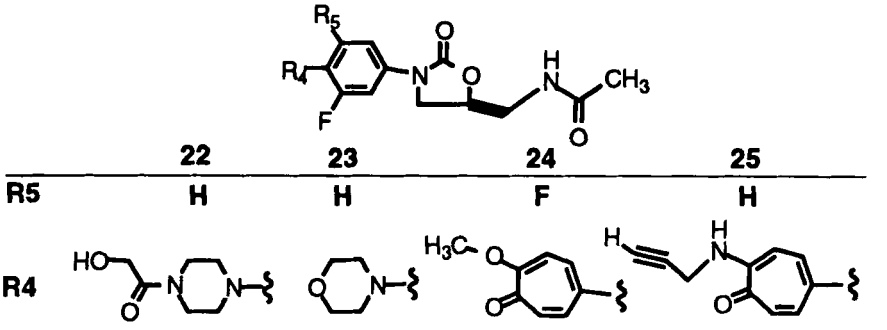


Macrolides - An erythromycin carbazate derivative (18) had *in vitro* activity against *S. aureus* strains with macrolide-lincosamide-streptogramin (MLS) resistance, erythromycin-(R) *S. pyogenes* and erythromycin-(R) *E. coli* with MICs of 0.01, 0.1 and 12.5 μg/ml respectively (100). The older ketolide, RU 64004 (19), had activity superior to clindamycin against oxacillin-resistant *staphylococci* and VRE (vanA, vanB and vanC) isolates (101).

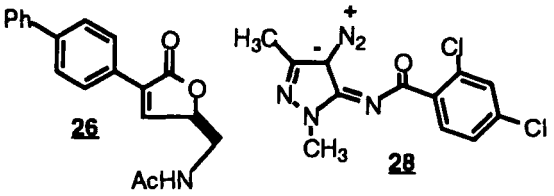


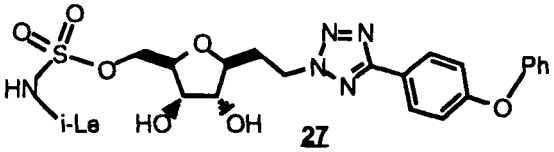
Glycopeptides - LY333328 (20, structure not shown, see ref. 102) has been previously reviewed (102) but extensive testing results were recently reported (103-108). This semisynthetic glycopeptide was active against *VRE-faecalis* and *VRE-faecium* with MICs against vanA and vanB strains of 0.03-2.0 and 0.016-1.0 μg/ml respectively. It was also very active against all *pneumococci* tested (MICs 0.125 μg/ml) including penicillin-(R) strains. In two studies (106,107), 20 had MIC_{90}s of 0.5 and 1.0 μg/ml against MRSA and MRSE. An eremomycin hydrazide derivative (21, structure not shown), a benzylamide derivative of amino acid 7 of eromycin, had activity equivalent to the parent except for *S. epidermis*, where it was 10-fold more active (109).

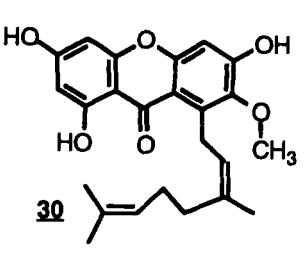
Oxazolidinones - Since the previous review of the oxazolidinones U-100592 (**22**) and U-100766 (**23**) (110), several reports detailing antibacterial activity have been published (111-116). The *in vitro* activities (MIC_{90}s) of **22** and **23** (112,113) were: MRSA (1.0), MRSE (1.0), ORS *haemolyticus* (1.0), PRSP, (0.5, 1.0), VRE-*faecalis* (1.0), and VRE-*faecium* (1.0). The *in vivo* efficacy, ED_{50}s (mg/kg), of **22** and **23** were: MRSA (0.9, 2.0), MRSE (1.9, 4.7), VRE-*faecalis* (1.3, 10), and gentamycin-(R) *E. faecium* (12.5, 24). The tropone derivatives **24** and **25** showed good MICs (µg/ml) (117) against MRSA (0.5), MRSE (0.5, 0.25), *E. faecalis* (1.0, 0.5) and *S. pneumoniae* (0.125). ED_{50}s (mg/kg) vs. MSSA for **24** and **25** were 2.3 and 1.3 respectively.

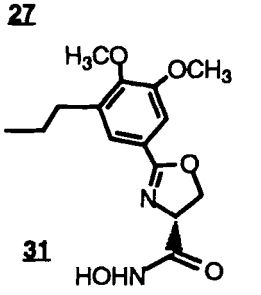


Miscellaneous - Furanone **26**, a carbon isostere of the oxazolidinones, was shown to have activity against penicillin-(R) *S. aureus*, MRSE and *E. faecalis* with MICs of 0.13 µg/ml (118). Nearly all the activity resided in the R-configuration. CB432 (**27**), a compound which targets isoleucyl synthetase (56), had modest activity (MIC of 25 µg/ml) against MRSA. However, it was chosen for further study because of its excellent selectivity and activity vs. the isoleucyl enzyme (IC_{50} 1.3 nM) and *in vivo* efficacy (PD_{50} 16.5 mg/kg) against MDR *S. pyogenes*. Compound **28**, a diazopyrazole derivative (119), showed selective activity against MRSA and *S. faecalis* of 3.1 and 1.0 µg/ml. SCH 27899 (**29**, structure not shown), an oligosaccharide belonging to the everninomicin family, showed good antibacterial activity against MRSA, CNS, VRE-*faecalis* and VRE-*faecium* with MIC_{90}s of 0.25, 0.5, 0.5 and 0.5 µg/ml respectively (120,121). It had an MIC_{90} of 0.5 µg/ml against all *enterococci* tested (~800 strains). A xanthone compound (**30**), isolated from *Garcinia mangostana* (122), had activity against MRSA superior to that of vancomycin (0.313-1.25









compared to 3.13-6.25 μg/ml). L161241 (31) is the optimized product of a Lipid A SAR (described earlier) with MICs (μg/ml) vs. *E. coli, E. cloacae* and *Pseudomonas* of 1.0, 6.0 and >100 respectively. The PD_{50} (ip) in septicemic mice (*E. coli*) was 12.5-50 mg/kg (53).

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Chapter 12. Bacterial Genomics and the Search for Novel Antibiotics

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Introduction - Emergence of bacterial resistance to a number of antimicrobial agents such as macrolides, quinolones, vancomycin and ß-lactam antibiotics is becoming a serious worldwide health problem (1-4). The single-most important concern in clinical practice is the increase in the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In addition to resistance to ß-lactam antibiotics, many MRSA strains are also resistant to macrolides, tetracyclines, aminoglycosides and fluoroquinolones. Recently, penicillin resistant *Streptococcus pneumoniae* strains have also appeared and multidrug-resistant strains of *Mycobacterium tuberculosis* have emerged in several countries including the United States (5). *M. tuberculosis* resistant to aminoglycosides, ethambutol, isoniazid, pyrazinamide, and rifampin have been isolated from drug abuse users. Resistance of *Bacteroides fragilis* to ß-lactams ranges from 3 to 30%; nearly all of them are resistant to tetracyclines. This rapid and extensive emergence of antibiotic resistant bacteria has resulted in a clear cut need to discover new antibiotics. Those with mode of actions on novel bacterial targets will offer the best chance to overcome resistance (6).

Recent advances in rapid gene cloning, PCR-based gene amplification and DNA sequencing technologies, along with associated sophisticated analysis software, have made bacterial genome sequencing both practical and efficient. As shown in Table 1, more than 20 bacterial genomes have been either fully or partially sequenced, most within the past two years. These sequences have or will appear in public databases. As will be described in this report, genomic sequencing enables the uncovering of previously unidentified genes and their corresponding proteins. To a first approximation, proteins found to be essential for the survival of the bacterial cell can be considered as potential targets. Whole genome sequencing will accelerate the identification of new targets for the discovery of new antibacterial agents. In addition, bacterial genomics should uncover unique or common sequences that can be used in diagnostic applications: in particular the rapid speciation of organisms that are difficult to cultivate and the rapid determination of drug resistance profiles. Vaccine applications arising from genome initiatives are presently being explored.

The apparent pharmaceutical potential that arises from bacterial genomics has driven a large part of the overall sequencing effort, particularly in the United States. Three companies, Incyte Pharmaceuticals, Human Genome Sciences and Genome Therapeutics, Inc. have each undertaken large efforts to obtain the genomic sequences of many important pathogens. These sequences will be provided to their customers, generally the large pharmaceutical companies, and are not likely to become publically available. It is certain, therefore, that a number of bacterial genomes will be sequenced more than once.

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TABLE 1. Partial list of bacterial genomes being sequenced.

Organism	Size (Mb)	Organization	Reference / Web Site
Completed:			
Aquifex aeolicus	1.5	RBI	
Escherichia coli	4.6	U. Wisconsin	www.genetics.wisc.edu
Haemophilus influenzae	1.8	TIGR	(7) / www.tigr.org
Methanobacterium thermoautotrophicum	1.7	GTI	
Methanococcus jannaschii	1.8	TIGR	(8) / www.tigr.org
Mycoplasma genitalium	0.6	TIGR	(9) / www.tigr.org
Mycoplasma pneumoniae	0.8	U. Heidelberg	(10 / www.zmbh.uni-heidelberg.de/ M_pneumoniae/MP_home.html
Synechocystis sp. PCC6803	3.6	Kazusa	www.kazusa.or.jp
In Progress:			
Archeoglobus fulgidus	2.2	TIGR	
Bacillus subtilis	4.2	Int"l Consort	www.ddbjs4h.genes.nig.ac.jp
Borellia burgdorferi	1.3	TIGR	
Caulobacter crescentus	3.8	TIGR	
Clostridia acetobutylicum	2.8	GTI	
Deinococcus radiodurans	1.6	TIGR	
Enterococcus faecalis	3.0	TIGR	
Halobacterium salinarium	4.0	Max-Planck Inst.	
Helicobacter pylori	1.7	TIGR	
Legionella pneumoniae	4.1	TIGR	
Mycobacterium avium	4.7	TIGR	
Mycobacterium leprae	2.8	GTI	www.pandora.cric.com/htdocs/leprae
Mycobacterium tuberculosis	4.2	TIGR/Sanger	www.sanger.ac.uk /pathogens
Neisseria gonorrhoea	2.2	U. Oklahoma	www.genome.uo.edu
Porphyromonas gingivalis	2.2	TIGR	
Pseudomonas aeruginosa	5.8	U. Laval	
Pyrobaculum aerophilum	2.2	UCLA/Caltech	
Pyrococcus furiosus	3.0	U. Utah	
Rickettsia prowazeckii	1.1	Uppsala U.	
Salmonella typhimurium	4.5	TIGR	
Shawanella putrefaciens	4.5	TIGR	
Streptococcus pneumoniae	2.5	TIGR	
Streptococcus pyogenes	1.8	U. Oklahoma	www.genome.uo.edu
Sulfolobus solfataricus	3.0	NRC Canada/ Europe	(17) / www. imb.nrc.ca
Thermoplasma acidophilum	1.7	TIGR/U. Texas	
Treponema denticola	1.0	TIGR/U. Texas	
Treponema pallidum	1.0	TIGR	
Ureaplasma urealiticum	0.8	U. Alabama	

GTI-Genome Therapeutics, Inc.; RBI-Recombinant Biocatalysis Inc.; TIGR-The Institute for Genomic Research.

Though many of the organisms listed in Table I are important human pathogens, it can be seen that a large component of the publically-funded bacterial genomic sequencing effort is not being driven by medical/pharamceutical considerations. Sequencing of *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*

and *Pyrococcus furiosus*, three members of the Archaea, has been funded by the US Department of Energy. Clearly, interest in these organisms, as well as the other extremeophiles in Table I, is based on energy or environmental implications. But the principal driving force behind publically-funded bacterial genome initiatives has been and remains the scientific interest of investigators. The ultimate prospect of determining the function of every gene in a free-living organism, and then understanding how the genes control all cellular behavior, as well as gaining insights into the evolution of genes, enzymes and metabolic pathways, is difficult for biologists to resist.

At this writing, there is very little reported information on the identification of novel targets for antibacterial drug discovery. Following a brief overview of what we have learned to date from bacterial genomic sequencing efforts, this chapter will focus on how the bacterial genome initiative is being conducted and on how the information is generated and used.

OVERVIEW: INFORMATION FROM GENOMIC SEQUENCING

Since nucleotide (and protein) sequences generated from genomic sequencing are matched to sequences in GenBank, EMBL and other databases, most of our understanding of the information generated from bacterial genomic sequencing is based upon our current state of knowledge. A typical example of global information gained from bacterial genomic sequencing is exemplified by the data generated from the sequence of *E. coli* (12). The circular genome is approximately 4.6 million base pairs in length and contains 4,286 ORFs (open reading frames). Of these, 1817 have assigned a known function, either by independent biochemical or genetic analysis or through matching of the sequence to a known gene. Of the remaining 2,469 ORFs, approximately half were found to have matches to sequences whose functions could not be assigned. The other 1200 ORFs were found to be unique. Considering the time and effort expended by thousands of scientists over the past 40 years, it is somewhat surprising to learn that our understanding to date has advanced to only about half of the genome of the best studied organism in biology. For the other, smaller organisms that have been sequenced, matches to known functions represent upwards of 60% of the total number of ORFs. The exception is *Methanococcus jannaschii* where 70% of the ORFs do not have an assigned function. Clearly, data generated from genomic sequencing has underscored how limited our understanding of diverse organisms is at the present time. At the rate at which bacterial sequences are currently being generated, Craig Venter predicted that more than 400,000 genes will be discovered in the next 10 years, 50% of which will have no matches in our rapidly expanding databases (13). The need to devote attention to biochemistry and genetics to understand function is apparent, even today.

METHODOLOGY

From the large scale genome sequencing efforts developed at The Institute for Genomic Research (TIGR) in Maryland, the Sanger Centre in Cambridge, UK and several companies specializing in high throughput, large scale sequencing, two general strategies have emerged. TIGR and others employ the shot-gun cloning approach of random genomic fragments (1 kb or smaller) into plasmids which are

then sequenced from the plasmid/insert junctions in both directions using universal primers based on plasmid sequences. The dideoxy chemistry used to generate the sequence employs fluorescent primers or terminators. In general, this procedure yields sequence of 500 bp or greater per template. Much of the procedure, including cloning, colony picking, template preparation and DNA sequencing has been automated. For a genome of approximately 2 million base pairs, 15,000 - 20,000 clones are prepared and sequenced. This approach generates upwards of 7.5 million bp of sequence yielding 3-4-fold coverage of each nucleotide in the genome. The sequence generated is assembled into linear fragments (contigs) ranging from a few kb to larger than 50 kb by computer programs such as PHRAP (14), SEQUENCHER (15) or the assembly program developed at TIGR (see Table 1 for Website) that match regions of overlap and discard vector sequences or sequence that is not considered to be reliable. Although, in theory, the sequence of the entire genome should be obtained by this procedure, in practice this approach yields the sequence of 90 - 95% of the genome. At institutions with large-scale sequencing capablilities, this process can take as little as 3 months. Sequence gaps and physical gaps are filled in by long range PCR sequencing of larger templates of the genome prepared in cosmids (ca. 40 kb inserts) or λ clones (10 - 20 kb inserts) or by sequencing directly from the ends of cosmid or λ templates. Gap closure can take a number of months to complete. Sequence is considered complete when the entire genome can be assembled into a single contig. Most bacteria have circular chromosomes; the single exception among the completed genomes is *Borrelia burgdorferi*, whose chromosome is linear.

The other strategy uses an ordered set of cosmids carrying sequences covering the entire genome. This was done for the *Escherichia coli* genome at the University of Wisconsin and is in progress for the *Bacillus subtilis* project where specific segments of the genome have been assigned for sequencing to the members of a consortium made up of European and Japanese laboratories. Sequencing of the *Mycobacterium tuberculosis* genome at the Sanger Centre also employs ordered cosmid sets. Cosmid DNA is either subcloned into plasmid or M13 vectors to facilitate efficient high throughput double-stranded or single-stranded sequencing reactions, respectively. Alternatively, sequencing can also be done employing the cosmid templates themselves. The latter yields shorter sequence reads and requires "primer walking" to get through a single cosmid. In general, sequencing from cosmids takes longer to complete and can only be used when ordered sets of cosmids are available through prior or unrelated work, but is the method of choice for highly repetitive genomes.

BIOINFORMATICS

DNA/Protein Analysis - Bioinformatics is the term generally used for the organization and analysis of biological data. In the context of genomics, the term applies to the DNA analysis software packages that are used to make sense of genomic sequence that can span several million nucleotides. Beginning with the raw sequence, genome analysis packages require a number of applications including searching the sequence for ORFs, providing translation of the ORFs, matching sequences (usually amino acid or short peptide motifs) to sequences in GenBank, EMBL or other sequence databases for determination of proposed gene function, aligning sequences,

searching for specified patterns (promoter sequences, ATP binding sites, NAD binding sites, etc.) within a sequence, providing restriction nuclease or other enzyme cleavage sites in a nucleotide or protein sequence, providing secondary structure predictions, codon bias, etc.

For a completed bacterial genomic sequence (e. g., *E. coli, H. influenzae*, etc), the following additional information is desired:

(I) A complete listing of all the genes indicating their proposed function. For clarity, the genes can be listed by category and sub-category (e.g. genes involved in amino acid biosynthesis, subdivided by amino acid; genes involved in DNA replication, etc.);

(II) A listing of the other genetic elements present in the genome (transposons, insertion sequences, repeated sequences, prophage sequences, etc.) and their relationships to other known genetic elements;

(III) Graphic representations of the genome end to end showing the relative positions and sizes of the genes. Color-coding and shading can be employed to illustrate gene function. Magnified regions of sub-genomic segments (up to 6- 10 kb) should show the organization of proposed operons, direction of transcription, cis-acting regulatory regions, etc.

(IV) A facile mechanism to perform whole genome comparisons. It would be of value to determine, with a single query, which genes are present in a number of genomes being compared, which are present in one but not in another, which is unique in one of a set of genomes, etc. Present sequence analysis packages permit comparisons largely on a gene to gene basis.

A bioinformatics software package designed to handle complete genomes is not currently commercially available. However, computer programs to address some of the needs listed above have been developed at several of the sequencing institutions mainly for use within or for their commerical partners. Some of the programs developed at TIGR are available and can be downloaded from their Website. Most of the software being developed uses a Web browser interface and is designed to link sequence data to homology or functional data. A limited example is found at the TIGR Website (Table 1) for the sequences *of Haemophilus influenzae, Mycoplasma genitalium* and *Methanococcus jannaschii*, all organized in a similar fashion. At the Web page containing the complete listing of the genes by functional category, one can click on function to obtain the list of sequences. The genes are organized in a functional hierarchy and, after choosing a function, a sequence is presented. The putative function for each, along with a score of its similarity to the best match in all available databases, is shown. Clicking on the ID number of the gene reveals the actual sequence of both the nucleotide and the corresponding peptide which may be copied and downloaded for further use. A query page is also present which allows one to search with a desired sequence for a match with the given genomic sequence. Internal Web pages at TIGR not available to the public display the graphic representation of the gene map shown in their publication in Science (7). A similar, but more robust analysis is available at NCBI (http//:www.ncbi.nlm.nih.gov/Complete_Genomes/).

The MAGPIE (Mutlipurpose Automated Genome Project Investigation Environment) program being developed by T. Gaasterland at Argonne National

Laboratory can be used to annotate and graphically represent genomic information (16). It is being used for analysis of the *Sulfolobus solfataricus* P2 genome initiative as well as for others (17). Readers can access this site at www.mcs.anl.gov/home/gaasterl/magpie.html for a particularly well though out analysis of genomic information.

Metabolic Pathways - In parallel to the rapid emergence of nucleotide sequence information has been the development of graphic representations of the genomic complement of metabolic pathways. This work was linked to the sequencing initiatives of the two genomes, first *E. coli* and then *H. influenzae*, (18,19). In EcoCyc a metabolic pathway can be chosen from a menu and then shown in overview (20). The user can follow the pathway in progressively more detail. Individual reactions are shown indicating the enzyme involved and its EC number, where known, with links to ENTREZ and GenBank as well as reference information on the enzyme and its corresponding protein (and gene) sequence. HinCyc follows the same format for the metabolic pathways present in *H. influenzae* predicted from the full genomic sequence and is based on the EcoCyc format. The pathway enzymes are linked to sequence reports present at the TIGR Website for the *H. influenzae* genome (21). As a consequence, HinCyc is currently more complete than EcoCyc with respect to sequence linkages. By linking genomic sequence to metabolism, EcoCyc and HinCyc allow one to make instant comparisons between biochemical and corresponding genetic organizations for any metabolic pathway. Another approach to integrating genomic data with biochemical pathways uses the Boehringer-Mannheim biochemical pathways chart (Http//:www.expasy.ch/cgi-bin/search-biochem-index). As shall be discussed below, this information will become valuable when attempting to understand novel pathways and their corresponding genes whose polypeptide products may be selected as future drug targets or which may play a role in bacterial pathogenesis.

FUNCTIONAL GENOMICS

Although bioinformatics can enable one to assess whether the function of a given ORF is known, it cannot assign function to a sequence for which there is no match in searchable databases. From a drug development point of view, it is important to determine which genes are essential for the growth of the organism *in vitro* and/or *in vivo*, as well as to know the function of the given gene in question. Knowledge of a gene's role in the virulence of the organism is also desirable. These subjects are grouped under the title of functional genomics and new developments in this area are described here.

Essential Genes *in vitro* - The absolute standard in determining whether a gene is essential for the survival of the host organism is to genetically disrupt the gene and assay for viability: survival indicates the gene is NOT essential. Simple gene disruption experiments employ homologous recombination between an internal segment of the test gene and the chromosome. The gene fragment is generally cloned in a 'suicide' vector (e.g., one that contains a temperature sensitive replication origin and cannot be cannot be maintained at elevated temperatures) that carries a selectable marker (e.g., an antibiotic resistance gene). Survival of the cells at the non-permissive temperature in the presence of the antibiotic is dependent upon

integration of the plasmid into the chromosome, thereby allowing the selective marker to be maintained. In bacteria, integration occurs through single reciprocal recombination between homologous sequences, that is the corresponding sequences common to both plasmid and chromosome: in this example, the segment of the gene on the plasmid and its full length chromosomal counterpart. Such homologous recombination results in the disruption of the chromosomal gene by integration of intervening plasmid sequences. If the cells carrying the integrated plasmid continue to grow, it can be concluded that the gene was not essential, at least under the conditions employed in the experiment. This approach is laborious and time consuming, requiring significant effort in plasmid construction and mutant analysis. In addition, an established genetic system is required for the bacterial species in question: plasmids must be available, as well as methods to move DNA into the host. Furthermore, if the knockout does not occur, sufficient controls must be done to be assured that failure to observe recombination was due to loss of the cells through loss of the essential gene. These procedures limit the number of genes that can be examined. Considering that upwards of 40% of the total number of genes in bacteria are not characterized, single gene disruption does not offer a practical methodology to assess genomic function.

Higher throughput methods to do gene disruptions make use of transposon-based mutagenesis. If the genomic sequence is known, identification of the location of the transposon after integration can be rapidly obtained in the following manner. An oligonucleotide (oligo) based on a sequence close to one end of the transposon is synthesized. Oligos specific for sequences at the start of desired genes (gene signatures oligos) are also made. After transposition has occurred, genomic DNA is prepared from samples of cells and used as templates in PCR reactions employing the gene signature oligo as one primer and the transposon-based oligo as the other. The PCR products are then analyzed by agarose gel electrophoresis. Appearance of amplified bands of discrete size corresponding to a segment of DNA from a given set of primers indicates that the transposon is present in the gene. The population of transposon-mutagenized bacteria can be analyzed repeatedly and the amplified band representing the insertion can be followed over time. Maintenance of the insertion through many generations indicates that the transposon is in a non-essential gene. Since many PCR reactions can be run on the DNA samples, it is possible to rapidly screen for the presence of transposons in a large number of genes. It should be emphasized that while one can use this procedure to rapidly determine which genes are non-essential, it is not necessarily correct to assume that genes that do not yield amplified bands are essential. Transposition is not completely random and some sequences are refractory to the transposition event. Furthermore, since each individual transposition event is analyzed in an population of cells in which random transposition has taken place, it is possible that some cells that have undergone multiple transpositions will be lost through transposition into an unanalyzed gene. In addition to the need for the genomic sequence from which to make the oligo primers, this procedure depends upon a transposon that can undergo high frequency, random transposition in the desired genetic background. Transposons of this sort are not yet available for all important pathogens. This methodology was first developed for the yeast *Saccharomyces cerevisiae* whose genome has been fully sequenced (22). Since the host is diploid, single transpositions into a single copy of an essential gene are not immediately lethal. Thus cells carrying such a transposition can be

propagated for a few generations until a diploid mutant is obtained. In fact, colonies carrying conditionally lethal transpositions could be obtained and examined for the loss of the signature amplified band when the cells are grown under different conditions. It remains to be determined whether direct visualization of band loss through transposition into an essential gene can be accomplished with bacterial genomes.

Essential Genes *in vivo* - An *in vivo* essential gene is defined as one that is required for maintenance and growth of the bacterium in an animal. If the gene is required for *in vivo* growth but is not required for standard growth *in vitro*, it is commonly referred to as a "virulence" or "pathogenesis" gene. These are distinguished from genes required for growth under all conditions, e.g. genes encoding ribosomes, RNA polymerase, DNA gyrase, cell wall enzymes, etc. Variations of the methodologies described above for determining *in vitro* essentiality can be applied to *in vivo* experiments. A library of 'signature tagged' transposon mutants that survive *in vitro* can be used to infect an animal host. The bacteria are then recovered and PCR is used to determine which individual clones are still present. Individual mutants that are lost during the infection must represent functions that are required for virulence. However, since growth of bacteria *in vivo* is difficult to measure, it may not be possible to visualize the loss of a particular gene. Parallel *in vitro* experiments are required to demonstrate the random nature of the transposition and the ability to determine the survivability of cells *in vitro* carrying insertions in true virulence genes.

Gene Expression *in vivo* - Another interesting approach to discovering genes that are specifically expressed during infection, termed "in vivo expression technology (IVET)", was developed in J. Mekalanos' laboratory at Harvard Medical School. Here, a promoterless reporter gene and a selectable marker (again, antibiotic resistance) were placed close to one end of a transposon that could integrate into the chromosome of several Gram negative bacteria (23, 24). For resistance to be expressed and the reporter gene to be active, the transposon will have to insert adjacent to a promoter sequence. After growth of the transposition mutants *in vitro*, a population not expressing the reporter gene is picked and used to infect animals that are given large doses of the antibiotic. Bacteria recovered under these conditions must have expressed the resistance gene in the animal: the promoter, therefore, must have been turned on *in vivo*. Characterization of the sequences upstream of the resistance gene will yield the identity of the promoter and its corresponding gene. This genetic approach could be useful in leading to the identification of selectively expressed genes.

MEDICAL/PHARMACEUTICAL APPLICATIONS

The increasing emergence of antibiotic resistance has necessitated the search for novel drugs that attack new bacterial targets, but which targets are the most suitable for drug intervention has not yet been settled upon. In this regard, genomic sequencing can provide the ultimate series of choices. However, as outlined above, sequencing and bioinformatics provide only the beginning of the drug discovery venture and require the applications of genetics, biochemistry and molecular biology to yield novel targets. However, certain benefits from the sequencing initiatives are already apparent. Where one was employing an enzyme isolated from *E. coli* or another non-pathogen as a surogate for the enzyme from the pathogen, the availability of the entire genomic sequence from the desired organism allows one to

rapidly find the gene in question, determine if it has close homologs and provides the rationale for its cloning and expression. Sequence comparisons at the genomic level can also enable one to determine the distribution of a putative target and its variation from strain to strain. Information accumulated from genomic efforts, therefore, will allow judicious choice of targets that will enhance the probablility of broad coverage of new drugs and, hopefully, for targets well differentiated from mammalian cells, with likely reduced toxicity.

Other, less apparent benefits also emerge from the genomic approach. It is a general rule in bacteria that associated biochemical functions are physically clustered along the chromosome. Consequently, when examining genes at a given locus where most functions are understood, it is highly probable that an uncharacterized ORF is also involved in a related function, thereby providing a hint for biochemical characterization. This has proven to be true for "pathogenicity islands" that contain large numbers of adjacent genes involved in virulence in a number of organisms.

We believe, therefore, that the analysis and use of bacterial genomic sequences (which represents a 'top down' approach) will rapidly lead to exciting discoveries of new genes and novel biochemical functions, which will have significant impact on new drug discovery. It is fair to state that the era of "reverse genetics" (isolate the protein, determine its N-terminal sequence, make probes and find the corresponding gene) is rapidly coming to an end. With the continued rapid appearance of new genes, the putative sequence of every bacterial enzyme is becoming a reality.

FUTURE DIRECTIONS

Incremental advances in sequencing technologies, PCR amplification and DNA isolation, and improvements in bioinformatics will continue to make the process of genomic sequencing more realistic to small scale sequencing institutions. With continued effort, it is very likely that the genomic sequence of every important pathogen as well as from a diverse array of microbes (bacteria, and fungi) will be obtained within the next 10 years. It is also likely that genomic sequences will be obtained from microbes that cannot be cutured: DNA will be obtained from its natural depository (i.e., the soil, an animal, etc.). This will necessitate genomic sequencing from mixed DNA samples.

With the sequence of the genome in hand, it should be possible to look at expression of every gene in the bacterium at both the RNA and protein level. This was accomplished recently in the yeast *S. cerevisiae* where absolute levels of mRNA for every gene was determined using ^{32}P-labeled oligos made for each gene and used as hybridization probes with total isolated mRNA (25). The ability to place gene probes in micro-arrays on chips or slides and then automate the quantitative determination of gene expression by hybridization is at most one or two years away. This powerful technology will enable a global understanding of bacterial cell biology including the linking of regulatory effects on distantly related operons and genes. Global visualization of gene expression at the protein level is also becoming a reality. Proteomics, the term used by some for genomic protein expression, employs large format 2-D gel electrophoresis to visualize, identify and quantify protein expression levels. Matching spots on gels to gene sequences will still require some effort, however. Thus, although the complete DNA sequence of an organism can be

considered a significant scientific advance, the full realization of the potential of the genomic approach for discovery of new drugs will become apparent when this information is coupled to the ability to monitor the activity of every gene in a cell.

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Chapter 13. Resistance to Antiretroviral Drug Therapy

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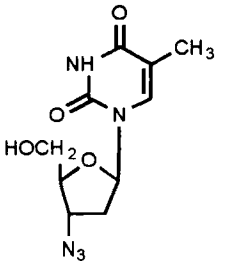
Introduction - The development of drug resistance during antiretroviral therapy has presented a formidable challenge that has limited the successful treatment of HIV-1 infection. In the ten years since the introduction of the first AIDS drug, zidovudine (AZT), overcoming clinical drug resistance has been a central focus of most drug discovery efforts. The development of highly potent combination regimens currently employed for viral suppression has benefited not only from a better appreciation of how HIV becomes drug resistant, but from a much deeper understanding of the dynamics of viral replication during HIV infection.



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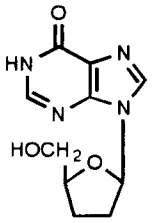

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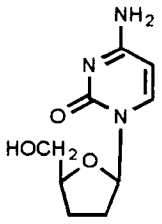

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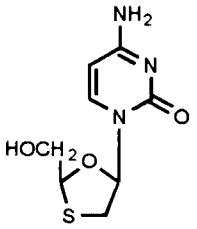

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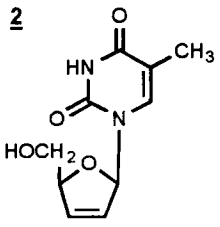

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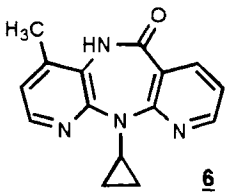

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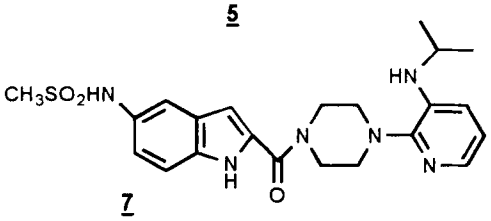

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There are a relatively large number of agents from several major drug classes that are currently marketed for treating HIV infection. The largest number of these drugs target the viral reverse transcriptase (RT), which is an essential enzyme that operates in the early phase of the replication cycle to generate proviral DNA from the RNA that is packaged with the virus. RT inhibitors fall into two general classes: nucleoside analogues that are phosphorylated by cellular enzymes (1) to triphosphate derivatives that subsequently act as chain terminators of the RT polymerization reaction; and, non-nucleoside reverse transcriptase inhibitors (known collectively as NNRTIs) which are derived from a diverse number of chemical classes that share the ability to inhibit the enzyme by binding at a common site on the reverse transcriptase that is remote from the catalytic center. The nucleoside analogues that are currently approved for the treatment of HIV infection include 3'-azido-3'-deoxythymidine (1,

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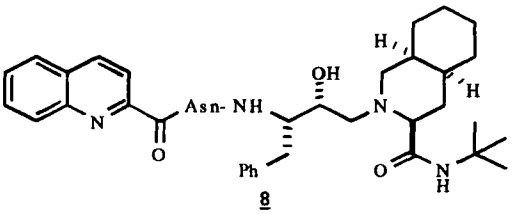
AZT, zidovudine), 2',3'-dideoxyinosine (2, ddI, didanosine), 2',3'-dideoxycytidine (3, ddC; zalcitabine), (-)-β-L-1-2',3'-dideoxy-3'-thiacytidine (4, 3TC, lamivudine), and 2',3'-didehydro-3'-deoxythymidine (5, d4T, stavudine). Nevirapine (6) and delavirdine (7) are currently the only NNRTIs approved for HIV therapy. Other compounds from both classes of RT inhibitors have been disclosed which are at various stages of preclinical and clinical development.



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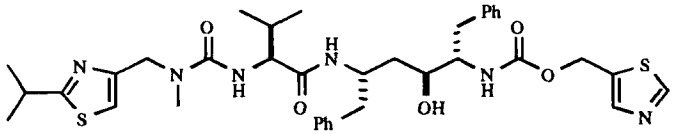

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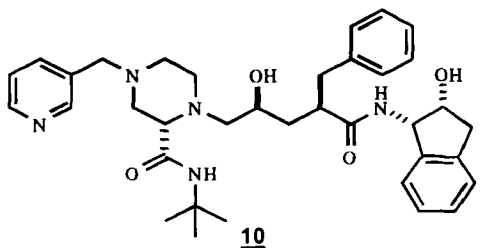

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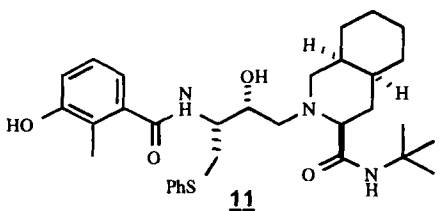

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HIV-1 protease has emerged more recently as an important target for chemotherapeutic intervention. To date, numerous peptidomimetic inhibitors of HIV protease have been reported, many of which are exceedingly potent inhibitors in biochemical assays. This has translated into potent IC_{50} values for inhibiting viral replication in cell culture, reflecting the key role HIV protease plays in cleaving the HIV *gag-pol* polyprotein during assembly and budding of HIV. Moreover, unlike RT inhibitors, which only block HIV replication in acutely infected cells, protease inhibitors also inhibit production of infectious virus from chronically infected cells (2). There is a growing body of information that describes the clinical efficacy of protease inhibitors as monotherapy and the highly suppressive effects of these drugs when used in combination. Presently approved protease inhibitors include saquinavir (8), ritonavir (9), indinavir (10), and more recently, nelfinavir (11). Other protease inhibitors are in clinical development (141W94, ABT-378, and U-140,690).

The considerable efforts that have gone into the discovery and development of antiretroviral agents have generated a large body of information on the drug

resistance that emerges during therapy. In turn, these agents have served as important tools to further investigate important aspects of viral replication in HIV-infected individuals. There is now a clearer picture of specific HIV mutations that are responsible for resistance development and the cross-resistance patterns seen with different drugs. In addition, genotypic characterization of viral isolates during drug therapy has provided a temporal view of the stepwise progression of resistance development that can occur with some drugs--observations which can be accommodated by current models of HIV viral dynamics in the infected patient.

Biological Basis of Rapid Resistance Development by HIV - It has long been known that the replication of retroviruses is accompanied by a high rate of errors due to the infidelity of the retroviral reverse transcriptase and the lack of proof-reading mechanisms (3). Mansky and Temin (4) recently assessed the replication fidelity in HIV and estimated a mutation rate in the range of 3×10^{-5} mutations per nucleotide per replication cycle. To appreciate the impact that this mutation rate has on resistance development, it should be viewed in the context of the dynamics of viral replication during the course of HIV infection.

The availability of sensitive viral load assays, as well as a variety of highly potent drugs, has provided the means to quantitatively assess the dynamics of HIV infection in humans. Work from several centers has disproved the long-held model for HIV infection that attributed the long clinical latency period typically seen after initial HIV infection to a true period of viral latency when minimal replication occurred. Rather, what appears to be the case is a continual high rate of viral replication which is concealed during the early stages of infection by a vigorous immune response. Initial estimates of virus production, obtained by modeling changes in viral load in response to antiretroviral agents, suggested a very high rate of virus turnover with a minimum production of 10^8-10^9 virions per day on average per infected individual (5, 6). More recently, it has been estimated that the average total HIV-1 production is 10×10^9 virions per day per infected individual using viral load data obtained during a clinical trial with ritonavir (7).

Many investigators have pointed out the vast number of HIV mutants that can potentially accompany this level of virus production. Given the size of the HIV genome (10^4 nucleotides), the error rate of reverse transcriptase, and the kinetics of viral replication, every point mutation can theoretically occur 10^4 to 10^5 times per day in an infected individual (8). This is underscored by several studies that document the occurrence of pre-existing drug mutations in drug-naive patients (9, 10). Clearly, a variety of additional factors affect the actual rate of emergence of HIV resistance during therapy, such as the fitness of different viral mutants, virion half-life, drug levels achieved and viral clearance by the immune system. This more accurate picture of HIV population dynamics readily explains the uniform failure of monotherapy for HIV infection and provides a stronger theoretical basis for devising combination therapies and for predicting the necessary pharmacological properties necessary for highly suppressive anti-retroviral therapy.

Scope of Drug Resistance Seen with HIV - The rapid replication rate and potential for genetic variability in HIV have led to the identification of a remarkable number of HIV variants that exhibit laboratory/clinical resistance to antiretroviral drugs. A recent compilation (11) lists nearly 150 mutations that render HIV less susceptible to various clinically-used or experimental agents. It is somewhat sobering to recall that the early interest in HIV protease as a therapeutic target was based in part on the hope that minimal resistance development would occur against protease inhibitors because of this enzyme's vital role in viral maturation and assembly. However, in spite of the highly coordinated and site-specific cleavages necessary for successful polyprotein

processing by HIV protease, over 40 distinct protease mutations have been identified in this 99 amino acid homodimer which provide significant protection against protease inhibitors (11).

In spite of the potential for viable mutations at many sites on the HIV genome, recent clinical experience with different antiretroviral agents (and combinations) suggests that distinct resistance patterns can predominate in patients during therapy with different agents. A considerable amount of data is being continuously acquired which allows a comparative assessment of mutation sites, durability of therapy before resistance development, and the genotypic changes occurring over time as a patient progresses from fully drug-susceptible to drug-resistant This information should be useful in devising optimal treatment regimens with current agents, as well as in developing novel therapies.

Resistance to Reverse Transcriptase Inhibitors - Reduction of drug efficacy due to the rapid emergence of mutations has long been associated with agents directed against HIV RT(12, 13). HIV-1 can develop high level resistance *in vitro* to many RT inhibitors due to a single mutation in the RT gene (see Table 1). For example, treatment with 3TC leads to the rapid acquisition of a mutation at codon 184 in the active site of RT and rapid loss of antiviral potency during therapy (14). For nevirapine, an NNRTI, a single point mutation can confer a 2- to 3-log decrease in drug susceptibility leading to the rapid development of clinical resistance (15). The binding site of all NNRTIs is remote from the catalytic center and is comprised of two stretches of amino acids from 100-110 and 180-190 of the RT sequence. As can be seen in Table 1, mutations that result in clinical resistance occur within these regions. Clinical trials have revealed that the viral suppression provided by monotherapy with many of these agents lasts only a few weeks, likely reflecting the requirement of only one mutation to achieve a significant drop in susceptibility and the likely pre-existance of such drug resistant viral populations prior to treatment. The re-emergence of high plasma viral levels occurs in all cases with drug resistant virus, providing important confirmation of the relationship between loss of drug potency and resistance in the infected patient.

By contrast, clinical resistance to AZT occurs more slowly. HIV-1 isolates obtained from individuals during AZT treatment show a decreased sensitivity to AZT resulting from the accumulation of several specific mutations in the HIV-1 RT gene. The following amino acid substitutions typically occur: M41L, D67N, K70R, T215Y/F, L210W, and K219Q (11, 16). Clinical experience with AZT suggests that ongoing viral replication must occur for many months against the selective pressure provided by AZT before HIV isolates with the appropriate complement of mutations emerge. These multiple mutations may be derived by the acquisition of additional mutations in pre-existing single or double mutants, or through genetic recombination events. Several studies have documented the shifts in viral genotype that occur over time during AZT therapy, including the demonstration of mixtures of mutations during the transition phase from susceptible wild type to fully mutated, resistant viral populations (17).

Similar correlations of mutant viral genotypes with diminished drug susceptibility have also been established for ddI and ddC (18, 19). After extended monotherapy with ddI the L74V mutation is the most abundant genotype observed; however, changes in susceptibility are less dramatic than those observed with AZT-resistant isolates.

Combination therapies with nucleoside analogues induce greater and more sustained improvements in markers of disease severity (higher CD4 counts and lower

plasma HIV RNA titers) than are typically seen with monotherapy regimens (20). Trials of AZT/nevirapine and AZT/3TC combinations confirm the clinical benefits of these regimens (14, 20). Ultimately, however, virus can be isolated from patients with both AZT and 3TC drug resistance mutations. Upon prolonged therapy with these agents, viral variants show amino acid changes in the RT gene that lead to gradually increasing resistance. It has been demonstrated that rebound of the HIV-1 RNA load in the case of AZT is due to both the outgrowth of wild-type virus and drug resistant mutants; whereas in the case of 3TC, viral RNA rebound can only be attributed to the emergence of drug-resistant mutants. Another study demonstrated that the evolution

Table 1. Mutations in HIV-1 RT associated with drug resistance *in vivo*

Drug	*In vivo* mutation	Cross resistance
AZT	M41L D67N K70R T215Y T215F L210W K219Q	
ddI	K65R L74V V75T M184V	ddC ddC ddC/d4T ddC/3TC
ddC	K65R T69D L74V V75T M184V Y215C	ddI ddI ddI/d4T ddI/3TC
d4T	V75T	ddI/ddC
3TC	M184V M184I	ddI/ ddC
Nevirapine	A98G L100I K103N V106A V108I Y181C Y181I G190A	 DMP-266 Delavirdine//DMP-266/HBY-097 Delavirdine/DMP-266
Delavirdine	K103N K103T Y181C P236L	Nevirapine/DMP-266/ HBY-097 Nevirapine
DMP-266*	L100I K101E K103N V108I V179D Y181C Y188L	Nevirapine Nevirapine/Delavirdine/ HBY 097 Nevirapine Nevirapine/Delavirdine
HBY-097	K103N	Nevirapine/ Delavirdine/DMP-266

*mutations developed *in vitro*

of drug resistance proceeds more slowly in the case of AZT because AZT is not as effective as 3TC, and the first AZT drug-resistant mutant is competing with the rebounding wild-type virus (14). HIV isolated from patients after prolonged therapy with AZT/ddC (41 months) showed resistance to AZT only (21). It was concluded that HIV-1 developed reduced susceptibility to AZT more readily than to ddC. In another study, patients who received long term AZT therapy and then ddI therapy developed reduced susceptibility to ddI and the emergence of ddI resistance was associated with reversion to a more AZT sensitive phenotype (19).

It is important to point out that different RT mutation combinations may not necessarily be compatible. *In vitro* studies suggest that in addition to being simply additive, these mutations may antagonize or synergize with each other (22, 23). Simple additivity was observed when the ddI resistance mutation, 74V, was introduced into AZT resistant virus such as 41L/215Y by site directed mutagenesis. The resulting strain was cross-resistant to both ddI and AZT (24). By contrast, 3TC resistance mediated by the M184V mutation can reverse AZT resistance (25). AZT resistance can be associated with modest reductions in susceptibility to ddI and ddC (18).

Resistance to Protease Inhibitors - Recent clinical studies have confirmed that protease inhibitors constitute a significant step forward in achieving viral suppression when used in combination with RT inhibitors. Protease inhibitor monotherapy typically demonstrates a rapid decline in mean plasma viral RNA with a concomitant increase in circulating CD4 cells (26, 27). A clear relation is observed between increasing drug doses and the duration of response. Phase III studies with ritonavir demonstrated marked delay in the progression of HIV-related disease and prolonged survival in patients with advanced AIDS. Although the plasma viral RNA in most patients initially falls beneath the detectable levels, this measure of viral load tends, over time and as a function of inhibitor dose, to return to pre-treatment levels in some patients (26, 27). The rebound in viral replication is associated with the emergence of virus with diminished *in vitro* susceptibility to drugs (10).

Drug	***In vivo* mutations**
Ritonavir	20/33/36/46/54/63/71/**82**/84/90
Indinavir	10/20/24/**46**/54/63/71/**82**/84/90
Saquinavir	10/**48**/54/63/71/84/**90**
Nelfinavir	**30**/35/36/46/71/77/88/90
141W94	10/30/36/**50***/82[1]

primary mutations are in bold numbers, other numbers reflect secondary mutations frequently seen with a given agent; *developed *in vitro*, [1]*preliminary results*

Interestingly, the resistance patterns seen after therapy with different protease inhibitors are distinct. Population and clonal sequencing of the protease genes from resistant isolates revealed that V82 is the primary mutation selected by ritonavir (Table 2 & ref. 10). Initial variation at position 82 was consistently observed,

regardless of time of therapy, followed most frequently by the stepwise accumulation of mutations at other positions such as 54, 71, 36, 46, 20, 33, 84 and 90 (10). Multiple mutations were observed only in HIV from patients who experienced either a lack of complete suppression of plasma viral RNA, or a rebound from undetectable serum levels. By contrast the mutations found consistently during saquinavir treatment were L90M and G48V, which reflected the mutations conferring reduced saquinavir sensitivity *in vitro* (28). The development of substitutions at 82 were also observed in the majority of the patients who received indinavir (9). Among indinavir treated patients, substitutions at other protease positions were detected (Table 2 and ref. 9). DNA sequence analysis of protease genes from nelfinavir-treated patients indicated that D30N is the primary mutation observed (Table 2 and ref. 29). The appearance of D30N was associated with the emergence of mutations at other positions that include: 35, 36, 46, 71, 77, 88, and 90.

Primary mutations are not sufficient to render the virus resistant to protease inhibitors. As observed with AZT where several mutations in the RT gene are necessary for clinical resistance, the transition from being fully susceptible to protease inhibitors to clinically-resistant requires the accumulation of a predictable complement of mutations in the protease gene (9, 10). For example, alterations at residue 82 are necessary but not sufficient for high level resistance to indinavir or ritonavir. The early mutants selected by ritonavir retain considerable sensitivity to ritonavir, and it is the selection of additional mutations that confers an increasing level of resistance. In general, primary mutations occur at the active site of the HIV protease; however, many of the secondary mutations that are necessary for full expression of drug resistance actually occur on the surface of the enzyme. It should also be pointed out that viral isolates harboring multiple mutations have been identified which retain sensitivity to ritonavir. Interestingly, the mutations did not include variations at V82. Further work is necessary to establish how alterations at sites remote from the catalytic center contribute to changes in drug susceptibility.

As would be predicted from these resistance patterns, the highly ritonavir-resistant multiple mutants displayed cross-resistance to both indinavir and nelfinavir, but retained wild-type sensitivity to saquinavir and 141W94 (10). Saquinavir post-treatment isolates containing the L90M mutation in the protease gene showed some loss of sensitivity to saquinavir and retained full sensitivity to ritonavir. Isolates from some patients containing a rare double G48V/L90M mutation showed some cross-resistance to other protease inhibitors; but retained full sensitivity to ritonavir (10). Highly indinavir-resistant virus showed appreciable cross-resistance to ritonavir, 141W94, and saquinavir (9).

In vitro passage against pairs of protease inhibitors resulted in a delay in the selection of resistant variants compared to passage against the individual inhibitors, suggesting that combinations of protease inhibitors with distinct resistance patterns may extend the durability of antiviral therapy. Ultimately, however, dual resistant variants can be isolated after combination passage with 141W94/ ritonavir (16E, 32I, 46I, 82I, and 84V) or 141W94/nelfinavir (32I, 46I, 54M and 71V). Resistant mutants were also isolated from dual protease inhibitor combinations such as indinavir plus saquinavir (10F, 48V, 54S, 71V, 77I and 90M) (30, 31). Clinical trials have demonstrated that ritonavir/saquinavir combination therapy results in a profound suppression of plasma viral RNA levels to below 200 copies/ml in 80-90% of the patients. Interestingly, in compliant patients suffering viral rebound (5 total), the primary mutations seen were at positions 82 and 54, which are frequently selected by ritonavir. However, one non-compliant patient, who failed therapy, had mutations associated with both drugs (32).

Several studies have demonstrated that combinations of agents which inhibit HIV at different stages of its growth cycle can result in synergistic inhibition of replication. The combination of ritonavir with nucleoside analogs such as AZT and ddC was shown to have more potent antiviral activity than single agent or combinations of other agents such as AZT and 3TC. In another study, a retrospective analysis showed that the number of sustained responders after one year was significantly higher in the saquinavir/AZT combination arm than with AZT monotherapy, and correlated closely with the absence of genotypic resistance (28).

Use of nucleoside analogs can result in a variety of serious side effects. AZT has been found to produce bone marrow suppression (33), ddI can cause pancreatitis (34), and both ddI and ddC can give rise to peripheral neuropathy (35). Combinations of other agents have yielded some promising results in human trials. In spite of the rapid resistance associated with NNRTI monotherapy, NNRTI/protease inhibitor combinations show considerable promise. After 12 weeks of combination treatment with DMP-266 and indinavir, a mean reduction of -3.2log (99% suppression) in HIV RNA was observed (36). These findings suggest that combination therapy with a protease inhibitor and an NNRTIs might extend the durability of antiviral activity.

Drug Pharmacokinetics and Virus Suppression - Clinical experience clearly indicates that maximal virus suppression requires high and sustained levels of antiretroviral agents. For example, with ritonavir monotherapy, the magnitude of the viral load decrease and the durability of virus suppression was clearly dose dependent (26, 27). Interestingly, the rate of accumulation of HIV mutations in infected individuals during therapy correlated inversely with the trough plasma concentrations of ritonavir. Thus, the lower that drug levels drop in between doses, the more likely it is that drug resistant quasispecies will appear. No relationship was observed between the selection rate of viral mutants and baseline plasma viral RNA levels or CD4 levels (10). An extension of these studies suggests that the duration of viral suppression is correlated inversely with the actual viral load achieved during therapy (37).

These data suggest that enhancements of plasma drug levels would extend the suppressive effects of antiretroviral therapy. The potent inhibition of cytochrome P450 (IIIA) by ritonavir (38, 39) provides an interesting approach to achieving this. Animal studies have shown that co-administration of ritonavir elevates the plasma concentration of peptidomimetic protease inhibitors by interfering with cytochrome P450-mediated clearance (39). This effect is most pronounced with ABT-378, a potent HIV-1 protease inhibitor currently under clinical development (40). Co-dosing of ritonavir considerably enhances the plasma levels and half-life of ABT-378 in rodents and dogs (41, 42). Clinical trials are necessary to confirm the extent of these interactions in humans and to assess the impact of sustained drug levels on viral load and the duration of viral suppression.

Summary - The definition of viral resistance mechanisms and the development of agents that target resistant variants will be critical to the design of successful new treatment strategies for HIV infection. Recent studies of HIV replication dynamics suggest variable, but finite half-lives for the elimination of HIV-infected cell populations--if mutant virus outgrowth can be completely suppressed (6). Such efficacious and durable antiretroviral therapy will depend, not only on the identification of highly potent inhibitors with activity against mutant isolates, but on the optimization of pharmacological properties to ensure continuous and maximal suppression of drug resistant variants and the widest possible distribution to body sites that harbor replication-competent virus.

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Chapter 14. Non-HIV Antiviral Agents

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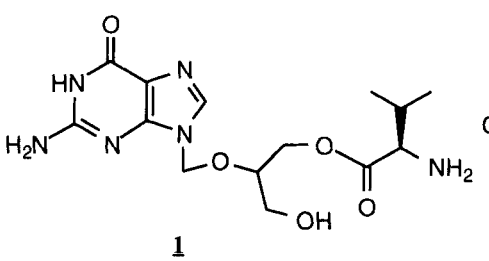
Introduction - In spite of the attention that the development of anti-HIV agents has garnered recently, the search for antiviral agents targeted against a variety of other viruses has continued to yield novel compounds for the treatment of non-HIV diseases.

Herpes Viruses - A detailed review of the most clinically advanced and newly approved anti-herpetic agents has appeared (1), also published was a review of the patent literature through 1995 (2).

Considerable effort is being expended pursuing analogs to improve the oral bioavailability of known, (or perhaps older) antiviral agents. The ganciclovir pro-drug RS-79070, mono-valine ester (**1**), was shown to be 61% bioavailable in humans based on the AUC of ganciclovir (3). In order to mitigate the nephrotoxicity of cidofovir (**2**), an orally biovailable prodrug, GS 3857 (**3**), the butyl salicylate ester of cyclic HPMPC (**4**) was developed. It exhibited 46% bioavailability in dogs based on cyclic HPMPC while reducing the systemic exposure to cidofovir at an equivalent dose (4). A lipid prodrug of phosphonoformate (**5**) was reported to be 93-fold more potent in cell culture than phosphonoformate itself (5).



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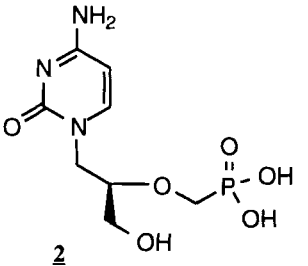

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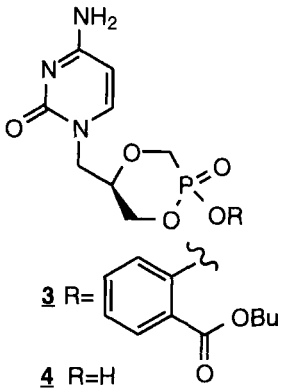
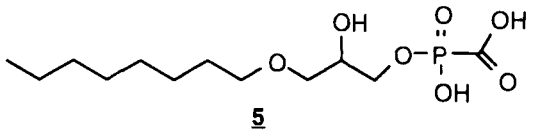


3 R=

4 R=H



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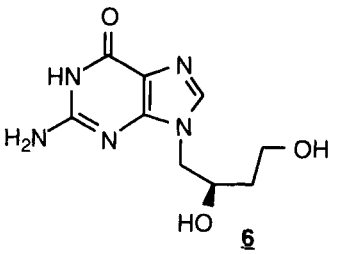

6

A study of the intracellular disposition of H2G ((R)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine) (**6**) showed that not only were H2G-triphosphate levels at least 200-fold higher in HSV-1 infected cells, but that the intracellular half-life of the

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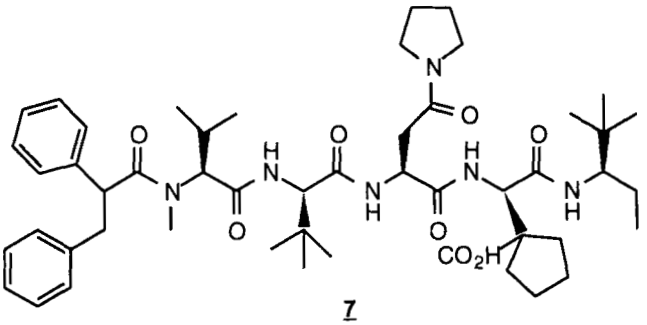
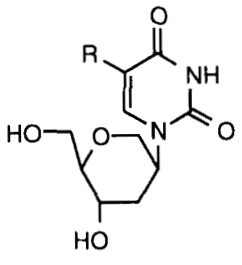
triphosphate was almost six-fold longer in HSV-1 infected cells (14 h vs. 2.5 h). The difference of triphosphate half-lives was much less pronounced in VZV infected cells (6).

Targeting HSV-1 ribonucleotide reductase by means of peptidomimetic agents afforded BILD 1357 (**7**), which exhibited an EC_{50} of 0.8 μM and 1.8 μM, respectively, in HSV-1 and HSV-2 in tissue culture (7). BILD 1357 was also found to be active in an acute murine HSV-induced ocular keratitis model (8). This class of compounds displayed significant potentiation of acyclovir and was active against acyclovir-resistant strains.

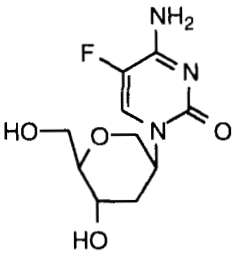


7

A series of 1,5-anhydrohexitol pyrimidine and purine nucleosides (**8-11**) showed activity against HSV-1 and HSV-2. Only the diaminopurine derivative was active against TK^- strains (9). A 2'-deoxy-2'-fluoroarabinofuranosyl pyrrolo[2,3-d]pyrimidine analog (**12**) was found to posess activity against HSV-1,HSV-2, HBV and CMV,and had comparable activity (EC_{50}=3.7 μg/ml) as ganciclovir against HCMV (10).



8, R=I
9, R=Et



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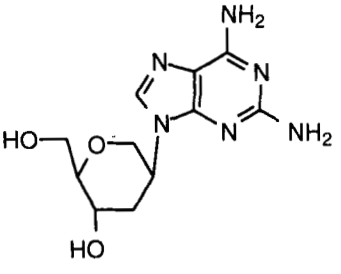

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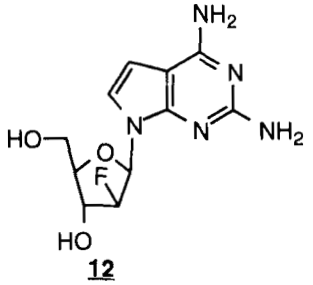

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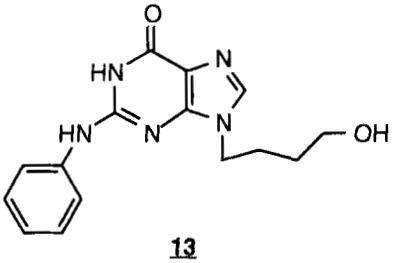
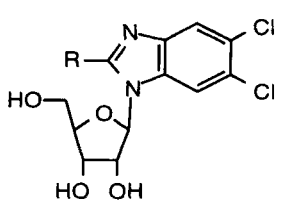
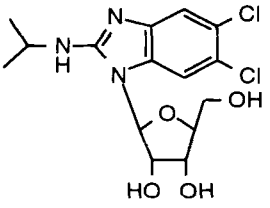


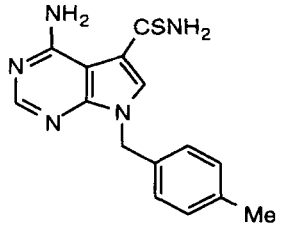
13

HBPG (**13**) , a thymidine kinase inhibitor, has been reported to posess an improved physicochemical profile as compared to 9-unsubstituted guanine analogs and was thus completely bioavailable when given to mice IP (11). Subsequent studies have shown HBPG to be effective in suppressing HSV-1 reactivation in a murine ocular

keratitis model of at doses of 200 mg/kg (12) as well as a squirrel monkey study at 150 mg/kg (13). HSV-Thymidine kinase is a potential target for interfering with herpesvirus reactivation.







14, R=H
15, R=Br

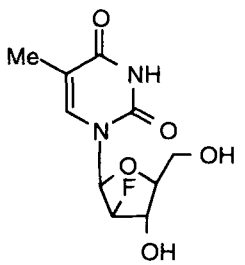
16

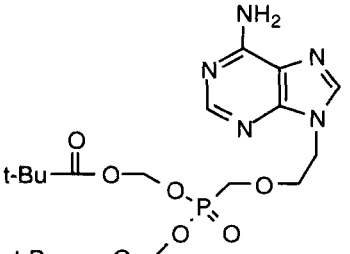
17

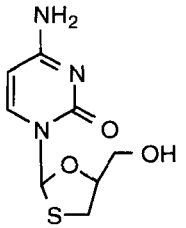
A series of analogs of DRB (**14**) was reported (14,15). The 2-bromo analog (**15**) displayed an EC_{90} of 200 nM against HCMV in a yield reduction assay. The clinical candidate chosen out of a large series of benzimidazole nucleosides analogs prepared was BW126W94, the 5,6-dichloro-2-(isopropylamino)-L-ribofuranosyl benzimidazole (**16**). This compound, suprisingly, was determined to have a mechanism of action distinct from that of the D-ribofuranosyl analogs, and exhibited several fold more potent activity *in vitro* than ganciclovir or cidofovir (16). Out of a series of 4-amino-5-thiocarboxamidopyrrolo[2,3-d]pyrimidine analogs, the 7-p-methylbenzyl congener (**17**) was significantly more potent than ganciclovir against HCMV by an ELISA assay (17).

A retrospective analysis of the association of anti-herpesvirus treatment with the incidence of Kaposi's sarcoma in HIV infected individuals has shown that treatment with foscarnet or ganciclovir, but not acyclovir, reduced the risk of developing Kaposi's sarcoma (18). Consistent with these results, HHV-8 (Kaposi sarcoma associated herpesvirus) was found to be sensitive to ganciclovir, cidofovir and foscarnet, but not acyclovir (19). Another herpesvirus that has been associated with human malignancies, Epstein-Barr virus (EBV), has been shown to be sensitive to the L-nucleoside L-FMAU (**18**) in H1 cells (20).

Among other antiviral targets, the X-ray structure of HCMV protease has been described (21). This serine protease appears to be unrelated to chymotrypsin, since it lacks a glutamate or aspartate residue normally present in the catalytic triad. Instead two histidine residues appear to activate the serine residue.







18

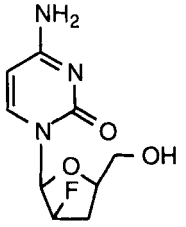
19

20

Hepatitis B and C - In a phase I/II study of adefovir dipivoxil (**19**), patients exhibited a 97% reduction in HBV DNA levels with once-a-day dosing at the end of the 28-day dosing schedule. HBV DNA levels returned to baseline within six weeks after the cessation of dosing. No seroconversions were observed and the elevation of liver transaminases was the most common adverse event (22).

A long term study of lamivudine (**20**) treatment for chronic hepatitis B has shown that suppression of HBV DNA was achieved in 20 of 23 patients after a median treatment of 52 weeks and 9 patients tested HBeAg negative (23). Lamivudine

resistant strains developed in two patients at week 36. Lamivudine resistant duck HBV mutants have been generated by site-directed mutagenesis of the duck HBV polymerase. These mutations where introduced at the methionine of the YMDD motif and are analogous to those that occur in lamivudine-resistant HIV polymerases (24). The activity of lamivudine against HBV has led to the continued investigation of L-nucleoside analogs for activity against this virus. (For a review of nucleosides see reference 25) (25). The previously mentioned L-FMAU (**18**) exhibited activity against HBV with no detectable effect on mitochondrial DNA synthesis (26). The synthesis of a series of analogs related to L-FMAU yielded the cytosine analog (**21**) as another active compound against HBV (27). The dideoxy-didehydro analogs of L-cytidine (**22**) and 5-fluoro-L-cytidine (**23**) were reported to exhibit potent activity against HBV and HIV (28).



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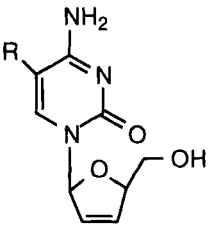
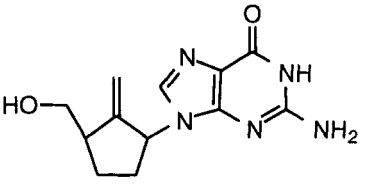


22, R=H
23, R=F

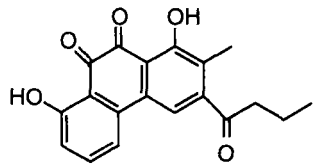


24

A structurally unique analog of dideoxyguanosine, BMS-200475 (**24**) was reported to be active *in vitro* (29) and *in vivo* against HBV (30). The metabolism to the triphosphate was shown to be carried out by cellular kinases (31). Doses of 20-500 µg/kg/day of **24** resulted in 2-3 log reductions of HBV DNA titers in chronically infected woodchucks (32).

The targeted delivery of ara-A to the liver for the treatment of hepatitisB via intramuscular administration of a lactosaminated poly L-lysine conjugate of ara-AMP was achieved without any reported immunogenicity or delayed neurotoxicity (33).

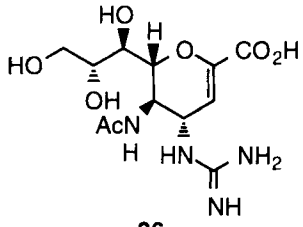
While interferon-α remains the only clinically approved treatment for hepatitis C, the recent determination of the structure of HCV NS3 protease by two independent groups has provided an opportunity for the exploitation of this target for the development of antiviral agents (34,35). One of the structures was determined with a synthetic NS4A cofactor peptide complexed to the protease. This serine protease displays some homology to trypsin and contains a zinc binding site. Due to the difficulty of developing a cell based antiviral assay for HCV, efforts have been made to develop surrogate assays for HCV protease activity. One assay coupled HCV protease activity to the enhancement of chloramphenicol acetyltransferase activity (36). An engineered poliovirus with a requirement for HCV NS3 protease activity has also been reported (37). Subsequently, a report of the characterization of a clone of a MT-2 cell line which supports HCV replication has appeared (38). This HTLV-1 immortalized cell line was found to support HCV replication for 30 days post infection. A naturally derived phenanthrenequinone inhibitor (**25**) of the NS3 protease has been reported (39).



25

Influenza Virus - Influenza virus replicates by using a number of unique proteins, many of which have been exploited as targets for chemotherapy. Influenza neuraminidase is an enzyme which cleaves terminal sialic acids off glyconjugates allowing for release of newly synthesized virus from infected cells and for efficient spread of virus from cell to

cell (40) GG167 (**26**) is a potent influenza neuraminidase inhibitor (41,42) and inhibits both influenza A and B virus *in vitro* (43,44). GG167 is also efficacious in mice (45) and ferrets (46) infected with influenza virus when administered intranasally, but is ineffective when administered orally due to its low oral bioavailability and rapid elimination. In a phase I/II clinical challenge study, intranasal administration of GG167 was well tolerated and was found effective for both the prevention and treatment of experimental influenza. GG167 prophylaxis was 95% effective in preventing febrile illness and early treatment with GG167 reduced the frequency of febrile illness by 85% (47).



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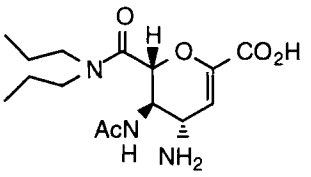
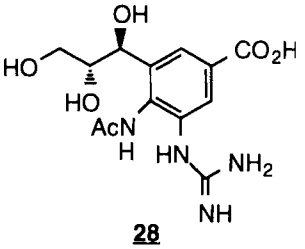


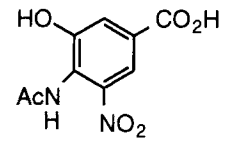
27



28

The emergence and nature of viral resistance to GG167 has been investigated. When influenza virus was passaged in the presence of GG167, a mutant emerged which showed 1000-fold less sensitivity *in vitro*. In an enzyme assay, the neuraminidase of the mutant virus was ~200-fold less sensitive to inhibition by GG167. Sequence analysis indicated a single amino acid change of the conserved Glu 119 to Gly. Crystallographic analysis suggested that the reduced efficiency to GG167 derived from the loss of the interaction of Glu 119 with the guanidine moiety of GG167 (48,49). In another experiment, influenza virus was cultured in the presence of GG167 by limiting dilution passaging. Viruses emerged which were 1000-fold less sensitive to GG167 *in vitro*. Sequence analysis indicated, however, that no changes had occured in the neuraminidase, but changes in the conserved amino acids in the hemmaglutinin were detected. These hemmaglutinin mutants seem to be allowing the virus to be released from cells with less dependence on the neuraminidase (50).

The success of GG167 and the validation of neuraminidase as a target for intervention has spurred research into potentially orally bioavailable analogs. The rationale for the design of analogs, the structural characteristics and modelling studies have been discussed (51,52). Carbocyclic analogs (53,54), modifications in the glycerol side chain (55) and modifications on the guanidine moiety (56) led, in general, to compounds with decreased potency relative to GG167. Compound **27**, where the glycerol side chain was replaced by a carboxyamide, showed *in vitro* potency against influenza A comparable to or better than GG167. It was, however, ~500X less active against influenza B (57). Similarly, the approach of utilizing a planar benzene ring for attachment of substituents has met with limited success. Compound **28** was found inactive (58), while compound **29** showed inhibition of the neuraminidase enzyme in the mM range (59). Compound **30** is a carbocyclic transition state analog with a lipophilic side chain and is a promising neuraminidase inhibitor. It exhibited *in vitro* inhibitory activity of both influenza A and B viruses comparable to GG167 (60).



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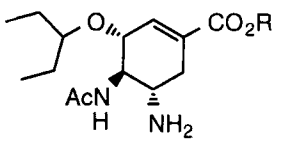
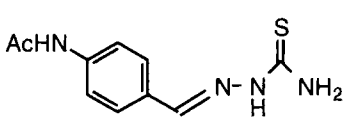


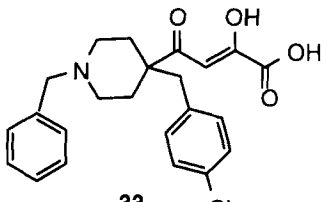
30, R=H
31, R=Et



32

X-ray crystallographic structure of **30** complexed in the neuraminidase active site revealed the existence of a large hydrophobic pocket. In order to accomodate the

large 3-pentyl group, the carboxylate of Glu 276 of neuraminidase is forced to orient outward from the hydrophobic pocket, whereas in the GG167 neuraminidase complex, Glu 276 interacts with the two terminal hydroxyls of the glycerol sidechain (60). The ethyl ester of **31** (GS 4104), exhibited good oral bioavailability (> 30%) in rats and dogs, and was efficacious on oral dosing in a mouse model of influenza infection with no toxicity observed in a 5 day repeat dose study in rats at an oral dose of 300 mg/kg/day (61). A non-sialidase inhibitor of influenza neuraminidase, thiosemicarbazone **32** was also reported (62).



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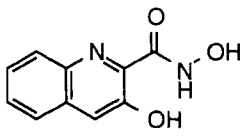

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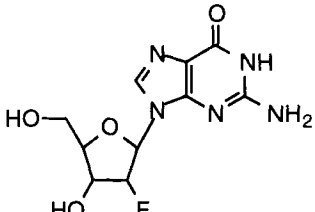
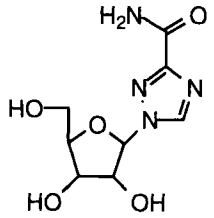


35

Another unique influenza virus enzyme is endonuclease, which cleaves capped and methylated RNA primers from RNA transcripts. These primers are subsequently used by the influenza polymerase for transcription. Screening against the cap-dependent endonuclease has resulted in the identification of inhibitors. Derivatives of the 2,4-dioxobutanoic acid **33** inhibited *in vitro* transcription and influenza viral replication with IC_{50} ranging from 0.18 to 0.71 μM (63). Other endonuclease inhibitors include flutimide (64) and the N-hydroxamic acid BMY26279 **(34)** (65). Other novel inhibitors of influenza virus which have been described include the polymerase inhibitor 2'-deoxy-2'-fluoro guanosine **(35)** and polyoxometalates which seem to act by inhibiting influenza virus fusion (66, 67).

Respiratory Syncytial Virus (RSV) - Currently, two products are approved in the U.S. for RSV infections: Ribavirin **(36)** for treatment of RSV infections and RespiGam, an immune globulin for the prevention of RSV disease in high risk infants. The chemotherapy of RSV infections has recently been reviewed (68). Doubts about the efficacy of ribavirin therapy persist (69). A monoclonal RSV antibody, which could be easier to use than RespiGam, entered into a Phase III clinical study (70). Peptides derived from conserved regions of RSV, measels virus (MV) and para influenza virus (PIV) were found to be potent and selective inhibitors of viral fusion. A peptide T118 showed an IC_{50} of 50 nM of inhibiting RSV *in vitro* (71). Other agents which have been identified recently as RSV inhibitors *in vitro* include polyoxometalates (67) and pyridobenzazoles such as **37** (72).



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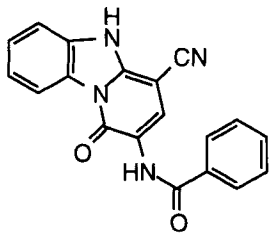

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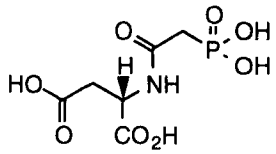
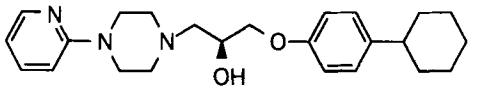


38

The aspartate transcarbamoylase inhibitor N-(phosphonoacetyl)-L-aspartate (ALA) **(38)** was found to reduce pulmonary RSV titers in RSV infected cotton rats when given at ≥ 10 mg/kg/day on days 1-3 postinfection (73).

Picorna Viruses - Human picorna viruses are divided into two subgroups: the rhinoviruses and the enteroviruses, which includes echoviruses, coxsackie virus and polio virus (74). SDZ 35-682 **(39)** was found to have potent activity *in vitro* against

several rhinovirus genotypes and against echovirus (75). It was also found efficacious in an echovirus model in mice (76). The crystal structure of a related analog, SDZ-880-061 (**40**) bound to human rhinovirus was also reported (77). SCH 47802 (**41**) and its derivatives are potent inhibitors of enteroviruses *in vitro* (IC_{50}=0.03 to 10mg/ml). Upon oral dosing, SCH 47802 was able to protect mice from polio virus induced encephalitis (78). A new analog, SCH 48973, was identified as a potent, broad spectrum anti-enterovirus compound with good oral bioavailability and *in vivo* efficacy for both the treatment and prophylaxis in a polio virus model in mice (79-81). Derivatives of WIN 54954 (**42**) were reported, which had increased metabolic stability or enhanced potency (82, 83). Reports on other anti picorna virus agents include benzimidazoles and flavanoids (84, 85). VP 63843 was evaluated in humans and was found to be efficacious following dosing in coxsackie virus infected individuals (86).



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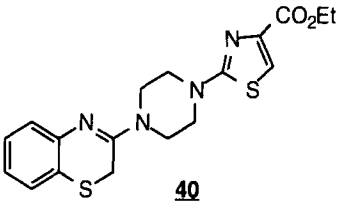
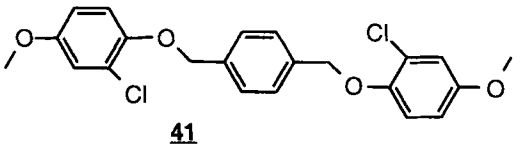


40

An *in vitro* translation assay was used to analyze inhibitors of rhinovirus 3C protease (87). The synthesis and evaluation of a number of isatin analogs led to the identification of potent inhibitors of rhinovirus protease (88). Compound **43** was found to have good selectivity versus other proteolytic enzymes, including chymotrypsin and cathepsin. The crystal structure of **43** bound to rhinovirus protease was solved (88). Compounds **44** and **45** were found to have *in vitro* anti rhinovirus activity equal to WIN 51711 but with improved therapeutic index (89).



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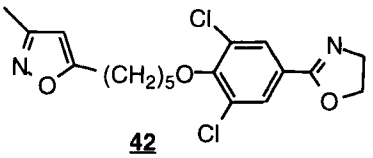

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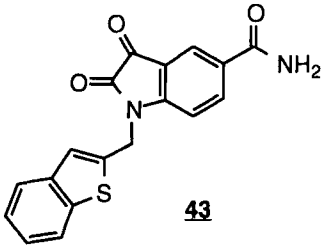

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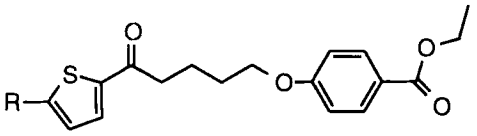

44, R=Me
45, R=Cl

Miscellaneous Viruses - The activity of topically applied cidofovir against papilloma virus infections in humans was reported (90). Cidofovir was also found to have activity *in vitro* against polyoma viruses and was efficacious *in vivo* in a murine model of polyoma virus infection (91). Various cysteine protease inhibitors were active against adenovirus replication (92). Foscarnet was found active *in vitro* against rotavirus transcription and replication (93).

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Chapter 15. Recent Advances in Antifungal Agents

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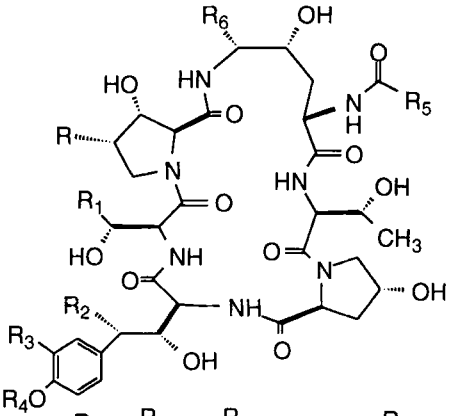
Introduction - Systemic fungal infections of immunocompromised hosts continue to be a major problem in infectious disease chemotherapy. The polyene amphotericin B remains the standard of therapy because of its broad spectrum and fungicidal activity. However, because of its safety and efficacy in candidiasis, fluconazole is now the most widely used agent. Since the last review of this area, a number of new compounds have been described and new antifungal targets defined.

Inhibitors of Cell Wall Biosynthesis - A review of the discovery, process development and isolation of the pneumocandins has appeared (1). Pneumocandin D_0 (**1**), a novel minor component from fermentation broths of *Zalerion arboricola* was reported to have the most potent activity against *Pneumocystis carinii* among the naturally occuring lipopeptides (2). A water soluble sulfonate derivative F-11899 (**2**) was isolated from *Coleophoma empedri* and shown to have *in vivo* activity against *C. albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii* in mice (3).



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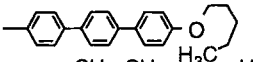
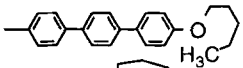
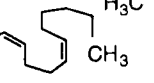
1	OH	CH_2CONH_2	OH	H	H	$-(CH_2)_9$ branched CH_3	OH
2	CH_3	CH_2CONH_2	OH	OSO_3Na	H	$-C_{16}H_{33}$	OH
3	CH_3	CH_3	OH	H	H		OH
4	H	CH_2CONH_2	OH	H	H	$-(CH_2)_9$ branched CH_3, N, NH_2	
5	CH_3	CH_3	OH	H	OPO_3HNa		OH
6	CH_3	CH_3	H	H	H	$-(CH_2)_7$ 	H

New semisynthetic derivatives of echinocandin bearing polyarylated acyl side chains have been described (4). One derivative LY303366 (**3**) has been shown to have good activity against *Candida albicans* and moderate activity against *Aspergillus* (5). LY303366 is orally bioavailable and was well tolerated in Phase I clinical trials (6).

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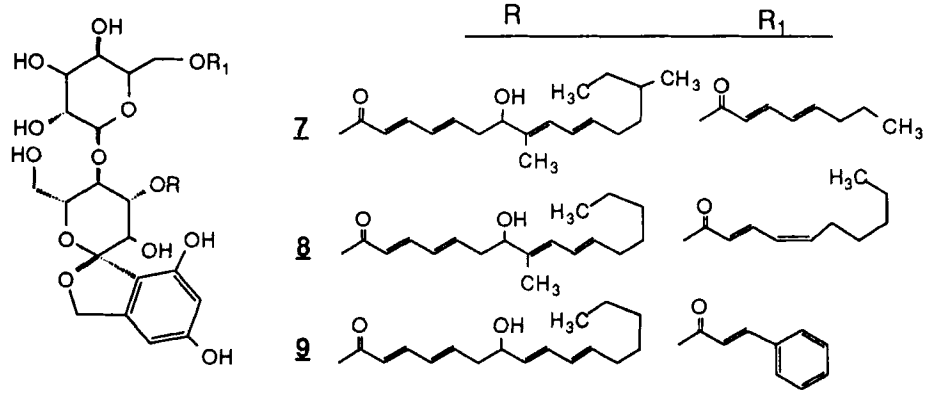
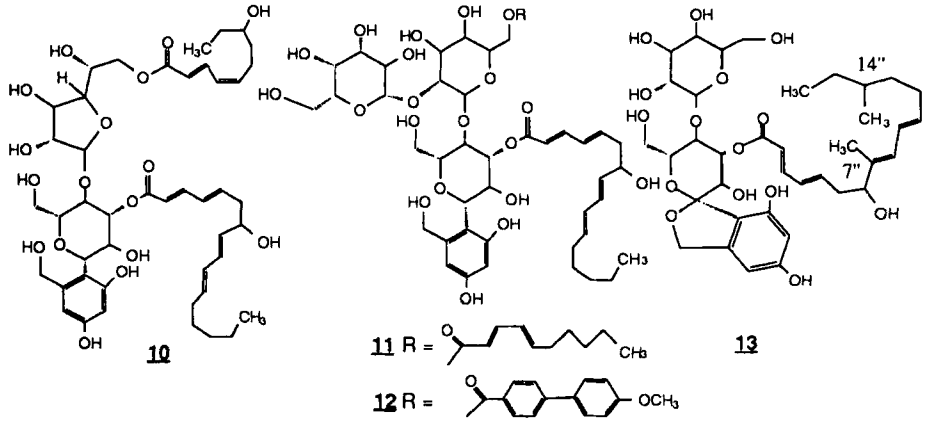
The SAR of semisynthetic derivatives of pneumocandin have also been described (7,8). Out of a series of water soluble amine containing congeners (9), L-743,872 (4) has undergone extensive preclinical examination and has been shown to possess efficacy in animal models of candidiasis and aspergillosis (10). L-743,872 (10), LY303366 and its water soluble phosphate derivative LY307853 (5) (12) are efficacious in murine models of *Pneumocystis* infections. The total synthesis of echinocandin D (6) was reported (13).

Five new glycolipids, with structural similarities to the spirocyclic disaccharide papulacandin, were described. Mer-WF3010 (7) was isolated (14) from *Phialophora cyclaminis*, BE-29602 8 (15) from a *Fusarium* strain, saricandin (9) (16) from a *Fusarium* species, furanocandin (10) (17) from a *Trichothecium* species and fusacandin (11) (18) from *Fusarium sambucinum*.



Furanocandin bears a galactofuranosyl residue as the second sugar instead of the usual pyranose, while fusacandin has a second galactose residue. Both 10 and 11 possess a C-glycosidic phenol instead of the spiroacetal moiety of papulacandin and, in that respect, are related to chaetiacandin.

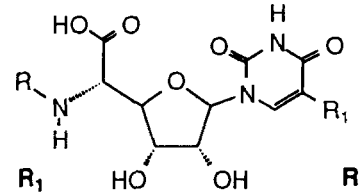


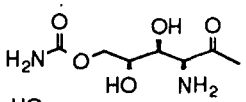
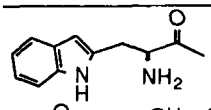
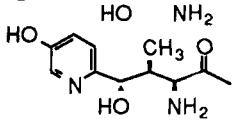
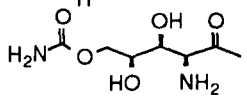
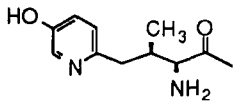
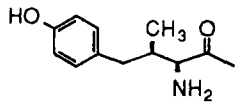
In spite of their potent inhibition of β-1,3-glucan synthase and good antifungal activities, the glycolipids have poor efficacies in animal models of infection. This may be due to inactivation through non-specific binding to serum proteins. A fusacandin derivative 12, which retained *in vitro* activity in the presence of serum, was identified from a series of analogs in which the 6'-O-acyl group had been replaced (19).

A total synthesis of the major papulacandin fragment, papulacandin D (13) was reported (20). A stereocontrolled synthesis of the side chain led to identification of the hitherto unassigned chiral centers at C-7" and C-14" as (*S*)-configurations (21).

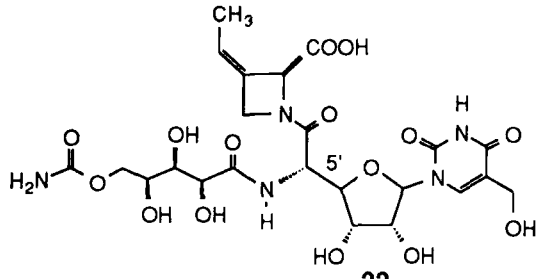
Papulacandin B was used to select for resistant mutants from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* Use of these mutants led to the identification of a gene Pbr1p which may be part of the glucan synthase complex (22). Echinocandin resistant mutants have also been isolated (23). This will likewise advance the course of molecular biological studies.

The peptidylnucleosides, polyoxin B (14) and nikkomycin Z (15) are inhibitors of fungal cell wall chitin biosynthesis. These compounds are, however, much more potent against filamentous fungi than against the human pathogen *C. albicans.* This disparity has been attributed to poor transport into yeasts. Nikkomycin Z has now been shown to be a specific inhibitor of the chitin synthase isozyme Chs3 but ineffective against the isozyme Chs2 from the yeast *S. cerevisiae* (24). This observation has led to the suggestion that the resistance of yeasts to nikkomyzin Z is largely due to its lack of inhibitory activity against the latter isozyme.

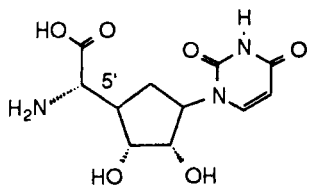


	R	R_1		R	R_1
14		HOH_2C—	18		H—
15		H—	19		H_3C—
16	H—	H—	20	H—	H_3C—
17		H—	21		H—

In an effort to modify the side chain peptide to yield compounds useful in human disease, a method employing tandem Edman reaction and decarboxylation was reported for the degradation of polyoxin D to yield the synthetic intermediate uracil polyoxin C (16) (25). Subsequent elaboration of 16 yielded 17 and 18 which retained chitin synthase inhibition and activity against *C. albicans.*



22



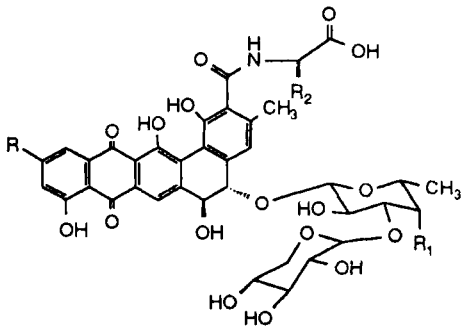
23

A total synthesis of 15, using an oxazoline based methodology to construct the pyridylaminobutanoic acid fragment, was reported (26). Aminohomologation of sugar

aldehydes was used in the total synthesis of polyoxin J (**19**) (27). Stereoselective synthesis of thymine polyoxin C (**20**) (28) using a [3.3] rearrangement of an allylic trifluoroacetimidate was reported. An enantioselective approach to synthesis of the N-terminal amino acid fragment of nikkomycins B (**21**) and Bx was described (29).

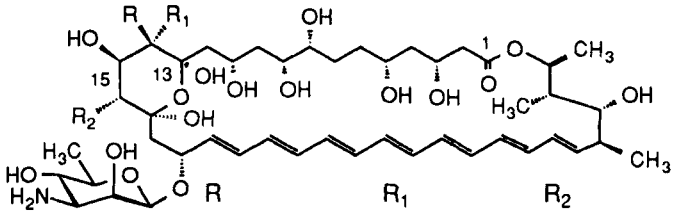
Stereochemistry of the exocyclic double bond of polyoxymic acid, the azetidinyl substituent on the 5'-carbonyl group of polyoxin A (**22**) was revised from *trans* to *cis* (30) and its total synthesis subsequently reported (31). A carbocyclic analog of uracil polyoxin C **23** was synthesized via a palladium-catalyzed nucleophilic substitution of the anion of uracil on an unsaturated bicyclic lactone (32).

The naphthacenequinone antifungals, pradimycin A **24** and benanomycin A **25** are broad spectrum antifungal agents which affect cell wall integrity by complexing with mannoprotein via Ca^{++} mediation. The 4'-amino group of **24** was modified to produce new water soluble analogs **26** and **27** (BMS-181184) which retained the antifungal activity of **24** (33). BMS-181184 is active against *C. albicans* (34), *Aspergillus* (35,36) and *C. tropicalis* (37) in animal models of infection. Compound **27** was subsequently produced from fermentation of *Actinomadura* sp. AB1236 (38). Synthetic modifications of the aglycone of **24** showed that C-11 is the only position which can be modified without loss of activity. Thus new analogs **28** to **30** were reported which had activities comparable to **24** (39).



	R	R_1	R_2
24	OCH_3	$NHCH_3$	CH_3
25	OCH_3	OH	CH_3
26	OCH_3	NHCHO	CH_3
27	OCH_3	OH	CH_2OH
28	H	$NHCH_3$	CH_3
29	OCH_2CH_3	$NHCH_3$	CH_3
30	OCH_2CH_2F	$NHCH_3$	CH_3

Compounds Affecting the Cytoplasmic Membrane - Dehydration of the polyene macrolide amphotericin B **25** gave a 13,14-anhydro derivative which was stereoselectively hydroxylated with MCPBA to afford epimeric C-14 alcohols. The 14*R* epimer **26**, was obtained when the 15-OH group was unprotected, while hydroxylation of the 15-O-silyl derivative gave the 14*S* congener **27** (40).



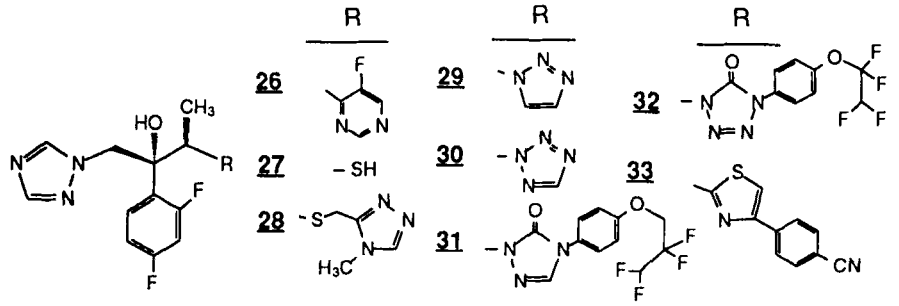
	R	R_1	R_2
25	H	H	COOH
26	OH	H	COOH
27	H	OH	COOH
28	H	H	CHO
29	H	H	$CH=CH-CH_2OH$
30	H	H	$CONH(CH_2CH_2O)_nCH_3$

A series of oximes and vinyl derivatives were synthesized (41) from the aldehyde **28**. Compound **29** retained its antifungal spectrum, but had less hemolytic activity than **25** against mammalian erythrocytes. Synthesis of the C1-C13 fragment of **25** through

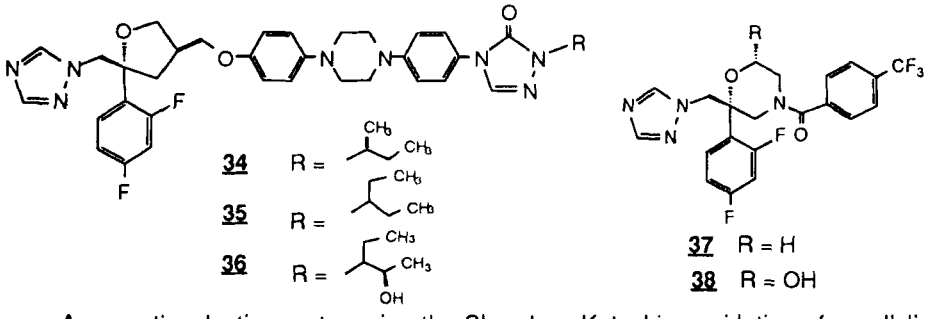
stereodivergent allylation of an unsymmetric β-amino aldehyde was described (42). Total synthesis of the same fragment was accomplished by employing a reductive ring opening of deoxyhalo sugars (43). A homologous series of oligo(ethylene glycol) substituted amides of **25** was synthesized. The critical micelle concentrations (CMCs) of these compounds were proportional to the lengths of the substitutents and to the concentrations necessary to cause hemolysis. There was only a modest reduction in antifungal activity with increasing CMC (44), thereby suggesting the feasibility of separating activity from toxicity in this class of compounds.

The mechanism by which **25** forms pores and causes ion leakage in lipid membranes has been extensively studied. Molecular dynamics simulations (45) of the current "barrel stave" model was performed to study ion coordination in the pores. Examination of a cross section of the simulated pore indicated that the polar substituents of the C1-C13 region may act as ligands for both cations and anions, in concert with water molecules.

Several reports appeared on the synthesis and evaluation of new azoles with activity against fluconazole resistant *C. albicans* and *Aspergillus*. Synthesis, preclinical and early clinical evaluation of a new triazole voriconazole **26** (UK-109496) was reported (46-48). Voriconazole has activity comparable to amphotericin B against *Aspergillus* (49) and efficacy in a model of *Aspergillus* endocarditis (50).

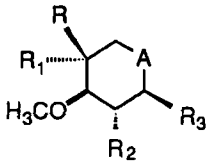


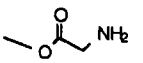
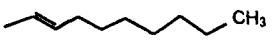
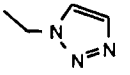
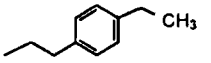
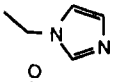
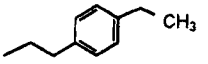
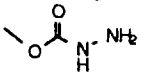
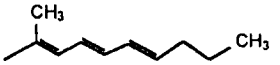
Using lactic acid as a synthon, a key optically active epoxide was synthesized which led to a number of enantiomerically pure azoles (51). Thus the sulfhydryl analog **27**, (51,52) thioalkyl triazole **28** (53), triazole **29** (54), tetrazole **30** (55), substituted triazolone **31** and tetrazolone (56) **32** were reported to have efficacies in animal models comparable or superior to fluconazole. Comparative studies of **31** (TAK-187) with fluconazole showed it to have superior activity against *Cryptococcus neoformans*, *A. fumigatus* and the non-albicans *Candida* species (57). ER-30346 (**33**), a thiazole derivative, was also reported to have broad spectrum activity (58-60).



An enantioselective route, using the Sharpless-Katsuki epoxidation of an allylic alcohol, was reported for the previously described Sch 42538, Sch 45012 and Sch 50002 (**34**) (61,62). Synthesis of two new triazoles Sch 51048 (**35**) and Sch 56592 (**36**) *via* chiral imide enolates was also described (63). These compounds have broad spectrum activities compared to fluconazole. New N-acylmorpholines, UR-9746 (**37**)

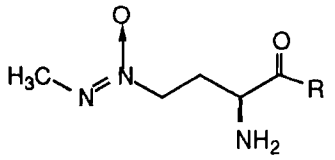
and UR-9751, (38) were reported (64,65) to be more potent than fluconazole and efficacious in animal models of cryptococcal meningitis (66) and histoplasmosis (67).

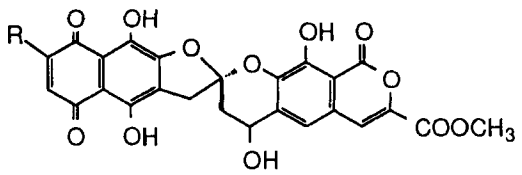


	A	R	R_1	R_2	R_3
39	O	CH_3	H		
40	CH_2	CH_3	CH_3		
41	CH_2	CH_3	CH_3		
42	O	CH_3	H		

Synthesis of new analogs of restricticin, Ro 09-1571 (39), Ro 09-2056 (40) and Ro 09-2127 41, were reported (68,69). These compounds, like the azoles, are inhibitors of lanosterol C-14 demethylase in the ergosterol biosynthesis pathway. Compound 41 showed more potent enzyme inhibitory activity than either fluconazole or itraconazole and had a broader spectrum of activity than fluconazole. The carbazate derivative 42 was effective in a mouse model of *C. albicans* infection (70).

Amino Acid and Protein Synthesis Inhibitors - Azoxybacillin (43) a novel azoxy-containing amino acid was isolated (71) from cultures of *Bacillus cereus*. The mode of action of 43 was determined to be inhibition of the sulfur fixation step in methionine biosynthesis, a process unique to fungi (72). The compound was subsequently shown (73) to inhibit at least two steps in the gene expression for sulfite reductase; transcriptional activation of *MET4* and post-transcriptional regulation of *MET10*. Azoxybacillin is antagonized by methionine and hence showed only weak activity *in vivo*. A number of derivatives (74) were evaluated, leading to identification of Ro 09-1824 (44), which is less antagonized by methionine (75).





43 R = OH

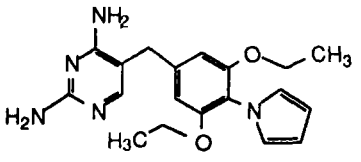
44 R = OBn

45 R = OCH_3

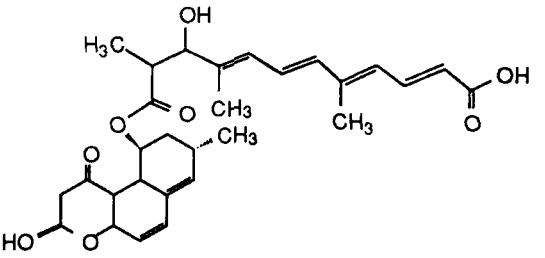
46 R = $NHCH_3$

Purpuromycin 45 and its analog MDL 63,604 (46) were reported (76) to have activities comparable to amphotericin B against *C. albicans*. These compounds inhibit protein and RNA synthesis in fungi, however, their poor solubilities limit their potential use in systemic infections.

Miscellaneous New Agents - Epiroprim (Ro 11-8958, 47) was identified as a potent inhibitor of *Pneumocystis carinii* dihydrofolate reductase (77). The chemistry and SAR of a broad spectrum agent calbistrin 48 were described (78). New 1,3,2-benzodithiazole S-oxides, such as 49 with more potent *in vitro* activity than amphotericin B against *C. albicans*, were reported (79). Design and synthesis of an alkylimidazole substituted dipeptide 50, a selective inhibitor of *C. albicans* myristoyl-CoA:protein N-myristoyltransferase were reported (80). A subsequent communication (81) discussed stereochemical and steric factors affecting potency and selectivity of these inhibitors.

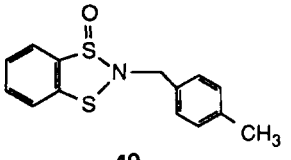


47

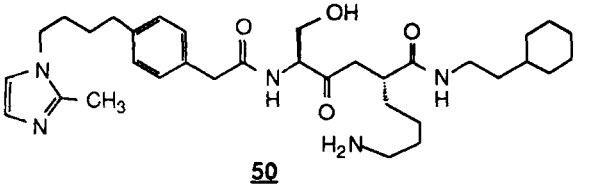


48

Although myristoyl-CoA:protein N-myristoyltransferase is a well characterized target (82), selective inhibitors may be more readily identified for the *Cryptococcus* enzyme as opposed to the *Candida* enzyme, as the former is more divergent from the mammalian enzyme (83,84).

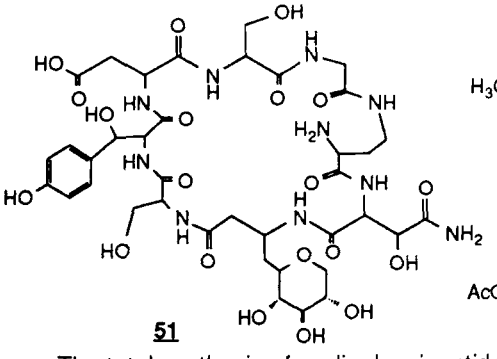


49



50

Cepacidine A (51), a heptapeptide from *Pseudomonas cepacia*, was described (85). The compound had broad spectrum activity. A dilactone from *Streptomyces* sp. 517-02 (52) was reported to have antifungal activity (86). BE-31405 (53) was isolated from *Penicillium minioluteum* and shown to have broad spectrum activity (87).



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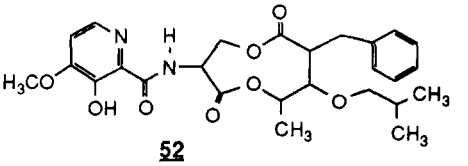

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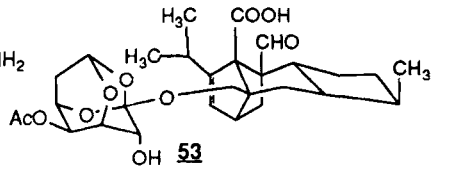
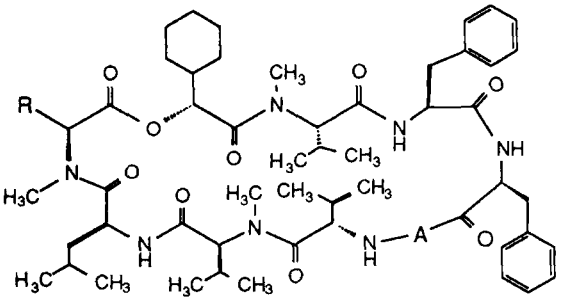
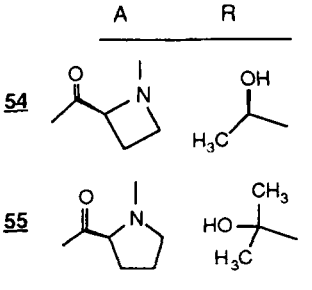


53

The total synthesis of cyclic depsipeptides Sch 56301 (54) Sch 57697 (55) and their progenitor, aureobasidin A, were reported (88,89). Compound 54 was reported to be more potent against *C. albicans* and *C. tropicalis*.





Emerging Antifungal Targets - β-1,6-glucan is a unique component of the fungal cell wall, hence its biosynthesis represents an attractive target. A number of mutants have been isolated (90) which are defective in the synthesis of this polysaccharide. This

development should help in further definition of this target. Further work on the fungal specific and essential protein, elongation factor 3 (EF-3), was reported (91).

The success of lanosterol C-14 demethylase inhibitors has stimulated interest in other steps of ergosterol biosynthesis (92). These targets include squalene epoxidase (93), 2,3-oxidosqualene-lanosterol cyclase (94) and Δ-7,5-desaturase (95). These targets have become more attractive in view of the number of different ways in which fungi may develop resistance to the azoles (96).

Another interesting target is the plasma membrane ATPAse. This enzyme is not unique to fungi, but differential responses of the mammalian and fungal enzymes to omeprazole and ouabain have been demonstrated (97,98). Similarly, topoisomerase I (99) of yeast has been shown to differ from the mammalian enzyme in terms of drug susceptibility (100). Other studies reported on mRNA transcription (101,102), checkpoints for mitosis (103) and the spindle pole body (104,105), the fungal equivalent of the mammalian centrosome, as possible targets for the development of selective antifungal agents.

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Chapter 16. Angiogenesis Inhibitors

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Introduction - Angiogenesis is required for the development of many proliferative diseases including rheumatoid arthritis, psoriasis, solid tumor growth, metastasis, as well as diabetic retinopathy and age related macular degeneration (1). At least 20 endogenous factors have been identified so far that either promote or inhibit angiogenesis. It is not the goal of this chapter to review these factors in depth, merely summarize those that may be useful as targets for therapy. For more in-depth discussions of the biology and biochemistry of angiogenesis, the reader is directed to a number of recent reviews on the subject (2-4). We will be focusing the majority of this review on the oncology aspects of anti-angiogenesis research since most of the effort in recent years has been spent there, even though several models for assessing antiangiogenic effects for oncology involve testing in eye models. For cancer, the inhibition of angiogenesis is a departure from traditional therapy in that it attempts to inhibit the process whereby a tumor recruits its own microvasculature to gain nutrients and rid itself of cellular by-products, rather than attempting to kill the tumor itself. Thus, antiangiogenic therapy should be viewed as cytostatic, rather than cytotoxic, and drugs that inhibit angiogenesis may need to be administered in conjunction with traditional anticancer agents.

Proteins and Large Molecular Weight Inhibitors - A central growth promoter in the neovascularization of solid tumors is a group of proteins known as Vascular Endothelial Growth Factors (VEGF), also known as Vascular Permeability Factor (VPF), coded by four mRNA splice variants that produce proteins containing 121, 165, 189, and 206 amino acids (5). Evidence for a fifth isoform of 145 amino acids also exists (6). All but $VEGF_{121}$ bind strongly to heparin, and only the two smaller forms are secreted from cells in soluble form. In a study where a large number of tumor cell lines were screened for VEGF production, only the SK-MEL-2 and M21 melanoma cell lines secreted minimal amounts of VEGF at normoxic and hypoxic conditions. When these lines were transfected with VEGF sense cDNA, they grew faster and larger in nude mice, producing many more lung metastases than the parental lines (7). In the clinic, expression of high amounts of VEGF correlates with a poor therapeutic outcome. For example, high-grade glioblastomas are more aggressive and better vascularized than the more benign gliomas; a significant factor in this difference is the amount of VEGF these tumors secrete (8). In a 10-year follow-up study of 328 patients with primary breast cancers, a direct correlation was noted between VEGF secretion and microvessel density, and an inverse correlation between microvessel density and relapse-free survival (9).

VEGF in dimeric form is the ligand that binds with high affinity to two tyrosine kinase receptors expressed exclusively on proliferating endothelial cells: KDR (the Flk-1 receptor in mice), also known as VEGFR-2, and Flt-1, also known as VEGFR-1 (10). A second ligand for Flt-1, placental growth factor (a homolog of VEGF) was recently discovered as well (11). The Flt-4 receptor with close homology to the Flt-1 gene has been described, but VEGF does not show specific binding to this receptor (5). In mice, disruption of the Flk-1 gene interferes with differentiation of endothelial cells, while disruption of the Flt-1 gene interferes with late-stage vasculogenesis and functions of maturing vessels not related to cell growth (12), implying that disruption of the binding of VEGF to KDR/Flk-1 is a more fundamental way to inhibit neovascularization. Binding of VEGF causes dimerization of the receptors and triggers a complex cascade ultimately leading to formation of new blood vessels in the growing tumor. A second family of endothelial cell receptors, termed Tie-1 and Tie-2

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(Tek), have been characterized; these receptors are also expressed on early hematopoetic cells (13). Tie-2 expression follows Flk-1 expression by 12 to 24 days during mouse embryonic development, implying an involvement in a later step of angiogenesis for this second receptor family (14). The function of these latter receptors in tumor angiogenesis has not been reported.

Disrupting the binding of VEGF to its receptor has been a major area of anti-angiogenesis research. A number of low-molecular weight inhibitors have been reported, and these are described in a later sections. However, several proteins have also been described in attempts to disrupt this interaction. Two chimeras constructed from the extracellular domains of Flt-1 and Flk-1, grafted to an IgG constant domain were studied in a mouse eye model of retinal neovascularization and inhibited this process by 100% and 95%, respectively, after 17 days (15). The anti-VEGF antibody A4.6.1 at 200 mg twice per week completely suppressed neovascularization and growth of human rhabdomyosarcoma A673 cells, even though the original tumor "seedlings" remained viable (16). An attempt to destroy proliferating endothelial cells has also been described. VEGF165 conjugated to the A-chain of diphtheria toxin DT385 through a disulfide spacer completely blocks angiogenesis in an eye vascularization model (17).

Two members of the Fibroblast Growth Factor family, bFGF and aFGF, stimulate angiogenesis. These proteins are fairly ubiquitous, they bind to heparan sulfate on the basement membrane, and a number of cells express receptors to these growth factors. Nevertheless, successful efforts to block tumor development with anti-bFGF antibodies in experimental models have been reported and reviewed (4). Interferons α and β (but not IFNγ) specifically block bFGF and suppress development of pancreatic eyelet tumors in mice, reducing their capillary density by about 40% (18). This effect has been attributed to a suppression of bFGF gene expression (19). IL-8 is another potent angiogenesis factor with endothelial cell chemotactic activity; depletion of this growth factor with neutralizing antibodies markedly reduces the growth and vascularization of the A549 non-small cell lung and Calu 1 squamous cell carcinomas in SCID mice (20).

An experimental antiangiogenic therapy has been reported whereby thrombosis was induced in tumor vasculature. A bi-specific antibody was constructed, one arm derived from the antibody B21-2 which recognizes an induced MHC class II antigen on the vasculature of the C1300 murine neuroblastoma, the other arm derived from antibody 10H10, specific for a truncated form of tissue factor (tTF). This construct localized tTF to the tumor vasculature, initiated the thrombogenic cascade, and completely occluded the tumor vasculature within 72 hr. No other sites of thrombosis were observed. Even in mice with large (up to 1 cm) tumors, treatment resulted in complete regression in 38% of the animals, lasting four months. Those tumors that re-grew did not express the induced MHC class II antigen (21).

Several new potent endogenous inhibitors of angiogenesis have been described. Since they are all large proteins and as such will probably not make good models for drug discovery, searching for small molecules that induce production of these proteins may be useful. IL-12 has demonstrated an inhibitory effect on the experimental Lewis lung tumor. This protein has no antiproliferative effect on endothelial cells, but induces the production of Interferon γ. IFNγ, a known antiangiogenesis agent with a similar inability to prevent endothelial cell proliferation, induces inducible protein 10 (IP-10), which does inhibit the proliferation (22, 23). IL-12 has advantages over INFγ as an antiangiogenesis agent because of its significantly greater biological half-life, so inducers of IL-12 may find a role as angiogenesis inhibitors.

Surgical removal of a primary tumor often produces rapid growth of metastases, both in humans and in animal models (24). Angiostatin, originally isolated from mice implanted with the Lewis lung carcinoma, inhibits angiogenesis in a number of primary

and metastatic tumors. The structure of angiostatin shows identity to an internal fragment of mouse plasminogen, comprising the first four disulfide-linked kringle domains (K1 to K4) (25). Fragments containing individual and multiple kringle domains of angiostatin have been prepared through additional enzymatic digestion or by recombinant methods, and showed that the presence of K4 was not required for full antiangiogenic activity. Angiostatin can also be produced by the digestion of plasmin with elastase, but elastase does not appear to be the enzyme that generates angiostatin *in vivo*. For now, this as-yet unidentified enzyme has been designated "plasminogen-angiostatin converting enzyme" (26).

Endostatin, a 20kD angiogenesis inhibitor has likewise been isolated from mice implanted with the murine hemangioendothelioma (EOMA) tumor. This material also inhibits the proliferation of endothelial cells but not the EOMA tumor, and shows identity to a C-terminal fragment of collagen XVIII. Endostatin has been produced in both baculovirus and *E. coli* expression systems. In addition to its *in vivo* activity against the EOMA tumor, endostatin has shown strong inhibitory activity against the Lewis lung, T241 fibrosarcoma, and B16F10 tumor cell lines, even when treating tumors of 100 to 200 mm^3 in size. Endostatin increases the apoptosis rate in tumors seven-fold even though the proliferation rate of the cells themselves is unchanged; 5 to 14 days after discontinuation of therapy, the primary tumors begin to regrow (27). Other endogenous antiangiogenic protein fragments will no doubt be discovered. One promising protein is thrombospondin-1. This complex, multimeric matrix glycoprotein is reported to function as both an angiogenesis promoter as well as an inhibitor (28).

SYNTHETIC AND SMALL MOLECULAR WEIGHT INHIBITORS

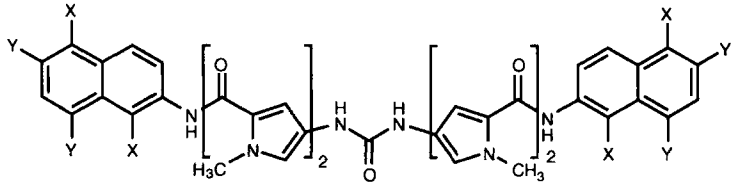
Sulfated analogs - Heparin and heparan sulfate are glycosaminoglycans which can modulate a wide range of cellular functions including growth, morphology and migration by interacting with extracellular matrix proteins, growth factors, growth factor receptors and proinflammatory mediators (29). In addition, several growth factors are sequestered and protected from proteolysis by cell surface heparan sulfate, an event which is modulated by the activity of heparanases. Heparin consists of a mixture of polysulfated polysaccharides with a molecular weight range of 6 to 20 kDa. Due to the critical role of heparin in activation and modulation of many growth factors important to angiogenesis (e.g. FGF, VEGF, PDGF, IL-3, IL-8, TNF-α, TGF-β), efforts continue to focus on defining the portions of heparin unique to angiogenesis in order to avoid its wide range of limiting toxicities. Heparin fractionated into low and high molecular weight species can inhibit or facilitate the binding of Heparin Binding Growth Factors (HBGFs) to their receptors. Recent studies have shown that low molecular weight heparin more effectively suppresses bFGF mediated angiogenesis than high molecular weight fractions (30).

Some of the most recent synthetic modifications of heparin have focused on enhancing the heparanase inhibitory activity of heparin analogs while reducing the anticoagulant activity. Removal of the 2-O and 3-O-sulfate groups enhanced the heparanase inhibitory activity of heparin (31). Removal of the carboxyl groups slightly lowered its heparanase activity while removal of both the carboxyl groups and 2,3-O-sulfates abolished the activity. Replacing the N-sulfates of heparin with N-acetyl groups reduced the activity. Periodate oxidized, borohydride reduced heparin had enhanced heparanase inhibitory activity. All of these modified heparins showed a marked reduction in anticoagulant potency relative to heparin yet at least some of the derivatives retained their anti-tumor and anti-metastatic activity.

GM1474 is a low molecular weight polysulfated oligosaccaride that also binds to bFGF (32). GM1474 inhibits tumor cell derived heparanase and bFGF-dependent proliferation of endothelial cells *in vitro,* is active in the CAM assay, but does not itself inhibit the proliferation of human or murine tumor cells *in vitro*. Systemic administration of GM1474 in a number of tumor models including human mammary

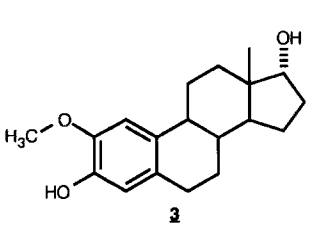
(MDA-231), prostatic (PC-3) and hepatocellular (SKHep-1) carcinomas, showed significant reduction in tumor growth. GM1474 was tested in a number of orthotopic models including a human renal cell carcinoma (SN12PM6) where it resulted in a >90% inhibition of tumor metastasis.

The most thoroughly studied sulfated small molecule inhibitor of angiogenesis, suramin, blocks the growth stimulating activity of VEGF, PDGF, acidic and basic FGF, IGF-1, and EGF (33). Studies in the past have shown that minor changes in the structure of suramin can effect its toxicity without abolishing desired activity. Several studies report on the replacement of the linking phenyl amino amides in suramin with N-methylpyrrole-2-carboxamides (34-36). Degree and position of sulfonation, as well as number of linking groups and attachment points relative to the urea core were investigated. The best activity was seen with tetrasulfonates containing two linking groups (FCE 27266, 1, and FCE 26644, 2). FCE 26644 inhibits the binding of bFGF, PDGFß, EGF and IL-1ß to cells (IC_{50}= 142, 55, 471, and 450 µM respectively). FCE 27266 inhibits the binding of bFGF, PDGFß, VEGF and IL-1ß to cells (IC_{50} = 145, 45, 275, and 15 µM respectively). Both compounds appear to bind to the ligands instead of the receptors as evidenced by competition assays. Both compounds were active in numerous tumor metastasis assays while at the same time showing less toxicity than suramin. In addition, 1 has shown activity in inhibiting hepatocyte growth factor activation (37).



1 X = SO_3Na, Y = H
2 X = H, Y = SO_3Na

Steroids, Flavinoids and Steroid Conjugates - Steroids were among the first small molecules to show an antiangiogenic effect *in vivo*. Some recent work has focused on the isolation and characterization of steroid metabolites and phytochemicals from dietary ingested substances (38). From the flavinoids, isoflavinoids and steroid metabolites characterized, genestein and 2-methoxyestradiol 3 were the most active at inhibiting angiogenesis *in vitro* and *in vivo*; 3 may exert its antiangiogenic effect by inhibiting tubulin polymerization through binding at the colchicine site on tubulin (39). Other microtubule affecting drugs, such as paclitaxel, have also shown antiangiogenic effects (40). The antiangiogenic effect of 3 is probably unrelated to its estrogen receptor binding (41).



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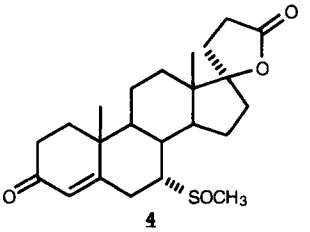

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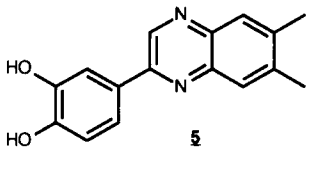
Spironolactone 4 is an orally active, renal aldosterone antagonist used clinically for the treatment of hypertension, congestive heart failure, acne, seborrhea and other diseases. Its potential as an antiangiogenic agent was suggested by its side effect of amenorrhea. Spironolactone inhibits bFGF and VEGF induced angiogenesis *in vitro* as well as in the CAM and bFGF induced rabbit corneal angiogenesis models (42). The antiangiogenic effect of 4 appears to be unrelated to inhibition of TPA (unlike other angiostatic steroids) or uPA, nor due to its antiandrogenic effects or through aldosterone antagonism.

The affinity of heparin for endothelial cells has been utilized to deliver angiostatic steroids by conjugating them to non-anticoagulating derivatives of heparin (43). Early conjugates utilized cortisone, which has glucocorticoid and mineralocorticoid effects (44). Current work examined the conjugation of ten additional steroids to high molecular weight fractionated heparin through a variety of acid labile linkers. Tetrahydrocortisone, tetrahydrocortisol and tetrahydro-S, all of which lack glucocorticoid and mineralocorticoid activities, were converted from non-inhibitory to inhibitory drugs by their coupling to heparin (43).

Tyrosine Kinase Inhibitors - The design of ATP mimics as inhibitors of specific tyrosine kinases was deemed impractical several years ago due to the high levels of endogenous ATP, high binding constants to overcome and the multitude of ATP dependent proteins. However, the recent success in designing selective inhibitors of epidermal growth factor (EGF) receptor kinase has engendered a strong interest in designing selective inhibitors of other kinases. SU1498 (**5**) and SU1433 (**6**) are representative of compounds that inhibit the autophosphorylation of Flk-1/KDR (IC_{50} = 0.7 μM and 9.3 μM, respectively) (45). SU1498 is selective for Flk-1 kinase with no activity at the doses tested for EGF, HER-2 and PDGF receptor kinases. SU1433 is somewhat less selective, showing activity (IC_{50} = 5 μM) with the structurally related PDGF receptor kinase, but no activity with EGF or HER-2. Both compounds showed activity in the CAM assay and in an *in vivo* VEGF induced permeability assay.



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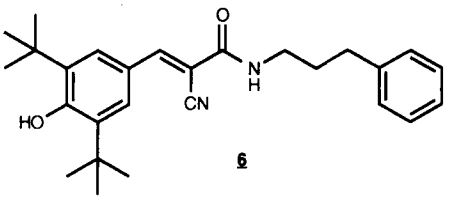

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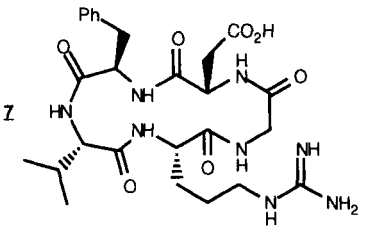
Integrins - The role of adhesion molecules (selectins, immunoglobulin supergene family, cadherins and integrins) in angiogenesis has been reviewed recently (46). Two distinct angiogenic pathways have been defined for α_v integrins. Use of anti-$\alpha_v\beta_3$ (LM609) and anti-$\alpha_v\beta_5$ (P1F6) monoclonal antibodies and cyclic RGD peptides active for inhibiting both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ demonstrated that angiogenesis in corneal or CAM models induced by bFGF or TNFα depended upon $\alpha_v\beta_3$, whereas angiogenesis initiated by VEGF, TGFα or phorbol ester depended upon $\alpha_v\beta_5$ (47). In addition, only $\alpha_v\beta_3$ was observed on blood vessels in ocular tissues with active neovascularization from patients with age-related macular degeneration of presumed ocular histoplasmosis, whereas both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were expressed on tissues from patients with proliferative diabetic retinopathy (48). These studies and others (49) suggest that VEGF is not a significant angiogenic stimulus in subretinal neovascular disease while both FGF and VEGF play a role in the neovascular response in retinal neovascular diseases.

Initial work on small molecule inhibitors of fibrinogen binding to vitronectin led to the discovery of the $\alpha_v\beta_3$ selective RGD cyclic peptide, c(RGDfV) **7** (50, 51), which is active in a number of angiogenesis models (52). Subsequent work has focused on replacing the Phe-Val dipeptide with β-turn mimics that utilize rigid building blocks. While most of these analogs did not adopt the desired conformation in the β-turn, one compound, c(RGD"R-ANC"), **8**, showed improved activity in inhibiting vitronectin binding to the $\alpha_v\beta_3$ receptor (IC_{50} = 8.5 x 10^{-4} μM) (53). Additional studies have investigated the SAR of cyclic retro, inverso, and retro-inverso peptides based on the RGD motif (54). The retro-inverso analogs of the highly active parents were dramatically less active but a retro-inverso analog [c(vfdGR)] of a poorly active parent peptide was found to be highly active and selective for $\alpha_v\beta_3$.



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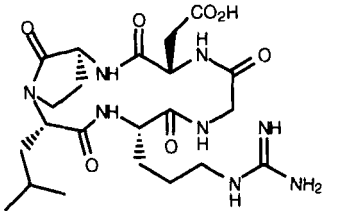

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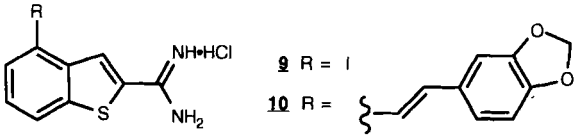
Urokinase Plasminogen Activator (uPA) and Matrix Metalloproteinase (MMP) Inhibitors - Angiogenesis requires a proteolytic digestion of the extracellular matrix and basement membrane. Invasive cells promote an extracellular protease cascade that includes uPA, plasmin and a number of MMPs. Previous work has shown that tumor cell invasion and metastasis can be blocked by anti-uPA antibodies (55, 56). In addition, peptides that block uPA binding to its receptor (uPAR) can reduce metastases (57). Recent work has shown that the EGF-like domain of murine uPA (mI-48) alone or fused to the Fc portion of human IgG (mI-48IG) is a potent inhibitor of the binding of mouse uPA to uPAR (mI-48IG, IC_{50} = 600 pM) (58). MI-48IG also inhibits capillary tube formation in fibrin gels, bFGF induced vascularization and B16 melanoma in syngeneic mice.

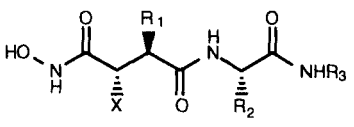
Using the potassium sparing diuretic amiloride as a starting point, B428 (9) and B623 (10) were developed as selective uPA inhibitors (0.32 μM and 0.07 μM respectively) (59). *In vivo* studies have shown that IP administration of these compounds blocked the tumor invasion of muscle and adipose layers of the subcutis and dermis in mice bearing F311 tumors. However, these compounds neither inhibited tumor-induced angiogenesis nor reduced the incidence of spontaneous lung metastasis (60).

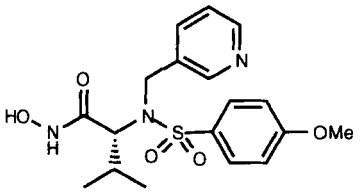


9 R = I

10 R =

The MMPs are a series of zinc-requiring proteolytic enzymes, secreted in latent pro-enzyme form which are involved in remodeling and degradation of extracellular matrices (61, 62). The role of MMPs in cancer (63-65) is complex and these enzymes may contribute at several stages in the tumorigenic processes, including invasion, progression, metastases, and angiogenesis. Though the gelatinases (MMP-2 and -9) are postulated to play a major role in tumorigenesis, other MMPs are implicated in specific cancers. In addition to the degradation of structural proteins, MMPs are hypothesized to be involved in the intricate interplay of tumor, stromal, and endothelial cells with each other and their matrix environments.





15

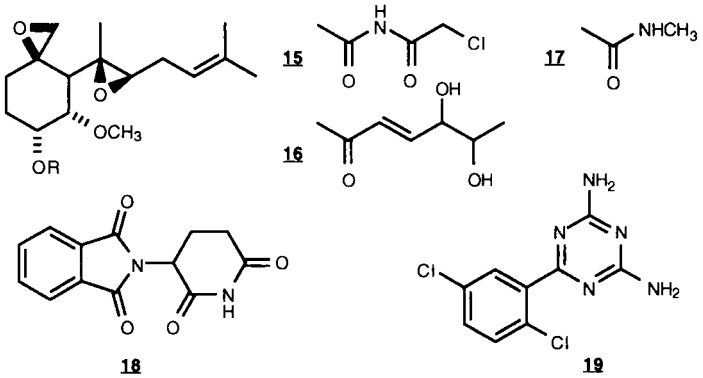
11 X = OH; R_1 = i-Bu; R_2 = t-Bu; R_3 = Me
12 X = CH_2S-(2-thienyl); R_1 = i-Bu; R_2 = benzyl; R_3 = Me
13 X = H; R_1 = i-Bu; R_2 = CH_2(3-indolyl); R_3 = Me
14 X = H; R_1 = $(CH_2)_3$-(4-Cl-phenyl); R_2 = t-Bu; R_3 = H

The design and SAR of MMP inhibitors have been reviewed (66-68). Batimastat (BB-94, 12) and marimastat (BB-2516, 11) are potent broad spectrum MMPIs (69-73),

reported to suppress or prevent the growth of various tumors in animal models. Batimastat has shown efficacy in clinical trials for the treatment of malignant pleural effusions and ascites on intrapleural administration. Marimastat, unlike batimastat, is orally active and in Phase III clinical trials for pancreatic, lung, brain, and stomach cancers (74). A related non-selective peptide-based hydroxamic acid, ilomostat (Galardin, GM-6001, **13**) is a topically active agent for treatment of corneal ulceration (Phase III) and has shown *in vivo* anti-angiogenic activity and efficacy against a number of human cancers in mouse models (75, 76). Selective gelatinase-A inhibitor CT-1746 (**14**) is reported to inhibit angiogenesis *in vivo*, and to be orally active in both a mouse orthotopic model (human colon cancer) and in the murine Lewis lung model in combination with cytotoxic agents (77-79). In a deviation from peptide-based compounds, CGS-27023A (**15**) is a potent, non-selective MMPI with oral activity in murine metastases and human tumor xenograft models (80). Another non-peptide orally active MMPI, AG3340 (structure not published), is effective in murine tumor models and has entered Phase I clinical trials (81-83). ACE inhibitor captopril is an *in vitro* inhibitor of MMP-2 and MMP-9 with anti-angiogenic and antitumor activity in the rat, though the effects on neovascularization may be derived from multiple pharmacological actions (84).

Miscellaneous Agents - Fumagillin and its analogs [TNP-470 (or AG-1470, **15**), FR-111142, **16**, FR-118487, **17**] are active in many models of angiogenesis. Despite extensive work, the molecular target of the fumagillins has yet to be determined. FR-118487 inhibits the growth of VEGF producing tumors but does not inhibit the production of VEGF mRNA (85). TNP-470 potently inhibits DNA synthesis stimulated by PDGF and IGF-I in smooth muscle cells (SMCs) (86). TNP-470 appears to arrest SMCs in G1 and inhibit the synthesis of mRNA for cdk2. Some recent work has focused on the use of TNP-470 in a number of tumor models including rhabdomyosarcoma (87), liver metastases (88), renal cell carcinoma by itself (89) and in combination with 5-FU (90), adriamycin, VP-16 and mitomycin-C (91). TNP-470, which is in clinical trials, is extensively and rapidly metabolized, leading to the speculation that the active species in animals is probably not the parent drug (92).

Thalidomide **18** is a well known, potent teratogen for which manifestation of toxicity has implicated an antiangiogenic effect (93, 94). Thalidomide is inactive in the CAM assay, yet active upon oral dosing in the bFGF induced rabbit corneal micropocket angiogenesis model. This may reflect a need for metabolic activation in the liver. Analogs of thalidomide which block metabolism are inactive in a corneal model of angiogenesis. Although thalidomide modulates TNF-α production, there is doubt that this is the mechanism of its antiangiogenic effect. Studies in the 1960's with thalidomide showed no clinical benefit in the treatment of solid tumors (95, 96). Recent animal studies have shown thalidomide to have activity in inhibiting the incidence of tumor metastasis but to have no effect on primary tumor growth (97, 98). Thalidomide is currently in clinical trials for treatment of age related macular degeneration.



Irsogladin **19** is used clinically as an orally active anti-ulcer agent in Japan and inhibits tubular morphogenesis and tissue type plasminogen activator synthesis in vascular endothelial cells (99). A recent report shows that orally administered irsogladin inhibits the tumor growth of human glioma cells in mice with a concomitant reduction in vascularization of those tumors (100).

Summary - The evidence that inhibiting angiogenesis will have a positive effect on controlling solid tumors has increased dramatically over the past few years. However, as the various biological factors that promote or inhibit this process are identified it is evident that angiogenesis may be difficult to control and no single target is fundamental over the rest. Intervention through a medicinal chemistry approach can involve blocking the secretion of angiogenic factors or potentiating the activity of suppressers, inhibiting the binding of growth factors to their receptors, blocking the enzymes involved in signal transduction, transcription, or the remodeling of extracellular matrix, through destruction of proliferating endothelial cells or even induction of thrombi in neovasculature. Even these broad targets have numerous points of intervention and plenty of room for innovation. Ironically, the precise antiangiogenic target of many of the agents that are presently in the clinic has not been defined, implying that more targets and additional points of intervention are yet to be identified.

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Chapter 17. Chemical Inhibitors of Cyclin-dependent Kinases

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Introduction - The eukaryotic cell cycle is regulated primarily at the G1/S and G2/M phase transitions or "checkpoints" with the cyclin-dependent kinases (CDKs) acting as key regulators of these checkpoints (1-4). CDKs are serine/threonine protein kinases which are composed of a catalytic CDK subunit and a regulatory cyclin subunit. Presently, eight CDKs and ten cyclins have been identified. CDK activity is regulated by many mechanisms, reflecting their critical role in cell cycle control. The cyclin subunit is the key regulator of CDK activity, with each CDK interacting with a specific subset of cyclins: cyclin A (CDK1, CDK2), B1-B3 (CDK1), C (CDK8), D1-D3 (CDK2, CDK4, CDK5, CDK6), E (CDK2), H (CDK7). Cyclin function is controlled by changes in cyclin levels at specific stages of the cell cycle. Passage through G1 and entry into S phase is sequentially regulated by cyclins D, E, and A, whereas cyclin B is the key regulator of the G2/M transition. In addition to cyclin binding, CDK activity is regulated by phosphorylation at specific sites of the catalytic subunit which can modulate kinase activity in either a positive or negative manner. Phosphorylation of Thr^{161} of CDK1 is necessary for maximal kinase activity and phosphorylation of Thr^{14} and Tyr^{15} residues negatively regulate kinase activity. The dual specificity phosphatase Cdc25 dephosphorylates Thr^{14} and Tyr^{15} to produce active kinase. Another major mechanism of CDK regulation involves a diverse family of CDK inhibitory proteins, termed the CDIs or CKIs, that bind and inactivate CDK-cyclin complexes. A number of excellent reviews that provide a more detailed analysis of the molecular machinery involved in cell cycle regulation are available (1, 3, 5-7).

Compelling evidence has begun to highlight the connections between CDK regulation and cancer (8, 9). Overexpression of specific cyclins, particularly cyclin D1, can contribute to cell transformation and CDIs have been identified as potential tumor suppressor genes. The p16 gene, in particular, is lost in the majority of tumor cell lines and in a significant number of primary tumors. The importance of CDKs and their regulators in cell cycle control coupled with their frequent deregulation in cancer makes them attractive targets for the identification of antineoplastic agents. In fact, a number of chemical inhibitors of CDK kinase activity have already been identified (10). Recent advances in the characterization of these inhibitors are discussed below.

CHEMICAL INHIBITORS OF THE CDKS

Six classes of chemical inhibitors of the cyclin-dependent kinases have been identified to date. Their structures are shown in Figure 1; their *in vitro* selectivity and relative potency are shown in Table 1. All of the compounds are derived from natural products and represent distinct chemical structures.

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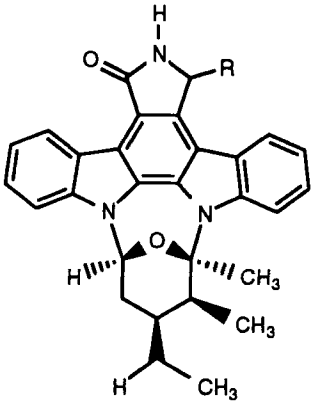

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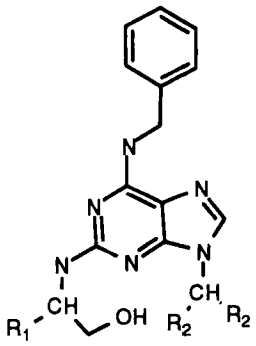
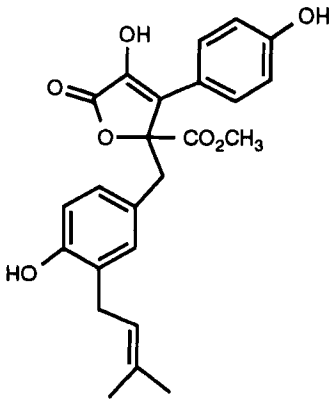
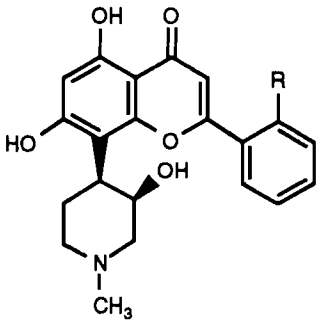
2 R = OH UCN-01



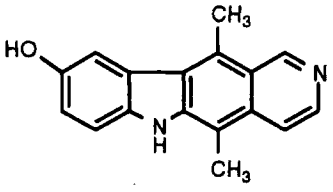
3 R1 = H, R2 = H olomoucine
4 R1 = C_2H_5, R2 = CH_3 roscovitine



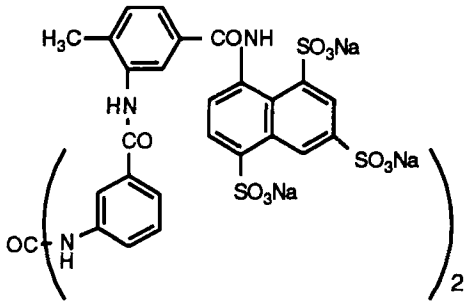
5 butyrolactone-1



6 R = H L868276
7 R = Cl flavopiridol (L868275)



8 9-hydroxy-ellipticine



9 suramin

Figure 1. Chemical Inhibitors of the CDKs

Table 1 - Selectivity of CDK inhibitors: IC_{50} (μM)

Target	7	3	5	2	8	9
CDK1	0.30-0.40	7	0.60	0.031	1	4
CDK2	0.10-0.40	7	1.5	0.030	ND	ND
CDK4	0.40	>1000	>1000	0.032	ND	ND
MAPK	ND	30	94	0.910	ND	ND
PKA	145	230	260	ND	ND	656
PKG	6	>2000	ND	ND	ND	ND
PKC	6	>1000	160	0.007	ND	29-50
EGF-R	25	440	>590	ND	ND	70

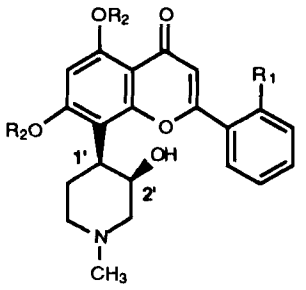
Refs. 10, 11

Flavopiridol (L86-8275) (7), an analog of a natural alkaloid isolated from the Indian plant *Dysoxylum binectariferum*, is a potent inhibitor of the cyclin-dependent kinases *in vitro*; IC_{50}'s for CDK1 (also named Cdc2), CDK2, CDK4 and CDK7 in immune complex kinase assays are 0.3, 0.1, 0.4 and 0.3 μM (11). Flavopiridol is competitive with respect to ATP (K_{iATP} = 0.04 μM) (12) and the crystal structure of a complex of CDK2 and L86-8276 (des-chloro-flavopiridol) reveals that the aromatic portion of the inhibitor binds to the conserved adenine-binding pocket of CDK2 (13). Interestingly, the phenyl group of flavopiridol interacts on a protein surface (CDK2 residues L83, H84, and D86) outside of the ATP-binding pocket. As another CDK inhibitor, olomoucine, makes similar contacts with CDK2 (14), this latter interaction appears to explain the specificity the flavopiridol has for the CDKs (see discussion below). While reported structural modifications on flavopiridol have not generated a more potent compound, structure/function analysis has provided useful information that may aid in the development of compounds with increased selectivity within the CDK family (11, 15). The 5,7 hydroxy and chlorine moieties are clearly important for inhibition of CDK2 activity (Table II). CDK inhibition by 1',2'-cis(-), cis(+) or trans(-/+) des-chloro analogs demonstrated that the cis(-) isomers are the most potent. While substitution of the chloro with either fluoro or bromo diminishes potency towards CDK4 and CDK7, these analogs have a two fold increase in selectivity for CDK2. In addition, substitution of a pyridyl for N-methylpiperidinyl ring in the des-chlorophenyl analog of flavopiridol results in a preferential inhibition of CDK4.

Flavopiridol inhibits cellular growth in either late G1 phase or in G2 phase, depending on the method of cell synchronization (16). Phosphorylation of the product of the retinoblastoma tumor suppressor gene (pRb), a substrate of cyclin D-dependent kinases and cyclin E-CDK2, is inhibited in flavopiridol-induced G1 arrested cells (17). CDK4 immunoprecipitates, derived from MCF-7 breast carcinoma cells (wild type for Rb) arrested in G1 by flavopiridol, possess decreased Rb kinase activity. Furthermore, the phosphorylation state of pRb is shifted from hyperphosphorylated to hypophosphorylated with the development of G1 arrest in living MCF-7 cells. Similar studies have also been performed on the Rb-negative MDA-MB-468 breast carcinoma cells. As these cells lack detectable CDK4 kinase activity, they provided a model to determine the effects of flavopiridol on cell cycle progression in the absence of CDK4 kinase activity. Following release from nocodazole synchronization, flavopiridol-

treated cells arrest in G1 phase and the *in vitro* kinase activity of CDK2 immunoprecipitates is inhibited (17). These data are consistent with the hypothesis that flavopiridol-induced inhibition of cell growth is due to the inhibition of CDK activity.

Table 2 - IC_{50} (CDK2) of Flavopiridol and its analogs



Compound	R1	R2	CDK2 IC50 (μM)
6 L86-8276	H	H	2.08
7 L86-8275	Cl	H	0.28
10	Cl	Me	7.00

Olomoucine (3) is a selective CDK inhibitor that displays selectivity within the CDK family and binds competitively with ATP (18). It inhibits the phosphorylation of Histone H1 by both CDK1 and CDK2 with an IC_{50} of 7 μM and by CDK5 with an IC_{50} of 3 μM. In contrast, it inhibits the phosphorylation of the retinoblastoma protein by CDK4 and CDK6 with IC_{50}s of >1000 μM and 250 μM, respectively. Comparison of the three-dimensional structure of the olomoucine-CDK2 complex with the ATP-CDK2 complex shows that the hydrophobic adenine binding pocket of CDK2 has a surprising ability to accommodate diverse structural moieties. Olomoucine binds in the ATP-binding pocket of CDK2, but in a different orientation from the adenine of the authentic ligand ATP (18). Furthermore, the N6-benzyl substituent in olomoucine interacts with amino acid residues that lie outside the conserved ATP-binding pocket (CDK2 residues 82 to 89). These molecular interactions are analogous to those observed between flavopiridol and CDK2 (13). As CDK2 residues 82-89 are conserved in other CDKs but not in non CDK kinases, they appear to play an important role in determining the selectivity of the CDK inhibitors.

Olomoucine has been shown to arrest cells in either G1 or G2 phase using a variety of cellular models ranging from unicellular algae to human tumor cells (17, 19, 20). Furthermore, a number of CDK substrates have been shown to be inhibited *in vivo* in olomoucine-induced growth arrested cells; (i) olomoucine inhibited Xenopus oocyte maturation and the *in vitro* and *in vivo* phosphorylation of the cyclin B-CDK1 substrate, elongation factor subunits γ and δ (19), (ii) the mitotic disassembly of the Golgi apparatus, a CDK1-dependent event, was inhibited in olomoucine-treated FT210 cells (21), and (iii) cyclin A expression, a marker of late G1 phase progression induced by the Myc protooncogene, was inhibited by the olomoucine analog, roscovitine (22). While these findings suggest that olomoucine inhibits CDK activity *in vivo*, the results must be interpreted cautiously since olomoucine inhibits of the phosphorylation of mylein basic protein by mitogen-activated protein kinase (MAPK) with an IC_{50} of 30 μM.

Butyrolactone-I (5), initially identified from *Aspergillus terreus var. africanus*, was isolated from *Aspergillus* strain F-25799 (23). It is an ATP competitor that selectively inhibits the activity of CDK1 and CDK2 (IC_{50} = 0.68 μM), but weakly inhibits other kinases including PKC, PKA, casein kinase, MAP kinase or EGF receptor tyrosine kinase. Butyrolactone-1 arrests cells in either late G1 phase or G2 phase at 17.7 μM

(24, 25). It also inhibits the phosphorylation of pRB in nuclear extracts and intact G1-arrested cells (23, 24). The nature of the butyrolactone-1-induced G2 arrest was examined by monitoring the effects of butyrolactone-1 on the phosphorylation of the CDK1 substrate histone H1. Butyrolactone-1 selectively inhibits the phosphorylation of both endogenous and exogenous histone H1 in nuclear extracts as well as in a purified cyclin B-CDK1 kinase assay. These findings suggest that the CDKs are the *in vivo* target of butyrolactone-1.

UCN-01 (**2**), an analog of the nonspecific protein kinase inhibitor staurosporine, was initially identified as a potent and selective inhibitor of Ca^{+2} and phospholipid dependent PKC. It possesses anti-tumor activity against a variety of rodent and human cancers *in vitro* and *in vivo*. Recently, it was determined that UCN-01 exhibits potent (0.03 μM) inhibition of histone H1 phosphorylation by CDK1 and CDK2 (26) and inhibits the CDK2, CDK4 and CDK6 mediated phosphorylation of pRb in cell lysates with IC_{50}s of 42 nM, 32 nM, and 58 nM, respectively. In addition, it inhibits the phosphorylation of pRb in whole cells with an IC_{50} of 100 nM. While these findings suggest that the anti-tumor activity of UCN-01 might be due to the inhibition of CDK activity, the relative lack of *in vitro* specificity and the high degree of structural similarity to staurosporine, increases the difficulty of accurately determining the *in vivo* target of UCN-01.

9-Hydroxy ellipticine (**8**), an analog of the alkaloid ellipticine, possesses significant anti-tumor *activity in vitro* and *in vivo* against a variety of tumor cell lines (27). Recently, it was shown that 9-hydroxy ellipticine inhibits the CDK1 mediated phosphorylation of histone H1 with an IC_{50} of 1 μM (28). Furthermore, phosphorylation of p53, a substrate of CDK1, is inhibited by 9-hydroxy ellipticine in cell lysates and whole cells. As the anti-proliferative effects of 9-hydroxy ellipticine on 3LL and SW480 cells coincides well with the inhibition of p53 phosphorylation, it was postulated that the anti-tumor activity of 9-hydroxy ellipticine may be due to the inhibition of phosphorylation of mutant p53. However, as it has previously been shown that 9-hydroxy ellipticine intercalates DNA and interferes with topoisomerase II function by stabilizing the enzyme-DNA complex (29, 30), it is difficult to determine its *in vivo* target.

Suramin (**9**), a symmetrical polysulfonated naphthlylurea, originally synthesized in 1916 at Farbenfabriken Bayer AG, Germany, has been used in the treatment of sleeping sickness and other parasitic diseases for almost 70 years. It was also tested clinically in the treatment of AIDS due to its ability to inhibit reverse transcriptase (31) and has shown promise as an antineoplastic agent (32). Recently, suramin has been shown to inhibit the activity of purified CDK1 with an IC_{50} of 4 μM (33), which the authors suggest may be the source of its antitumor activity. However, due to its general lack of specificity it is difficult to determine the *in vivo* target of Suramin in tumor cells.

As it is estimated that 2,000-3,000 protein kinases are present in a mammalian cell (34), a critical issue for any kinase inhibitor is its specificity. Thus, a specific CDK inhibitor should block cell cycle progression and CDK substrates should not be phosphorylated in intact cells. While the known CDK inhibitors (Table 1) all possess some degree of anti-tumor activity, the correlation between cell growth inhibition and inhibition of CDK activity is strongest for flavopiridol, olomoucine, and butyrolactone-1.

CDK INHIBITORS AND APOPTOSIS

Apoptosis is a genetically encoded cell death program defined by characteristic cellular morphologic and biochemical changes (35). A multitude of extracellular and intracellular factors have been implicated as modulators of apoptosis, including the cyclin-dependent kinases (36-38). As increasing evidence suggests that cancer therapy operates most potently through induction of apoptosis (39), it is of considerable interest to examine the effects CDK inhibitors have on apoptosis.

Recent studies have shown that, dependent upon cell type, the inhibition of CDK activity either blocks or induces apoptosis. Inhibition of CDK activity in differentiated cells, such as neurons and myotubes, protects them from apoptosis (40-42). Neuronally differentiated PC12 cells and sympathetic neurons undergo apoptosis upon removal of trophic support. Addition of flavopiridol and olomoucine blocks the death of these trophic factor-deprived neuronal cells (40) and attenuation of $p21^{WAF1}$ (a CDI) mRNA expression in differentiated SH-SY5Y neuroblastoma cells induces apoptosis (41). Furthermore, forced expression of $p21^{WAF1}$ or $p16^{INK4A}$ inhibits apoptosis during myoblast differentiation (42). These findings suggest that inappropriate activation of CDKs (by abnormal re-entry into the cell cycle) induces apoptosis and that terminally differentiated, non-dividing cells die after attempting to re-enter the cell cycle. Inhibition of CDK activity has also been shown to induce apoptosis (40, 43, 44). Olomoucine and butyrolactone significantly stimulate apoptosis in cells that have been blocked in G2 by DNA-damaging agents suggesting that CDK1 plays an active role in suppressing apoptosis (43). Flavopiridol has also been shown to induce apoptosis in a variety of human tumor cell lines during exponential growth (44, 45). Furthermore, while flavopiridol and olomoucine suppress the death of post-mitotic neuronally differentiated PC12 cells, they induce apoptosis in mitotic PC12 cells (40). Taken together, these results suggest that CDK inhibition in actively dividing cells may trigger apoptosis. It is worth noting, however, that expression of dominant-negative mutants of CDK1, CDK2, and CDK3 or constitutively active CDK1 in HeLa cells suppresses apoptosis. Furthermore, Myc-induced apoptosis was shown to be insensitive to forced expression of $p16^{INK4A}$, $p21^{WAF1}$ or $p27^{KIP1}$ or treatment with roscovitine (**4**) (22). Thus, the ability of CDK inhibition to induce apoptosis in tumor cells appears to be dependent upon cell type and genetic background.

THERAPEUTIC APPLICATIONS OF CDK INHIBITORS

Due to their ability to inhibit cellular proliferation, CDK inhibitors may find therapeutic utility in hyperproliferative diseases, such as cancer, psoriasis, and fungal infections. The strongest argument for developing CDK inhibitors as anti-tumor agents is provided by overwhelming evidence that suggests that at least one member of the "RB pathway" (pRb, p16, cyclin D1, and CDK4) is altered in nearly all human tumors (8, 9). pRb has the ability to suppress cell cycle progression and this activity is controlled by its cell cycle dependent phosphorylation. Phosphorylation of pRb is initiated by cyclin D-dependent kinases and accelerated by cyclin E:CDK2. p16 blocks cellular proliferation by inhibiting cyclin D-dependent kinase activity. The majority of human tumors frequently lack functional pRb or p16 or contain elevated levels of cyclin D1. All three genetic lesions have the same phenotype, namely, the loss of G1 checkpoint control. CDK inhibitors provide the opportunity to restore this critical cell cycle control point in tumor cells.

Flavopiridol is presently the most promising CDK inhibitor with regards to the potential of becoming a useful antineoplastic agent. It displays significant antitumoral effects against a variety of tumor types *in vitro* and *in vivo* (46), with its greatest activity being in prostate tumors (11). In fact, flavopiridol is the first CDK inhibitor to enter clinical trials. Suramin has also entered clinical trials as an antineoplastic agent (32), but its lack of specificity makes it difficult to attribute its antiproliferative effects to inhibition of CDK activity.

In addition to proliferative disorders, CDK inhibitors may be useful in other clinical settings. Due to their ability to protect neuronal cells from cell death, CDK inhibitors may find utility as protective agents in neuronal disorders. Of particular interest is Alzheimer's disease. One of the most striking molecular changes that has been linked with the etiology of Alzheimer's disease is the aberrant phosphorylation of the microtubule-associated protein tau. Several kinases have been implicated in tau phosphorylation, including CDK5:p25 (47). Thus, inhibition of CDK5 activity may find utility in the treatment of Alzheimer's disease. As Alzheimer patients are likely to require long term drug treatment, the need to identify a CDK5:p25-specific inhibitor is more critical than it is for CDK inhibitors used to treat cancer patients. A specific inhibitor is more likely to avoid toxicity issues that are likely to arise with long term treatment regimens.

FUTURE DIRECTIONS

One of the primary challenges of future work will be to increase the specificity of CDK inhibitors by structure-function studies. The clinical usefulness of a CDK inhibitor may improve by increasing its specificity against non CDK family members, as well as, within the CDK family. The co-crystallization studies between CDK2 and olomoucine and flavopiridol have already begun to offer some insights with regards to the specificity of the CDK inhibitors. CDK2 residues that are conserved within, but not outside, the CDK family are involved in the molecular interaction with the CDK inhibitors (14, 13). Utilization of these data in conjunction with molecular modeling studies may aid in the development of more specific inhibitors.

An alternative approach to improving the specificity of inhibitors of CDK activity, is to target other aspects of CDK regulation. The complexity of the regulatory machinery that regulates CDK activity, provides a number of intriguing targets. These targets include: (i) CDK activating kinase, (ii) Cdc25 phosphatase, (iii) cyclin degradation machinery, (iv) substrate binding domain, (v) cyclin binding domain, and (vi) CDI mimetics. The latter three targets offer the greatest possibility of identifying inhibitors that are CDK specific, but all would involve protein:protein interaction assays. Protein:protein interactions that occur over a large surface area, as with CDK2 and cyclin A (48), may be difficult to disrupt with small molecule inhibitors. It is worth noting, however, that a molecular interaction assay has recently identified a number of peptide aptamers that bind to and inhibit the activity of CDK2 (49). The authors suggest that the protein aptamers bind to a face of the CDK2 molecule and block its interaction with one of its protein partners or substrates. While these peptides are too large to be considered "drug-like", they do offer powerful tools to assess the effects of inhibiting CDK2 activity. Furthermore, co-crystallization studies with CDK2 and the protein aptamers may offer an opportunity to use molecular modeling for directed drug design.

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

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Chapter 18. T Lymphocyte Potassium Channel Blockers

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Introduction - Over the past 15 years, advances in molecular biology and patch-clamp technology (1, 2) have allowed detailed characterization of numerous ion channels. In the early eighties, two groups working independently discovered the type-*n* voltage-gated potassium channel (Kv1.3) in T lymphocytes (3-12). By the early nineties, the Kv1.3 gene was shown to encode the unique type-*n* channel, and the cloned channel had been biophysically and pharmacologically characterized (13-19). The functional channel in T cells is thought to be a homotetramer of Kv1.3 subunits with an approximate mass of 270 kDa and x-y dimensions of 65 x 65 Å (20-22). Expression of this gene is highly restricted to T cells, although Kv1.3 mRNA has also been detected at low levels in some other cell types (22-29). Only in T cells, however, does the Kv1.3 channel dominate control of the membrane potential (30-35).

Early studies showed that small-molecule blockers of Kv1.3 inhibit mitogen-induced activation of T cells. Because these agents were weak inhibitors and displayed low selectivity, it was evident that more potent and specific blockers were necessary to convincingly define the role of Kv1.3 in T cell activation. Several such selective high-affinity blockers, both peptide and small-molecule, have been discovered in recent years (36-44). Armed with these pharmacological tools, several investigators have established a critical role for Kv1.3 in lymphocyte activation. It is now thought that Kv1.3 blockers chronically depolarize the T cell membrane, attenuate calcium entry via calcium release-activated calcium (CRAC) channels in the plasma membrane, and consequently abrogate the calcium signaling pathway, which is essential for lymphocyte activation (30-35). Several excellent reviews have dealt with the electrophysiological and pharmacological properties of Kv1.3 and its functional role in T cell activation (10,25,26,30-33).

The vital role of Kv1.3 during T cell activation has stimulated a search for potent and selective channel blockers that can be used as immunosuppressive agents. Due to its distinct mechanism and restricted tissue distribution, a Kv1.3 blocker would not likely display the same toxic side-effects of currently used immunosuppressants such as cyclosporin and FK-506, and therefore may prove useful in the treatment of chronic autoimmune diseases as well as transplantation therapy.

NON-SELECTIVE SMALL-MOLECULE BLOCKERS OF Kv1.3

Classical K^+ Channel Blockers - Kv1.3 channels in both human and mouse T cells are reversibly blocked by several non-specific potassium channel antagonists such as resiniferatoxin **1** (IC_{50}=3 μM), quinine **2** (IC_{50}=6 μM), capsaicin **3** (IC_{50}=26 μM), flecainide **4** (IC_{50}=53 μM), 4-aminopyridine **5** (IC_{50}=190 μM) and tetraethylammonium **6** (IC_{50}~10 mM) (3-12,15,19,25,45). Many of these drugs require channel opening to gain access to a binding site, a phenomenon termed *use-dependent* (sometimes referred to as *open channel*) block. The Kv1.3 residues that interact with these drugs have not been specifically identified, although 4-aminopyridine has been shown to interact with an intracellular site on the channel (45).

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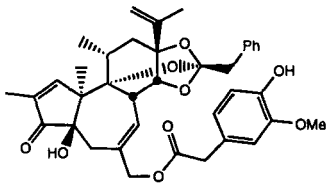
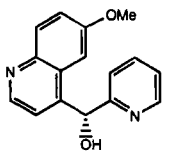
Early studies using **2**, **5** and **6** probed the role of Kv1.3 in T cell activation (3,6,9,21). These compounds all inhibited ^{3}H-thymidine incorporation in T cells stimulated by mitogen or alloantigens (3,9) with a potency paralleling their effects on Kv1.3. The absolute functional potencies of these compounds, however, were lower than those observed for channel blockade, suggesting that >50-90% of K^+ current blockade is necessary to inhibit T cell proliferation. In addition to blocking T cell activation, more detailed studies showed that these agents inhibited mitogen-induced IL-2 production, but not expression of the IL-2 receptor (9,12,46). Consequently, exogenous IL-2 was capable of overriding the inhibitory effects of these blockers.



Resiniferatoxin (1)



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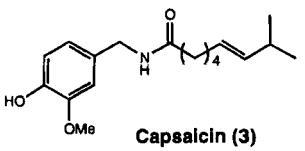

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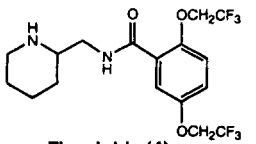
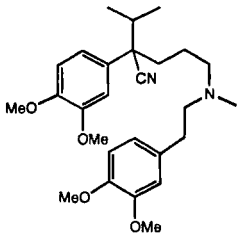
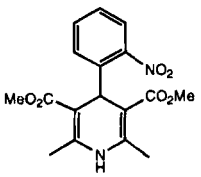


Flecainide (4)

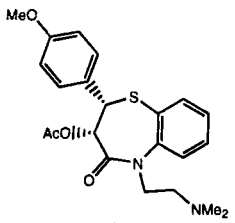
Calcium Channel Blockers - Known calcium channel antagonists exhibit use-dependent block of Kv1.3 with IC_{50} values in the micromolar range: verapamil **7** (6 μM), nifedipine **8** (5-24 μM) and diltiazem **9** (27-60 μM) (5,6,9-11,15,19,47). These drugs also inhibit mitogen-induced activation of T cells at concentrations that block Kv1.3 (9-11,46,47). Recent investigations suggest that verapamil interacts with an internal binding site on Kv1.3 (48).



Verapamil (7)

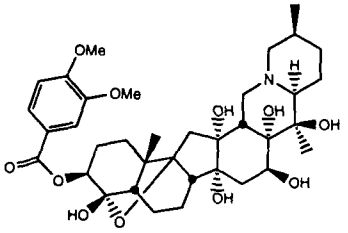


Nifedipine (8)

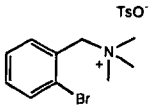


Diltiazem (9)

Sodium Channel Modulators - Veratridine **10** is a known sodium channel opener and was employed in studies investigating the role of sodium channels in T cells (49). Rather than inducing a sodium current in lymphocytes, this compound was found to block Kv1.3 K^+ efflux at high concentrations (IC_{50} = 97 μM). Patch-clamp studies with **10** showed that this molecule blocks Kv1.3 when applied to either the inside or outside of the cell, and, unlike use-dependent blockers such as verapamil, displays rapid block of current on the first depolarizing pulse, indicating that it blocks the closed state of the channel. In contrast to **10**, bretylium tosylate **11**, a sodium channel opener, reversibly blocked Kv1.3 in a use-dependent manner with an IC_{50} of 0.5-5 μM (50). At similar concentrations, this drug inhibited ^{3}H-thymidine incorporation in mitogen-stimulated T cells. Unlike tetraethylammonium ion, bretylium tosylate is thought to interact at a site other than the channel pore.



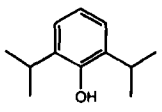
Veratradine (10)



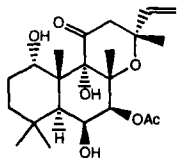
Bretylium Tosylate (11)

Anesthetics - The i.v. anesthetic, propofol **12**, was also found to exhibit effects on T lymphocytes, blocking Kv1.3 current in a use-dependent manner with an IC_{50} of 40 μM, a physiologically relevant concentration for anesthesia (51). Anesthetics have previously been shown to have effects on potassium channels other than Kv1.3 (52). The currently accepted hypotheses regarding anesthetic mechanism of action (53) are consistent with the effects observed on Kv1.3, although it is not clear whether propofol interacts with the cell membrane or with the channel itself.

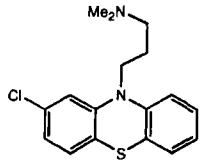
Forskolin - One of the more unusual examples of small-molecule blockers of Kv1.3 is forskolin **13**, which was discovered during a study intended to show the effects of increased intracellular cAMP levels on lymphocyte channel function. The addition of forskolin at a concentration of 60 μM diminshed peak current by 50% (54). Current blockade by forskolin was rapid and, surprisingly, was not due to increased cAMP *levels but rather to a direct action of forskolin itself. 1,9-Dideoxyforskolin (DDF), an* analog that is 100-fold less potent than forskolin at raising cAMP levels, also blocked Kv1.3 current in the same potency range as forskolin. The manner in which these analogs inhibit channel function is unknown. However, it is interesting to note that if forskolin and DDF interact directly with the channel, they are structurally unique blockers because they do not possess a basic amine or tetraalkylammonium salt.



Propofol (12)



Forskolin (13)



Chlorpromazine (14)

Other Agents - A variety of other small molecules have also been reported to block Kv1.3 current. Calmodulin antagonists such as chlorpromazine **14** and the closely related trifluoperazine block Kv1.3 at low micromolar concentrations (46,47). At similar concentrations, these two agents also inhibit IL-2 production by the human Jurkat T cell line and mouse thymocytes (55,56). The relative contributions of calmodulin inhibition and Kv1.3 blockade to the in vitro immunosuppressive effects of these phenothiazines remain to be determined. Additional small molecules that have shown weak blockade of Kv1.3 current include juglone, progesterone(46,47) and the neurotransmitter, acetylcholine (58). The known immunosuppressive agents, cyclosporin A (CsA) and FK-506, also are reported to partially block the channel (59).

SELECTIVE BLOCKERS OF Kv1.3

Peptides From Scorpion Venom - Venom from insects and reptiles has historically been the source of ion channel blocking peptides (19-22,25,60-62). Scorpion venoms in particular have provided numerous peptides specific for the blockade of various potassium channels. One of the best studied blockers is a 37 amino acid peptide,

charybdotoxin (ChTX), which was reported in 1985 as a blocker of the high-conductance calcium-activated potassium channel (Maxi-K) found in smooth muscle and neurons (63-65). In 1989, ChTX was shown to reversibly block Kv1.3 with 1:1 stoichiometry (IC_{50} ~1 nM)(66). ChTX was also found to block other voltage-gated potassium channels as well as the biophysically distinct class of small-conductance calcium-activated K^+ channels (19-22,25,37,64,67-69).

Subsequently, four related peptides isolated from scorpion venom (margatoxin, kaliotoxin, noxiustoxin, agitoxin 2) have been shown to potently and reversibly block the closed (resting) state of Kv1.3 with IC_{50} values in the low picomolar to nanomolar range and with 1:1 stoichiometry (19-22,37,70). All five of these toxins contain 37-39 amino acids and are highly basic, possessing a charge of +5 or +6 at neutral pH (19-22,64-66). NMR structures of the peptides show that they all share a similar tertiary fold arising from 6 highly conserved cysteine residues that form three disulfide bonds (21,71-74). These disulfides provide a rigid and compact tertiary structure comprised of an α-helix laying atop a small 3-strand antiparallel β-sheet. Early studies using mutants of ChTX showed that the majority of residues which strongly interact with both voltage-gated and calcium-activated K^+ channels, project off the β-sheet down into the channel pore region (64-66,75-78). One of the most important residues is a central lysine (K27 in ChTX) that has been shown to interact with a highly conserved tyrosine (Y400 in Kv1.3) in the "signature sequence" (GYGD) thought to contribute to the potassium-specificity of the channel pore (22,79).

Because of their rigid structure, these small peptides have been used to map the outer-pore region of Kv1.3 and its closely related *Drosophila* homologue, *Shaker* (21,22,71,72,75-83). Using mutants of both toxins and the Kv1.3 channel, several toxin-channel interactions have been mapped. This information, together with distances derived from toxin NMR solution structures, predicts that the external region surrounding the ion conduction pore forms a saucer-shaped vestibule that is ~30 Å wide and ~6 Å deep. The outer entrance of the pore forms a 5 Å trough at the center of the saucer where the critical lysine residue of the toxin is thought to interact.

In addition to their role as structural probes, the toxins have been pivotal in confirming the important functional role of Kv1.3 in T cell activation. ChTX, in addition to blocking Kv1.3 current, also inhibits T cell proliferation (IC_{50}=2-10 nM) and IL-2 production (IC_{50}=0.8 nM). This functional data, although compelling, did not unequivocally establish the role of Kv1.3 as the primary K^+ channel involved in regulation of T cell activation. Subsequent to its discovery as a Kv1.3 blocker, ChTX was shown to block the small-conductance calcium-activated K^+ channels that are also found on T cells (37). These findings made it unclear as to which channels were responsible for the immunosuppressive effects of ChTX.

Studies with two toxins which display greater Kv1.3 selectivity clearly indicate that blockade of Kv1.3 leads to inhibition of T cell activation (37,38). The first, noxiustoxin, blocks Kv1.3 currents with an IC_{50} value of 0.2 nM (19,31,37) and suppresses T cell proliferation with an IC_{50} of 8 nM (37,38). This effect is most likely due to Kv1.3 blockade because the toxin is only a weak inhibitor of the small-conductance calcium-activated K^+ channels in lymphocytes (IC_{50}=1 μM). Although noxiustoxin blocks Kv1.2 (IC_{50}=41 nM) (19) and the Maxi-K channel (IC_{50}>450 nM) (84), these proteins are not expressed in T cells (25). The second peptide, margatoxin, a significantly more potent and selective blocker of Kv1.3 (IC_{50}=0.04 nM), depolarizes T cells, and inhibits the rise of intracellular calcium and IL-2 production triggered by anti-CD3 mAb (19-22,37,38).This immunosuppressive effect is mediated via Kv1.3 blockade, since margatoxin is 2000-fold selective for Kv1.3 over Maxi-K and small-conductance calcium-activated K^+ channels (37). The only other channel

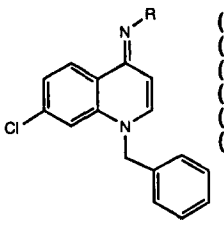
blocked by margatoxin, Kv1.2, is not expressed in T cells (85). Margatoxin is currently being pursued as an injectable immunosuppressive agent (86).

Other Toxins - Scorpion venom has not been the only source of Kv1.3 blocking peptides. α-Dendrotoxin, a highly basic 59 amino acid peptide derived from snake venom, inhibits Kv1.3 with moderate potency (IC_{50}=250 nM), but is more potent against the closely related voltage-gated channels Kv1.1 and Kv1.2 (IC_{50}~20 nM)(87-90). Several species of sea anemone have also yielded Kv1.3 blocking peptides (91-93). These sea anemone toxins range in size from 37-39 amino acids, contain 3 disulfide bonds, and are also highly basic (94,95). NMR analysis of one of these toxins, stichodactyla toxin (ShK), reveals a tertiary fold distinct from that of the scorpion toxins (96). ShK is an extremely potent blocker of Kv1.3, blocking both ^{125}I-ChTX binding (IC_{50}=32 pM) and channel current (IC_{50}=130 pM). The selectivity and functional activity of ShK has not yet been reported. Additional peptide blockers of Kv1.3 continue to be discovered. Recently, another scorpion toxin, maurotoxin, has been shown to block Kv1.3 (97). This 34 amino acid peptide has four, rather than three, disulfide bonds. Maurotoxin blocks Kv1.3 current with moderate potency, but is actually more potent against Kv1.1 and Kv1.2.

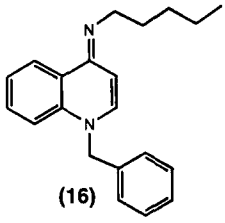
Selective Small-molecule Blockers - Increased interest in Kv1.3 as an immune suppression target has prompted high-throughput screening of pharmaceutical files for potential Kv1.3 blockers. The implementation of two different screening strategies has resulted in the discovery of two novel and structurally distinct series of potent and selective Kv1.3 blockers.

4-Imino-dihydroquinolines - Using an assay measuring ^{125}I-ChTX binding to T lymphocytes, a series of molecules was discovered that displayed vastly improved potency over previous small-molecule agents. WIN17317-3 **15**, was found to competitively inhibit ChTX binding to human peripheral blood T cells (IC_{50}=65-83 nM) (40,41). Detailed analysis of the binding interaction revealed that WIN17317-3 interacts with Kv1.3 at the ChTX binding site. Furthermore, patch-clamp analysis showed that this compound blocks Kv1.3 current (IC_{50}=250-335 nM) by binding to the open or inactivated state of the channel in a use-dependent manner. In a T cell functional assay, WIN17317-3 inhibited IL-2 production stimulated by anti-CD3 plus anti-CD28 mAbs (IC_{50}=1 μM). The Kv1.3 selectivity of WIN17317-3 was demonstrated by testing for blockade of calcium-activated K^+ channels in smooth muscle (150-fold less potent) and delayed-rectifier K^+ channels in cardiac myocytes (20-fold less potent).

A closely related analog to WIN17317-3, CP-339818 **16**, has also been reported to block ChTX binding (IC_{50}=120 nM) as well as channel current (IC_{50}=150 nM) (42). Similar to WIN17317-3, CP-339818 exhibits use-dependent blockade and inhibits T cell proliferation (IC_{50}=4.7 μM). Extracellular blockade of Kv1.3 currents by CP-339818 occurs with 1:1 stoichiometry. Detailed patch-clamp studies with Kv1.3 mutants demonstrated that this analog appears to bind to the inactivated state of the channel. CP-339818 also displays a high level of selectivity for Kv1.3. When tested against a panel of 10 related voltage-gated potassium channels, comparable activity to Kv1.3 was only observed with Kv1.4 (IC_{50}=0.3 μM), a channel found in heart and brain (98,99).



(15) R= n-pentyl
(17) R= n-hexyl
(18) R= n-propyl
(19) R= n-octyl
(20) R= $(CH_2)_2CO_2H$
(21) R= $(CH_2)_2NEt_2$



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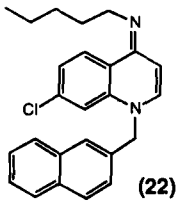
Optimal potency and selectivity in the 4-imino-dihydroquinoline series is obtained with the pentyl and hexylimino derivatives, **15** and **17**, which are roughly equipotent (Table 1). Analogs with shorter **18** or longer **19** alkyl side chains on the C4 imino nitrogen show 10-20-fold decreases in potency and/or selectivity. Terminal substitution with polar residues on the alkylimino side chain results in a much more pronounced loss of Kv1.3 potency. For example, an analog with a terminal carboxylic acid **20** is completely inactive against Kv1.3 at >10 μM, and the diethylaminoethyl derivative **21** does not displace ^{125}I-ChTX binding to Kv1.3, but rather displays low nanomolar activity against the cardiac delayed-rectifier K^+ channel blocked by dofetilide (100).

TABLE 1

Compound No.	ChTX Binding (IC_{50})	Kv1.3 Current Blockade (IC_{50})	Kv 1.3 Selectivity vs Cardiac Channel
15	65-83 nM	250-335 nM	20-fold
17	32 nM	335 nM	25-fold
18	544 nM	3160 nM	N.D.
19	280 nM	351 nM	9-fold
20	>10000 nM	N.D.	N.D.
21	7680 nM	N.D.	57-fold

The 7-chloro substituent on the dihydroquinoline ring provided both optimal potency and selectivity. Analogs of **15** with simple groups such as 6-methoxy, and 7-trifluoromethyl retained potency but these derivatives showed decreased selectivity for Kv1.3 over the cardiac K^+ channel. At the N1 position, a benzyl substituent was favored, although the 2-napthylmethyl derivative **22** was also very potent (ChTX IC_{50}=75 nM) and selective (100-fold). In contrast, the 1-naphthylmethyl derivative **23**



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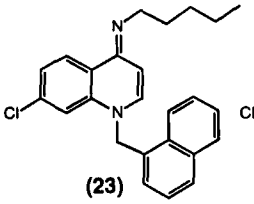

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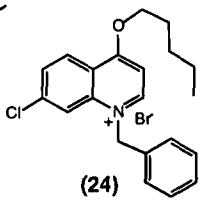

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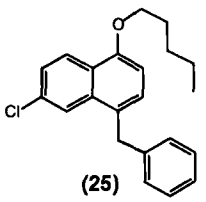
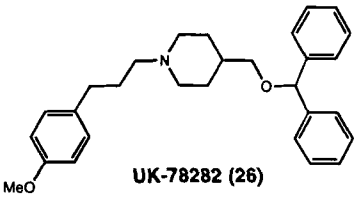


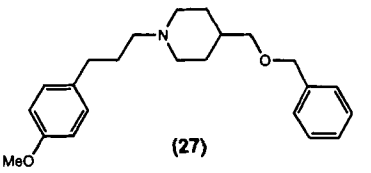
(25)

retained potency but not selectivity. These SAR studies also revealed that the combination of both nitrogens played an important role in the potency of these analogs. The quinoline ether **24** was found to display reduced inhibition of ChTX binding (IC_{50}=828 nM), but removal of both nitrogens in the naphthyl ether **25** was found to abolish activity (IC_{50}>5 μM).

Benzhydryl Piperidines - A second series of novel blockers, exemplified by the piperidine, UK-78282, was discovered using a rubidium efflux assay in which $^{86}Rb^{+}$ serves as a radioactive surrogate for K^{+}. Kv1.3 channels are opened by depolarizing test cells in a high-K^{+} buffer. UK-78282 **26** inhibits rubidium efflux (IC_{50}=0.4 μM), blocks channel current (IC_{50}=0.2 μM), and inhibits proliferation and IL-2 production of human T cells (IC_{50}=2-4 μM)(43,101). As with earlier, less potent blockers, expression of the IL-2 receptor was not affected and the compound did not inhibit total protein synthesis. Blockade by UK-78282 was use-dependent and persisted after attempted washout. Block could, however, be overcome by membrane hyperpolarization.

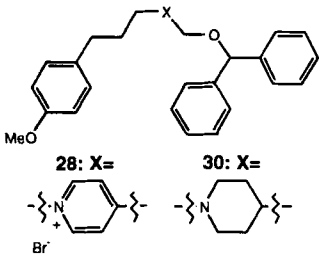


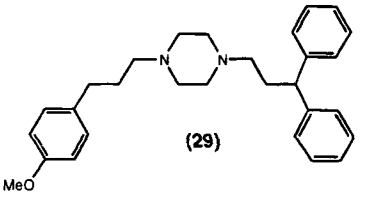
UK-78282 (26)



(27)

The selectivity of UK-78282 was examined by testing against a panel of potassium channels. In these studies, UK-78282 was found to be 20- to 200-fold selective for Kv1.3 over four closely related voltage-gated potassium channels . It was suggested that interactions with a unique histidine residue in the outer-pore region of Kv1.3 are responsible for the observed selectivity of UK-78282. Point mutations of several channel residues in the outer vestibule of Kv1.3 were generated to test this hypothesis. Mutant studies, as well as variations in pH, strongly suggested an interaction between the benzhydryl moiety of UK-78282 and the H404 residues in the tetrameric channel pore region. A similar interaction has been proposed for the benzhydryl-containing substance P antagonist, CP-96345 (102,103). The interaction seems to be specific for benzhydryl piperidine derivatives as the potency of analog **27** was not affected by mutations at H404. UK-78282 is the first relatively selective small-molecule Kv1.3 channel blocker reported to have biological activity in vivo. UK-78282 inhibits T cell activation in a murine allogeneic sponge matrix model (101).





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Exploration of the SAR of these benzhydryl piperidines was performed using the rubidium efflux assay. UK-78282 was found to contain three critical elements for Kv1.3 activity: a benzhydryl "head" group, a central basic nitrogen, and a lipophilic "tail". Analogs which contained smaller, less lipophilic replacements for the benzhydryl group (**27**) were 5-fold less active. The need for a single, central basic nitrogen was highlighted by the synthesis of pyridyl analog **28**, which was inactive (IC_{50}>20 μM), and the piperazine derivative **29** (IC_{50}=2.7 μM). Adding conformational restriction to the central piperidine ring, however, was tolerated, as tropane analog **30** was found to be equipotent to UK-78282. Finally, the necessity for lipophilicity in the p-

methoxyphenpropyl tail unit was explored. Variation of the lipophilic character of the tail revealed optimal Kv1.3 potency was obtained by analogs with a cLogP of 6.5.

Conclusion - Structurally diverse blockers of Kv1.3 have been identified. A common feature for most of these compounds is the presence of a basic amine or quaternary ammonium salt, which is thought to bind in the channel pore. Some recently identified blockers have shown marked increases in selectivity and potency, however, none of the small-molecule blockers identified to date display levels of potency and selectivity that will likely be required for Kv1.3 based immunosuppression. Thus, despite the rapid increase in knowledge regarding lymphocyte ion channels, the field of Kv1.3 blockers remains immature. Looking toward the future, further mapping of toxin-channel interactions may provide key insights into the design of agents suitable for testing in man.

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Chapter 19. Male Contraception

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Introduction - Approaches to the development of a safe and effective male contraceptive will be reviewed from 1986 when it was last reported in this series (1). There have been only 34 patents issued claiming methods of male contraception over the last ten years but there are nearly 400 references in the general literature, showing a continuing interest in the subject. Logistically, inducing infertility in the male is more challenging than in the female due to the production of sperm in the billions per month and fact that it takes but one motile gamete to fertilize an egg cell. In addition, males produce sperm continuously and store them in various stages of maturation, unlike the female's monthly cycle rendering one or two eggs at a time. While it is relatively easy to stop the production of sperm, it is difficult to do so without affecting levels of testosterone, a hormone necessary to maintain libido. Sperm can also be incapacitated or killed by toxic chemicals but not without deleterious side effects. For this reason, a more selective mechanism for blocking sperm production has been the object of reproductive research for over 30 years.

The social issues surrounding male contraception may be as challenging as the scientific ones. Cultural biases concerning male virility and trust issues between partners are insurmountable barriers for some couples. Nevertheless, a need exists for effective population control and a "male pill" would be a welcome addition to the diverse list of human anti-fertility options.

SMALL MOLECULE THERAPY

Steroids - Progestins, antiestrogens and androgens as well as various combinations of the three have all be used to alter fertility in the male. This is an active area of research which allows for quick entry into human clinical trials due to the comfort level of working with compounds of known toxicity, many of which have been used in women for years. Direct methods for the inhibition of spermatogenesis through suppression of secretion of gonadotrophins must be combined with replacement of testosterone (2). These methods include the use of combinations of progestogens or LH-releasing hormone antagonists and testosterone derivatives, or high dose testosterone. Though effective contraception can be obtained using steroid therapy, side-effects and/or the high cost of treatment limit the widespread use of these approaches.

Solasodine **1**, a steroid of plant origin, was administered to rhesus monkeys for 150 days and was found to interfere with spermatogenesis at stage XII of late spermatids (3). A decrease in sperm count was observed and sialic acid, cholesterol and glycogen were depleted following treatment. Oleanolic acid **2**, isolated from the flowers of *E. jambolana*, was also evaluated in male albino rats. The administration of **2** for 60 days decreased the fertilizing capacity of the animals without any significant changes in body weight or reproductive organ weights. It produced arrest of spermatogenesis but did not cause any abnormality to spermatogenic cells, Leydig interstitial cells, or Sertoli cells. (4)

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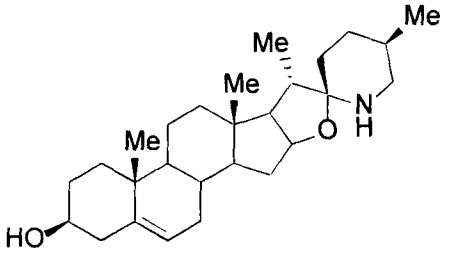

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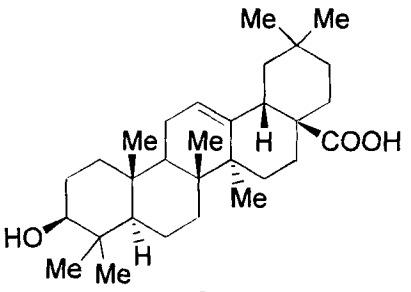

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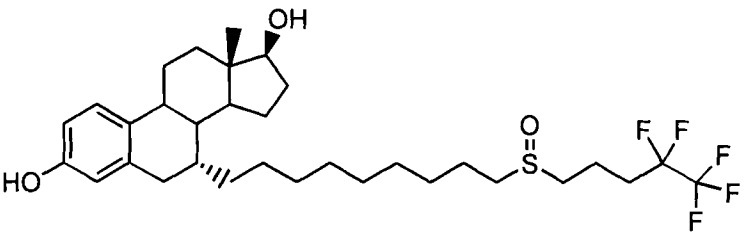
Administration of progestins has little effect on sperm count or viability. There are reports that antiprogestins, such as RU486, are useful in the control of male fertility and/or treatment of disorders such as prostate hypertrophy or prostate carcinoma. RU486 caused marked decreases in ejaculate volume and sperm concentration and motility in bonnet monkeys (5). On the other hand, the antiprogestin CDB 2914 has no antifertility effect in male rats (6). These reports fail to consider that all known antiprogestins are also potent antiglucocorticoids and the observed activity may be better explained by that mechanism of action. Progestins given in combination with testosterone do seem to enhance the action of androgens (7).

Antiestrogens are useful as chemical contraceptives for males and exhibit reversible action. Administration of the antiestrogen 7-α-9-((4,4,5,5,5-pentafluoropentylsulfinyl) nonyl)estra-1,3,5(10)-triene-3,17β-diol **3** to male rats induced a premature acrosome reaction and impaired sperm motility without altering the reproductive tract weight or sperm count (8). More work needs to be done to verify the efficacy of this therapy.

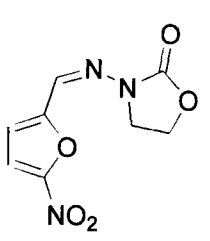


3

Non-steroids - New structures have emerged recently but there are still no small molecules nearing clinical trials at this time. Much of the literature contains either anecdotal accounts or toxicological reports on existing drugs or industrial chemicals and their ability to affect the male reproductive system. While it is interesting to note these side effects, unless they are made the target of a dedicated drug discovery effort, such compounds will not be useful as contraceptives. Some of the agents reviewed were not actually tested in males or even *in vivo*, rather, they would be better characterized as spermicides.

Two antimicrobials were examined for their effect on the male reproductive system. Furazolidone **4** caused a reversible infertility without affecting testosterone levels and ornidazole **5** reduced the ability of rat sperm to fertilize egg cells without affecting sperm number or motility (9,10). The calcium channel antagonists Nifedipine **6** and Verapamil **7** were found to modulate human spermatozoal functions (11,12). Carbaryl **8** has been examined for spermatological effects with the finding of both a

dose and an age-dependent decline in epididymal sperm count and sperm motility with younger males more severely affected by the therapy (13). An increase in sperm with abnormal morphology was also found.



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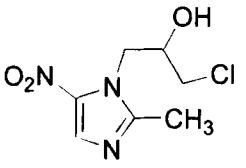

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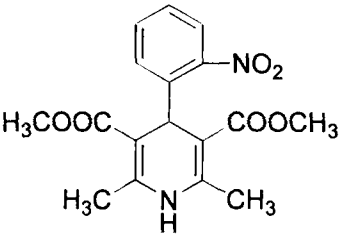

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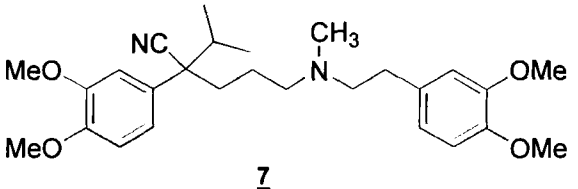

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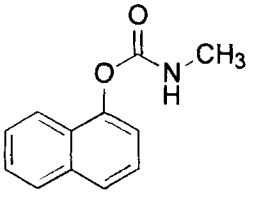
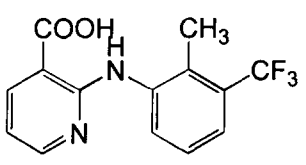


8

Flunixin 9, an anti-inflammatory-analgesic, has been examined as an anti-fertility agent in male rats (14). Prolonged exposure of male rats to flunixin significantly decreased the weights of genital organs, sperm cell concentration, sperm motility and the percentages of dead and morphologically abnormal spermatozoa. Decreases in the plasma testosterone level and increases in plasma LH and FSH levels were also observed in the treated groups. Administration of the drug to male rats decreased their ability to mate females and the number of viable fetuses was significantly decreased. 1,6-Dichloro-1,6-dideoxy-D-fructose 10 is cleaved to 3-chloroacetaldehyde and 3-chloro-1-hydroxypropane *in vivo*. Production of these chloro-trioses leads to the strong inhibition of glyceraldehyde-3-phosphate dehydrogenase in boar sperm (15). Etoprine 11 has a contraceptive effect on male rats thought to be due to its activity as an inhibitor of dihydrofolate reductase (16).



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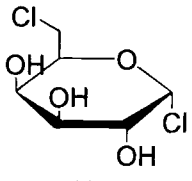

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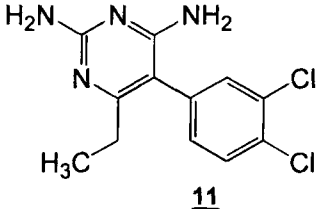
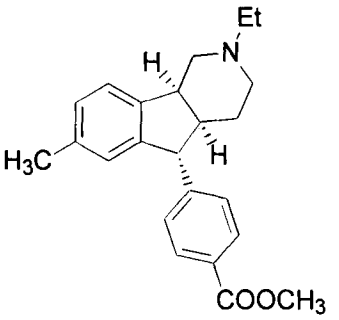


11

2,3,4,4a,5,9β-Hexahydroindeno(1,2-c)pyridines such as the ester shown 12, have potent antispermatogenic activity in rats (17,18). This is perhaps the best example of how making an observation concerning male reproductive activity as a side effect in an existing compound can lead to a synthetic effort aimed at compounds with the antispermatogenic effect as the major activity. About ten compounds were reported with definable structure activity relationships for changes in testicular weight. DL-204 13, is postulated to have a twofold mechanism of action, working to both reduce testes weight and decrease the production of gonadotropins (19,20).



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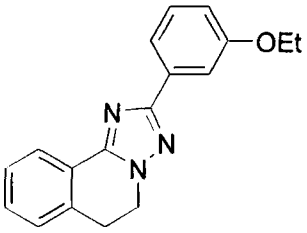
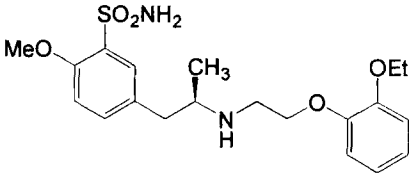


13

Tamsulosin 14 and terazosin 15, both highly potent and selective α-1-adrenoceptor antagonists, were administered subcutaneously to male rats causing a rapid and reversible reduction in fertility due mainly to impaired ejaculatory competence (21,22).



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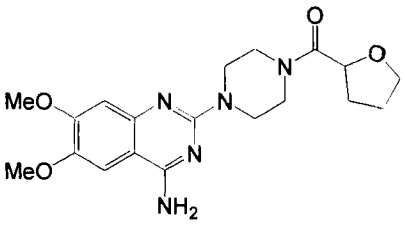
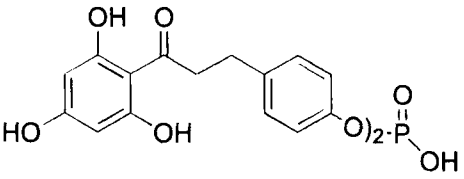


15

Di-4-phloretin phosphate (DPP 16), a prostanoid receptor antagonist, induced detrimental effects on reproduction of the male rat in high doses (23). This seemed to be caused mainly by failure of the rats to copulate and not by any physiological effect on sperm. The dye, malvidin chloride 17 also had antispermatogenic activity (24).



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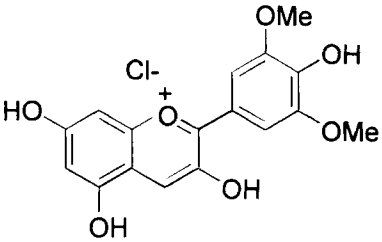
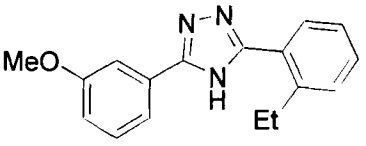


17

DL-111 18 may work by blocking androgen biosynthesis and was accompanied by a loss in testicular weight (25,26).

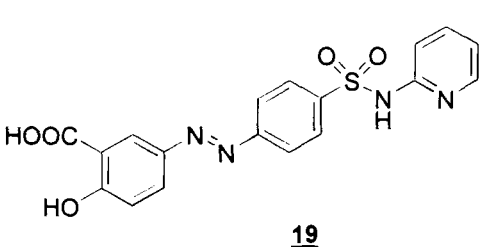


18

There are several compounds that have been studied for many years without gaining acceptance as drugs but which paradoxically, continue to enjoy the scrutiny of

workers in this field. Sulfasalazine **19** and gossypol **20** fall into this category. A review has appeared which concludes that sulfasalazine, taken in tolerable doses, has insufficient antifertility activity and too many serious side effects to be accepted as a contraceptive (27). Several studies appearing after the review both on sulfasalazine and metabolites came to a similar conclusion. (28-33)

There were 82 references to gossypol both alone and in mixtures. Although there is no compelling evidence to support the use of this drug in man, it continues to be of interest (2).



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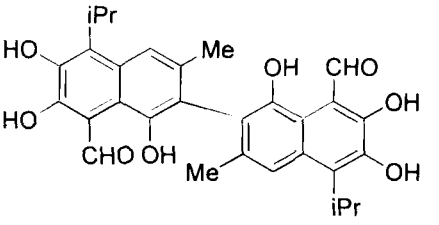
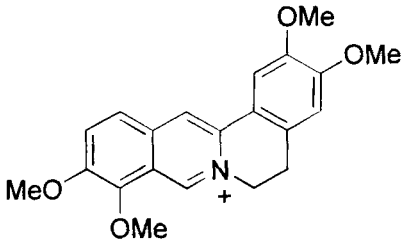


20

A number of fatty acids have been examined as potential antifertility agents in the male. In an *in vitro* study, 0.125% sodium morrhuate, an extract of cod liver oil composed principally of fatty acids such as arachidonic acid, completely abolished human spermatozoa motility. In male rabbits, dogs, and rhesus monkeys, a single injection of up to 2 mL *via* vas deferens into each cauda epididymis induced azoospermia or oligospermia on days 3-14 after treatment. By using the same procedure, a single injection of 1-2 mL of the mixture was studied in 1061 fertile male volunteers for 7-28 months (34, 35). It is highly unlikely that an injectable drug would be developed for this purpose.

The Berberis alkaloid palmatine hydroxide **21** was administered orally to dogs at 30 mg/kg/day for 60 days causing reduction but not a cessation of spermatogenisis. Although the Sertoli cells remained unaltered, Leydig cell numbers were decreased. The weights of the testes and epididymides were also reduced leading the authors to conclude that the antispermatogenic action of palmatine hydroxide may be mediated by disturbances in Leydig cell function (36).

Phenoxybenzamine, **22** an antihypertensive, reduced fertility in male rats after 1 week with no effect on general health or libido and was reversible. However, a diminished ejaculatory ability resulting from phenoxybenzamine blockade of α-adrenoceptors may contribute to the infertility since many normal sperm were present (37).



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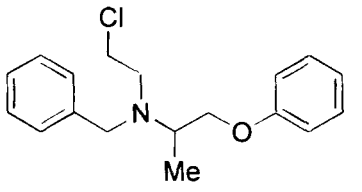
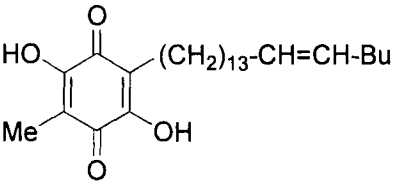


22

Maesaquinone **23** and embelin **24**, naturally occurring plant benzoquinones, have been investigated as possible male antifertility agents in animals. Marked changes were noted in the levels of testosterone or glycogen, protein, nucleic acids and certain carbohydrate constituents in reproductive tissues. These treatments, which affect levels of hormones or other blood constituents vital to the health of the user cannot be seriously considered as human drugs (38-41).



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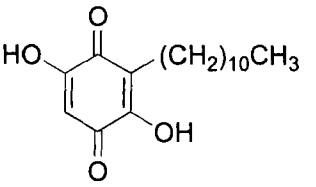
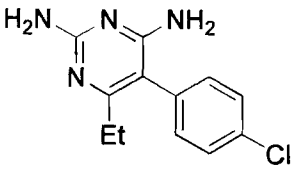


24

Pyrimethamine **25** caused spermatogenic arrest and male infertility in mice in a dose-dependent manner. Furthermore, upon cessation of drug administration all animals returned to normal fertility status. It is suggested that the action of **25** is due to its antifolate action. Thus, pyrimethamine represents another approach toward development of a male contraceptive (42).

The flavonoid kaempferol **26**, was screened for antifertility activity in male rats. Oral administration caused an inhibition of spermatogenesis and reduction in Leydig cell size and number thereby affecting fertility (43). 5,7,3'-Trihydroxy-6,8,4'-trimethoxyflavone **27** isolated from the seeds of *V. negundo* caused disruption of latter stages of spermatogenesis, but without affecting Leydig cell morphology. The weights of the testes and epididymis decreased. Azoospermia was claimed to be achieved without altering the metabolism or libido (44).



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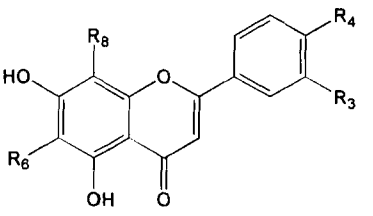
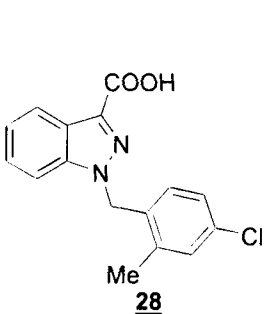


26 R3=R6=R7=H, R4=OH
27 R3=OH, R4=R6=R7=OMe

Tolnidamine **28** and the glycoside isolated from the leaves of *Aloe barbadensis*, Aloin **29**, were both administered to adult male langur monkeys (45-47). While **28** inhibited spermatogenesis, it caused irreversible testicular damage. Changes to Leydig cell number due to Aloin seemed to be reversible.



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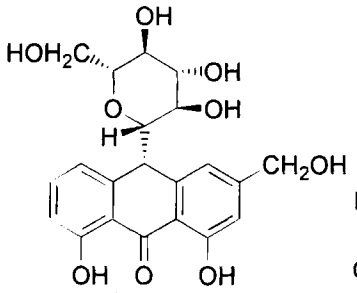

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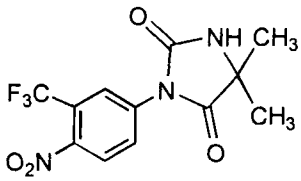


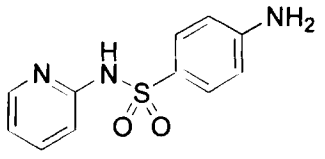
30

Administered at a daily oral dose of 10 mg (per adult male rat) for 30 days, the non-steroidal antiandrogen nilutamide **30**, increased plasma testosterone levels and caused spermatogenic arrest and stimulation of Leydig cells. Although a reduction occurred in accessory sex organ weights, no parallel reduction was evident in the secretory indexes. Females mated with treated males showed postimplantation loss, indicating an adverse effect of **30** during spermiogenesis. Apparently, the antiandrogenic potency of the compound in intact animals is insufficient to completely neutralize the elevated levels of androgens. A small dose of estradiol efficiently neutralized the central stimulatory effect of nilutamide and potentiated its antiandrogenic action (48).

The effects of P2-α-purinoceptor antagonists on ejaculatory competence and fertility were detected by injecting α,β-methylene ATP directly into the epididymis of rats inhibiting the fertility of the rats in serial matings (49).

Twenty-four polyphenolic compounds extracted from Chinese herbal medicines were studied for their effects on human sperm motility. A structurally related set of compounds, showed strong dose-dependent inhibition of sperm motility (50). Many of the common polyphenolic compounds which are naturally present in abundance in plant extracts exercise inhibitory effects on mammalian sperm motility *in vitro*, which could be the cause of the anti-fertility effects of the crude plant extracts (51).

Twenty sulfa drugs and other Sulfonamides were tested for antifertility activity by natural mating in male rats. Sulfapyridine **31** was the most potent of the compounds tested causing a 26% reduction in fertility at a dose of 450 mg/kg after 5 weeks and complete recovery by three weeks after drug withdrawal (52-54). The spermatic cords of rhesuses monkeys were injected with the long-acting local anesthetic, procaine **32**. Although the testes changed markedly, no change was observed in interstitial cells and the secretion of testosterone was not decreased. There was no toxic action to the liver and kidneys (55).



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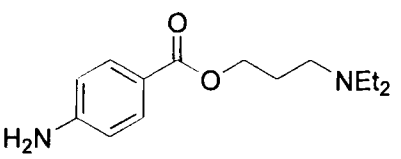

32

Investigations on the effect of a number of common chemicals on the male reproductive system have been motivated by reports of toxicity and not as part of a search for a clinically useful male contraceptive. It is not surprising that these toxic

solvents or metals would be disruptive to sperm or other reproductive tissues. Ethyleneglycol monomethyl ether has been tested on male rats (56). 2-Bromopropane caused azoospermia in humans exposed to the compound (57). Cadmium chloride was found to decrease motility in bovine sperm (58). Copper, long used in the manufacture of IUD's, will kill sperm (59,60). Neem oil an insect repellent and antifeedant, is also toxic to sperm (61,62). Aqueous glycerol injected into the testis causes infertility (63,64). The effects of glycine on the fertility of male rats was investigated by applying glycine locally to the epididymis using silastic rods. Glycine treatment had no effect on sexual drive, but reversibly impaired male fertility by causing a disruption of sperm function (65).

PEPTIDES

Hormones - The use of peptide hormones (LH-RH, FSH) and their analogs is a major area of study but the effectiveness of these therapies is somewhat controversial and is beyond the scope of this article.

Immunization - Use of antibodies to follicle stimulating hormone (FSH), sperm surface proteins and epididimal proteins has provided a fruitful area of research. Immunization against human sperm may have utility in the female as well as in the male. While too lengthy to review in detail, the references for this area are included. (66-88)

PHYSICAL METHODS

Voluntary sterilization by vasectomy is still the most commonly used method of male contraception despite the drawbacks of its surgical nature and non-reversibility. Non-surgical occlusion using new polymeric materials which are injected as a solution of monomer into the vas deferens and are allowed to set have been used to effect the blockage of the lumen. A Phase I clinical trial was undertaken on 38 volunteers who were injected with between 5 and 140 mg of styrene maleic anhydride (SMA) into each vas deferens. The treatment is well tolerated with only minimal side effects in a few cases and no long-term adverse effects. (89-96) No data have been presented on the possibility of reversing of this procedure by removal of the polymer. N-Butyl-α-cyanoacrylate also acts as a male contraceptive by sealing the vas deferens. This polymer was mostly retained in the injection site with monomer that appeared in the blood and organs rapidly cleared without accumulation (97). This technique may overcome some of these problems associated with mechanical methods but the possibility of reversing the procedure has not been investigated.

Summary - While physical methods continue to be the most prevalent form of male contraception, efficacious and safe chemical methods are under active investigation. Strategies that include inhibition of sperm maturation in the epididymis or direct interference with spermatogenic cells have not been generally successful because of unacceptable toxic side-effects. While there is optimism for a small molecule approach, no effective therapy has emerged. The most promising new approaches have begun to focus on interfering with highly specific aspects of spermatogenesis such as enzymic processes unique to sperm, interference with intercellular communication through cytokines, or application of antibodies against antigens of the epididymis or the spermatozoa. Research into the use of steroidal regimens and the peptide hormones LH and GnRH are actively being pursued.

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Chapter 20. New Dermatological Agents for the Treatment of Psoriasis

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Introduction - Psoriasis is a common, recurrent, inflammatory skin disease which affects approximately 2% of the population. There are 150 -250,000 new US cases diagnosed annually and the associated health care costs are estimated to be between $1.6-3.2 billion per year (1). The thickened, scaly, erythematous lesions of psoriasis are characterized by epidermal hyperproliferation, abnormal keratinocyte differentiation and infiltration of immune cells into the epidermis and dermis (2). Although the mode of inheritance or the genetic loci harboring the psoriasis gene(s) are unknown, several factors strongly suggest that psoriasis is a heritable disease. Some of these factors are as follows: (a) one-third of psoriasis patients have at least one family member afflicted with psoriasis; (b) a 65% concordance for psoriasis in monozygotic twins; and (c) an association of the psoriatic phenotype with HLA antigens HLA-Cw6 and HLA-DR7 (2,3).

Conventional topical treatments for psoriasis include emollients, keratolytics, coal tar, antharalin and corticosteroids. Systemic treatments include cytotoxic and immunosuppressive agents and retinoids. Various treatments for psoriasis, their modes of administration and their potential mechanisms of action along with their limitations are listed in Table 1. This chapter will initially discuss the emerging topical treatments of psoriasis and the mechanisms of action of these agents in inhibition of keratinocyte proliferation, normalization of keratinocyte differentiation and down-regulation of the inflammatory component associated with the disease. The second area of review will be selective immunosuppressive agents such as a diphtheria toxin-IL-2 conjugate, FK-506, a topical ascomycin analog, a CD28 action blocker and cell proliferation inhibitors such as kinase and IMPDH inhibitors.

Table 1. Past and Current Treatment

Treatment	Mode	Mechanism of Action	Limitations
Emollients	Topical	Moisturizing Agent	-Less effective -Large quantities required
Salicylic acid (2-4%)	Topical	Keratolytic	-Less effective
Coal Tar	Topical	Inhibiton of DNA synthesis	-Messy to use/stains clothes -Cosmetically unacceptable
Dithranol	Topical	Inhibition of DNA synthesis	-Messy to use/stains clothes -Cosmetically unacceptable -Irritant and stains clothes
Corticosteroids	Topical	Anti-inflammatory	-Steroid side effects (skin atrophy, tachyphylaxis, fast disease relapse, and worsening of the disease in some).
UVB Phototherapy	----	Anti-proliferative	-Erythema -Inflammation -Risk of cutaneous malignancies -Less effective as a monotherapy
Methotrexate, Azathioprine or Hydroxyurea	Systemic	Cytotoxic	-Side effects (bone marrow suppression, liver toxicity, immunodeficiency)
Cyclosporin	Systemic	Immunosuppresive	-Side effects (nephrotoxic, hypertension, immunosuppresion).
Acitretin	Systemic	Retinoid	-Side effects (teratogenic, liver and bone toxicity)

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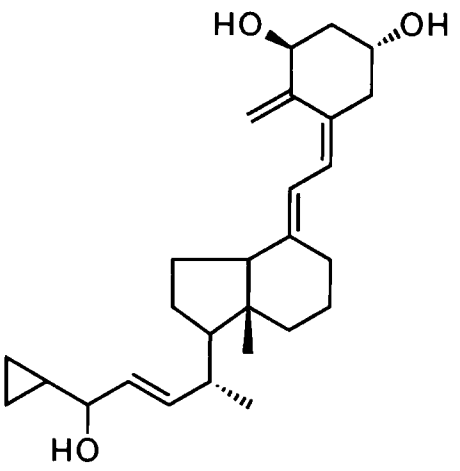
Pathogenesis of Psoriasis - The pathogenesis of psoriasis involves abnormal differentiation and hyperproliferation of keratinocytes, angiogenesis, lymphocyte activation, activation of dermal macrophages and neutrophil infiltration. The primary defect(s) in psoriasis may reside in epidermal keratinocytes which when triggered secrete the cytokines responsible for initial recruitment of the inflammatory cells of the immune system. Alternatively, the abnormal differentiation and hyperproliferation of the keratinocytes may occur secondary to triggering effects of cytokines produced by inappropriately skin targeted immune cells. It is assumed that both keratinocytes and the immune cells contribute to either initiation and/or maintenance of the disease. This realization is critical from a therapeutic viewpoint because psoriasis can be treated by interrupting the cycle of primary and secondary events by either modulating the hyperproliferative differentiation program of the keratinocytes and/or by inhibiting the inflammatory component.

At the molecular level, there is an overexpression of differentiation markers such as involucrin, TGase I, migration inhibitory factor related protein-8 (MRP-8), and skin derived antileukoproteinase (SKALP) in psoriatic keratinocytes (4-8). The psoriatic epidermis displays a loss of granular layer with absence of filaggrin expression (4). Further, the normal suprabasal keratins K1 and K10 are inhibited and expression of hyperproliferative keratins, K6 and K16, is induced (9,10). There is also an increased expression of IL-8 receptor, cytokines IL-6 and IL-8 as well as that of inflammatory markers HLA-DR and ICAM-1 (11-15). Moreover, the levels of transforming growth factor-a (TGF-a) and its receptor epidermal growth factor receptor (EGF-R) are also increased in psoriasis (16,17). Psoriasis also involves a major inflammatory component which is characterized by the presence of microabscesses of neutrophils in stratum corneum and influx of T lymphocytes in the epidermis and dermis. The majority of the T cells present in the dermis are CD4+ and those infiltrated in the epidermis are of CD8+ phenotype. These CD4+ cells are type I T-helper cells which secrete the inflammatory cytokines IL-2, IFN-g and TNF-a (for references, see 18).

NEWER TREATMENTS

Vitamin D3 Analogs - The observation that keratinocytes and dermal fibroblasts, contain receptors for 1a,25-dihydroxyvitamin D3 ($1,25(OH)_2$ D3) (19), and the observation that 1, $25(OH)_2$ D3 is a potent stimulator of epidermal cell differentiation (20) provided a rational basis for the clinical use of vitamin D3 receptor (VDR) agonists in psoriasis. Improvement in psoriasis was observed in a patient during a study of oral 1a-hydroxyvitamin D3 (1a (OH) D3) in osteoporosis (21). Oral treatment with 1a (OH) D3 (1 mg/day) in 7 patients resulted in complete remission in 4 and minimal improvement in 2 patients (22). Topical treatment with the natural VDR ligand, 1, $25(OH)_2$ D3, for 8 weeks in a study involving 19 patients with psoriasis vulgaris resulted in 84 % clearance or marked improvement. In contrast, no improvement was observed in control contralateral lesions treated with vehicle only (23). However, the use of $1,25(OH)_2$ D3 in dermatology is hampered by its hypercalcaemic activity. Calcipotriol (1) was identified during a systematic effort to synthesize non-calcipotropic VDR agonists and was shown to be 100-200 times less potent than 1, $25(OH)_2$ D3 in hypercalcaemic activity (24). The clinical efficacy of calcipotriol has been demonstrated in a number of studies (24). Calcipotriol ointment has also been compared with betamethasone 17-valerate and was slightly superior to the steroid (for references, see 24). In a study of long term use of calcipotriol, chronic plaque psoriasis patients (n=167) were treated twice daily with calcipotriol (50 mg/g). Complete clearing was observed in 26 % of the patients (25). In various clinical studies approximately 20 % of the patients reported cutaneous irritant reactions to topical calcipotriol (25).



1

Regulation of Gene Expression by VDR Agonists - Many of the biological effects of 1a,25-(OH)2 D3 and its natural and synthetic analogs are probably mediated by VDR, which is a ligand dependent transcription factor belonging to the superfamily of steroid/thyroid hormone nuclear receptors (26). VDR can form VDR-VDR homodimers and VDR-retinoid X receptor (RXR) heterodimers (27). The VDR-RXR heterodimer binds to the vitamin D3 response elements (VDREs) present in the promoter region of responsive genes. The VDREs are direct repeats of 5'-AGGTCA-3' motifs separated by 4 nucleotides (DR4) for VDR-RXR (28). Genes which are known to contain VDREs, such as, osteocalcin, osteopontin, are involved in calcium homeostasis and metabolism (29-31). However, there are no known VDRE containing genes whose expression could play a role in the resolution of psoriasis by VDR agonists. In contrast, the negative regulation of the expression of genes such as IL-1a, IL-2, IL-6, TNF-a and IFN-g, which are involved in exacerbation of inflammation, could to some extent account for the therapeutic effects of VDR agonists.

Effect of VDR Agonists on Proliferation and Differentiation of Skin Cells - 1a,25-(OH)2 D3 promotes terminal differentiation and inhibits proliferation of murine and human keratinocytes in culture (20,32). Importantly, the suprabasal cells are driven towards the differentiation pathway as exemplified by increased cornified envelop formation. These effects are accompanied by increased levels of transglutaminase I (TGase I) and involucrin (33). Keratinocytes from lesional and uninvolved psoriatic skin were inhibited as potently as normal keratinocytes by 1a, 25-(OH)2 D3 (34). Calcipotriol also inhibits proliferation and induces terminal differentiation of human keratinocytes (35). Some markers of cell proliferation which are down-regulated by VDR agonists in various skin systems are epidermal growth factor receptor (EGF-R), protooncogene c-myc, Ki-67, keratin 16 (K 16), TGase I and involucrin (Table 2).

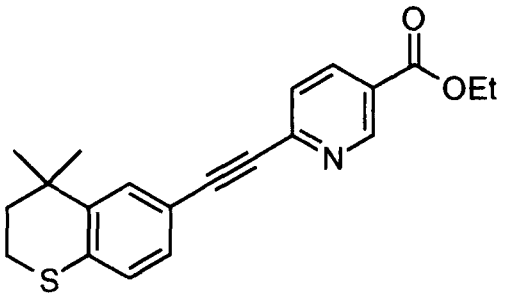
Table 2. Targets of VDR Agonists

Marker	Regulation	VDR Agonist	Systems	Biological Pathway	Ref.
c-myc	Down	$1,25(OH)_2D_3$	Keratinocytes	Proliferation	(36)
Ki-67*	Down	Calcipotriol	Psoriatic plaque	Proliferation	(37)
K16	Down	Calcipotriol	Psoriatic plaque	Proliferation	(37)
EGF-R**	Down	$1,25(OH)_2D_3$	Keratinocytes	Proliferation	(36)
TGase I	Up	$1,25(OH)_2D_3$	Keratinocytes	Differentiation	(33)
Involucrin	Up	$1,25(OH)_2D_3$	Keratinocytes	Differentiation	(33)
IL-2	Down	$1,25(OH)_2D_3$	T-cells	Inflammation	(38)
IL-6	Down	$1,25(OH)_2D_3$ Calcipotriol	PBMC Psoriatic plaque	Inflammation	(38, 39)
IFN-g	Down	$1,25(OH)_2D_3$ Calcipotriol	PBMC	Inflammation	(38)
LT	Down	$1,25(OH)_2D_3$	PBMC	Inflammation	(38)
TNF-a	Down	$1,25(OH)_2D_3$	PBMC	Inflammation	(38)
IL-10 receptor	Up	$1,25(OH)_2D_3$ Calcipotriol	Keratinocytes	Inflammation	(40)

*Ki-67 positive cells represent actively cyclang epidermal cells.
**High affinity EGF-R numbers were decreased.
Down -Down regulation, Up- Up regulation

Modulation of Immune System by VDR Agonists - The presence of VDR in monocytes, leukemic cells, and activated B and T lymphocytes suggested a role for VDR agonists in the immune system (41,42). 1a,25-(OH)2 D3 is a potent inhibitor of proliferation of mitogen-activated T lymphocytes (43). 1a,25-(OH)2 D3 also potently inhibits the activity of the T-cell growth promoting lymphokine, interleukin-2 (IL-2) (38,43). The activities of a number of pro-inflammatory cytokines, namely, IL-1a, IL-6, TNF-a (tumor necrosis factor a), IFN-g (interferon g), and lymphotoxin (LT) have been shown to be inhibited by 1a,25-(OH)2 D3 (38). Calcipotriol also inhibited the release of IL-6 and IFN-g from activated peripheral blood mononuclear cells (PBMCs) (24). In a study with 5 patients, calcipotriol inhibited IL-6 activity in psoriatic lesions after 1-2 weeks of topical treatment (39). The anti-inflammatory effects of VDR agonists are exemplified by decreased T-cell and polymorphonuclear cell numbers in psoriatic lesions after topical treatment (37). Interestingly, VDR agonists could also exert anti-inflammatory effects by upregulation of IL-10 receptor expression in keratinocytes (40). IL-10 is an anti-inflammatory cytokine and it is known to inhibit the cytokine response of pro-inflammatory Th1 cells. Various immune cell markers which are regulated by 1a,25-(OH)2 D3 or calcipotriol are summarized in Table 2.

Vitamin A Analogs - Topical retinoic acid (RA) was of little therapeutic value in psoriasis and its use was accompanied by significant irritation (for references, see 12). Etretinate (Tigason) and acitretin are effective systemic therapies but are contraindicated in women of child bearing age because of teratogenicity and their use is also limited by a high incidence of other side effects. Thus, they are generally used only for severe forms of psoriasis. Tazarotene (**2**, AGN 190168; ethyl 6-[2-(4,4-dimethylthiochroman-6-yl)-ethynyl] nicotinate), an RAR b/g selective synthetic analog of RA, is effective as a topical anti-psoriatic agent and is suitable for the treatment of mild to moderate plaque psoriasis, which constitutes approximately 80 % of psoriasis cases (12). In a double blinded, placebo controlled Phase III study of 324 patients with mild-to-moderate plaque psoriasis, once daily tazarotene (0.1 % or 0.05 %) gel or vehicle gel was administered for 12 weeks and patients were monitored for another 12 weeks post-treatment. Target lesions (trunk/limbs and knees/elbows) were chosen and assessed for clinical response in terms of reduction in plaque elevation, scaling and erythema. Treatment success rates ranged up to 70 %. Even on the difficult to treat knees/elbows target lesions, a treatment success rate of 60 % was obtained with both 0.05 % and 0.1 % tazarotene gels. For the most part, the clinical response was effectively maintained during the 12 week post-treatment period (44).



2

Regulation of Gene Expression by Retinoids - Retinoic acid (RA) and its synthetic analogs exert their biological effects through two families of nuclear receptors, retinoic acid receptors (RARa, b and g) and retinoid X receptor (RXRa, b and g), which belong to the super family of steroid/thyroid hormone nuclear receptors (45,46). RARs and RXRs upregulate gene expression by binding to the promoter regions of retinoid responsive genes as transcriptionally active RAR-RXR heterodimers (47) or RXR homodimers (48). The retinoic acid response elements (RAREs) of retinoid-responsive genes consist of a direct repeat of the sequence 5'-AGG/TTCA-3' separated by 2 (DR2) or 5 (DR5) base pairs (46). Although retinoids are therapeutically effective in the treatment of various dermatological diseases (49.50), their mode of action in skin is poorly understood. A number of retinoid-induced genes have been identified in various systems (28) but CRABP II is the only marker known to be induced by RA in normal skin (51). However, CRABP II is overexpressed in psoriasis (52), a disease which responds to retinoid treatment and is therefore not an appropriate efficacy marker of retinoid action in diseased skin. Retinoids also inhibit the expression of a number of genes which are associated with both cell proliferation and inflammation. Some of these genes contain AP1 as their major enhancer factor and retinoids inhibit the expression of these genes by antagonizing the action of AP1 components c-Jun and c-Fos (for references, see 53). These genes include metalloproteases (stromelysin-1, collagenase and gelatinase), proto-oncogenes (c-fos, c-myc and oct-3/4), growth factors, cytokines and their receptors (TGF-b1, EGF-R, IL-6 and IL-6 receptor), markers of cell differentiation (Keratins 5, 6, 14 and 16, TGase I and loricrin) and pro-inflammatory proteins (JE/MCP-1, iNOS, TNF-a and IL-2) (28).

Molecular Mechanisms of Retinoid Action in Psoriasis - Tazarotene binds to all three RARs but activates gene expression effectively only through RARb and g (53). However, it inhibits AP1-dependent gene expression equally well through all three RARs (53). The antagonism of AP1 dependent gene expression is probably important for the anti-inflammatory and anti-proliferative activities of tazarotene. Topical tazarotene and systemic etretinate both resulted in the inhibition of inflammation associated proteins HLA-DR and ICAM-1 in psoriatic lesions (12,54). Topical tazarotene also inhibited the expression of keratinocyte differentiation markers TGase I and involucrin, both of which are highly expressed in psoriatic lesional skin as compared to the normal epidermis. Further the level and pattern of expression of filaggrin was normalized by tazarotene (12). The expression of migration inhibitory factor related protein-8 (MRP-8) and skin derived anti-leukoproteinase (SKALP) is also negatively regulated by tazarotene in various skin based systems including psoriatic lesions (55). MRP-8 (or

calgranulin A) is expressed in chronic inflammatory diseases (psoriasis, primary chronic polyarthritis and cystic fibrosis) (56-58) and is abundant in neutrophils, monocytes and psoriatic keratinocytes. MRP-8 is present in epidermis in all inflammatory diseases tested so far (59,60). MRP-8 displays a striking amino acid homology (73%) to a murine cytokine CP-10, which is a chemotactic factor for polymorphonuclear cells and macrophages (61). Although falling into the general category of differentiation markers characterized in cultured keratinocytes, MRP-8 is absent in normal epidermis and is highly expressed in psoriatic epidermis. Therefore, MRP-8 appears to be a marker of the abnormal, hyperproliferative differentiation of the psoriatic keratinocytes and is also a marker for the inflammatory process associated with psoriasis. The differentiation normalizing activity of tazarotene is further evidenced by its inhibition of other differentiation markers which are overexpressed in psoriasis such as TGase I (55). Tazarotene also inhibits the interferon-g (IFN-g) induced expression of MRP-8 and TGase in cultured keratinocytes (55). Since IFN-g is overexpressed in psoriatic epidermis, it might play a role in inducing the abnormal, hyperproliferative keratinocyte differentiation pathways in psoriasis. The selective interdiction of certain IFN-g mediated gene expression pathways might be an important therapeutic mechanism of action of retinoids in psoriasis.

SKALP is also a hyperproliferative differentiation marker expressed in the suprabasal layers of psoriatic epidermis (8) and is incorporated into psoriatic scales by TGase (62). Thus, tazarotene by inhibiting SKALP, might directly reduce the scale formation associated with psoriasis. Further, tazarotene inhibits the levels of both hyperproliferative keratins K6 and K16 in psoriatic lesions (12,63). A list of the genes negatively regulated by tazarotene is shown in Table 3.

Table 3. Targets of Tazarotene Action in Psoriasis

Marker	Regulation	Skin Systems	Function/Biological Pathway	Ref.
TGase I	Down	Skin rafts Psoriasis lesions	Differentiation	(12, 55)
K 16	Down	Psoriasis lesions	Proliferation	(12)
EGF-R	Down	Psoriasis lesions	Proliferation	(12)
Involucrin	Down	Psoriasis lesions	Differentiation	(12)
Filaggrin	Up	Psoriasis lesions	Differentiation	(12)
ICAM-1	Down	Psoriasis lesions	Inflammation	(12)
HLA-DR	Down	Psoriasis lesions	Inflammation	(12)
MRP-8	Down	Keratinocytes Skin rafts Psoriasis lesions	Inflammation/ Differentiation	(55)
SKALP	Down	Skin rafts Psoriasis lesions	Differentiation	(55)
Stromelysin-1	Down	Keratinocytes	Inflammation	(53)
IL-6	Down	Keratinocytes Kaposi's sarcoma	Inflammation/ Proliferation	(64)
K6	Down	Keratinocytes Skin rafts Psoriatic lesions	Proliferation	(63)
TIG1	Up	Keratinocytes Fibroblasts Skin rafts Psoriatic lesions	Cell Adhesion?	(65)
TIG2	Up	Skin rafts Psoriatic lesions	Cell Adhesion?	(66)

TIG - Tazarotene-Induced Gene.
Down - Down regulation, Up - Up regulation.

Cyclosporin - The established therapeutic efficacy of cyclosporin in psoriasis (for references, see 67) identified a primary role for immune system activation in the pathogenesis of the disease. Cyclosporin is a potent immunosuppresive agent which also exhibits anti-proliferative effects on cultured keratinocytes at higher doses (2-10 mg/ml), which are thought to be achievable in skin after oral administration (for references, see 68). Cyclosporin is used in a number of inflammatory and autoimmune disorders in dermatology including severe plaque and erythrodermic psoriasis. Cyclosporin selectively acts on the *de novo* activation of T cells (69) and inhibits the expression of lymphokines IL-2, IL-4, GM-CSF (granulocyte macrophage-colony stimulating factor) and IFN-g (70). The presence of activated T cells has been demonstrated in psoriatic lesions (71,72). Further, psoriatic lesional keratinocytes phenotypically resemble IFN-g treated keratinocytes since they overexpress HLA-DR, ICAM-1, Cdw60, IP-10 and MRP-8 (55,73). Therefore, IFN-g produced by intralesional activated T cells may induce keratinocytes to produce cytokines, which in turn are capable of potentiating T-cell activation. Thus, cyclosporin appears to break the nexus between lesional T cells and keratinocytes by inhibiting the activation of T cells. Cyclosporin inhibits expression of IL-2, the major growth factor for T-cells. The IL-2 gene is silent in resting T-cells and it is induced after antigen stimulation. Cyclosporin blocks the antigen-mediated activation of T-cells via inhibition of IL-2 transcription by affecting the activities of the enhancers of IL-2 gene, namely AP-3, NF-kB and NF-AT (74). The activity of the major enhancer of IL-2, NF-AT (nuclear factor for activated T-cells), is most dramatically altered by cyclosporin. NF-AT is composed of two subunits, and cyclosporin blocks the translocation of the cytosolic subunit to the nucleus by inactivating cyclophilin, a peptidyl-prolyl cis-trans isomerase, which is required for correct protein folding (69,74,75). Topical cyclosporin preparations have not been therapeutically useful in humans (76).

FK-506 - FK-506 is a powerful, new immunosupressant, which also acts by blocking the antigen-induced activation of T-cells via inhibition of IL-2 transcription (75,77). FK-506, like cyclosporin, binds to and inactivates the activity of an immunophilin (another cis-trans isomerase), FK-506 binding protein (FKBP or FKBP12) (69). In a small clinical trail, all 7 patients with severe recalcitrant psoriasis experienced a complete remission of psoriasis upon systemic treatment with FK-506 (Tacrolimus) (78). However, it was effective only at doses comparable to that required for prevention of allograft rejection.

SDZ-281240 - SDZ-281240 is a newly developed immunosuppressive macrolide of the ascomycin type whose mechanism of action appears to be similar to that of FK-506. In a randomized, double blind and placebo-controlled clinical study, topical SDZ-281240 was tested on microplaques in 15 patients with severe, recalcitrant psoriasis. All patients showed significant improvement after 10 days of application. Histopathological and immunostaining (Ki-67, involucrin, HLA-DR, ICAM-1, CD3+, CD4+ and CD8+ antibodies) analysis exhibited almost complete reversion of epidermal hyperplasia, parakeratosis, hyperkeratosis, acanthosis and inflammatory infiltrate in the test microplaques (79). SDZ-281240 blocked the proliferation of stimulated lymphocytes but not HaCaT keratinocytes *in vitro*. Therefore, it appears to inhibit epidermal hyperplasia by selectively blocking T-cell activation rather than by inhibition of keratinocyte proliferation (79).

NEW TARGETS

Fusion Proteins - The major involvement of the activated T lymphocytes in psoriasis and the therapeutic success of immunomodulating agents such as cyclosporin, FK-506 and CD4 monoclonal antibodies (for references, see 72), have prompted the use of fusion proteins in psoriasis. $DAB_{389}IL2$ is a fusion protein of the membrane-translocation and cytotoxic domains of diphtheria toxin (DAB) and human IL-2. The rationale behind the fusion toxin ($DAB_{389}IL2$) is that it will selectively destroy the cells expressing the high-affinity IL-2 receptor. Thus, activated T-cells, and not keratinocytes or resting T cells, will be killed by the hybrid toxin. In a clinical study of 10 patients with chronic, extensive plaque-type psoriasis, intravenous $DAB_{389}IL2$ resulted in striking clinical improvement in 4 and moderate improvement in another 4

patients. Clinical improvement was accompanied by a marked reduction in epidermal CD3+ and CD8+ lymphocytes (72).

CTLA4-Ig is a fusion protein of human CTLA4 (homologous to CD 28) and immunoglobulin (Ig) G1 Fc region. CTLA4 (CD28) is a T-cell receptor, which binds with high affinity to a co-stimulator molecule B7 present on the surface of antigen presenting cells. B7-CD28 interaction results in T-cell proliferation. CTLA4-Ig is a soluble antibody which binds with high affinity to B7, thereby blocking B7-CD28 interaction and T-cell proliferation. CTLA4-Ig is an immunosuppressant, under development for prevention of transplant rejection, autoimmune diseases and psoriasis. It inhibits T-cell dependent immune responses and Th1 (IL-2 and IFN-g) cytokine production in animal models (80). Results of an early clinical trial indicate that it is well tolerated and holds promise for the treatment of psoriasis (81).

IMPDH Inhibitor (VX-497) - VX-497 is an inosine monophosphate dehydrogenase (IMPDH) inhibitor which is designed to treat transplant rejection, autoimmune diseases and psoriasis. IMPDH catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent oxidation of inosine-5'-monophosphate to xanthosine-5'-monophosphate, which is the crucial step in the de novo biosynthesis of guanosine nucleotide. Since proliferating B- and T - lymphocytes depend on the IMPDH catalyzed pathway for purine biosynthesis, this enzyme appears to be good target for immunosuppressive drugs. Further, increased IMDPH activity has been demonstrated in leukemic cell lines and tumor tissues (82).

Kinase Inhibitors - Epidermal growth factor (EGF) transduces its proliferating signal to keratinocytes by binding to and inducing the tyrosine phosphorylation of its membrane receptor (EGF-R). Theoretically, an agent which would block the activation and ligand dependent phosphorylation of the receptor would also inhibit the proliferation of keratinocytes. Since epidermal hyperproliferation is a hallmark of the psoriatic phenotype, a small synthetic molecule (SU5271) which specifically inhibits EGF-R activation in preclinical models is now in clinical trail for the treatment of plaque-type psoriasis (83, Sugen Internet Release).

Conclusion- The pathogenesis of psoriasis suggests that this disease can be treated by agents which either modulate the immune system or which affect the differentiation program of keratinocytes. Calcipotriol and tazarotene are significant additions to the dermatologists armamentorium of topical anti-psoriatic drugs. However, it would be desirable to decrease the side effects associated with these drugs and increase their efficacy. This can be achieved by the systematic synthesis of future generation of receptor or function selective retinoids or vitamin D3 analogs. In the case of retinoids, the activity through RARg is desirable since it constitutes more than 90 % of the total RAR repertoire in skin. Therefore, RARg selective retinoids may exhibit an increased therapeutic index. Alternatively, function selective compounds such as retinoids with only AP1 inhibitory activities but lacking in transactivation capabilities may exhibit less side effects. Since, retinoids, steroids, vitamin D3 analogs and immuno-suppressive agents affect psoriasis by different mechanisms, a combination of some of these agents at low doses may give a synergistic therapeutic effect. It may also be possible to significantly decrease the toxic side effects associated with each of these therapies by such a combination approach. Larger, well-controlled clinical trails are required to judge the usefulness of the newer agents, such as, fusion proteins and agents targeting IMPDH, kinases and PNP.

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Chapter 21. Selective Cyclooxygenase-2 Inhibitors

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Introduction - Cyclooxygenase (prostaglandin synthase) catalyzes the oxygenation of arachidonic acid to prostaglandin H_2 as a first step in the synthesis of prostaglandins, prostacyclins and thromboxanes. The enzyme is expressed in mammalian cells as two distinct isosymes that show about 60% amino acid sequence identity. The two isozymes are very similar with respect to their catalytic properties, affinities for arachidonic acid and structures of the active site, with minor differences in their requirements for activation by hydroperoxides and preferences for fatty acid substrates (1, 2). Cox-1 is the major form expressed in healthy tissues and plays a role in thrombogenesis and in the homeostasis of the gastrointestinal tract and kidneys. Cox-2 synthesis is inducible in many cell types by bacterial endotoxin, cytokines, including IL-1 and TNFα, mitogens and growth factors, and is repressed by dexamethasone. Both enzymes are sensitive to inhibition by conventional nonsteroidal anti-inflammatory drugs (NSAIDs). The observations that Cox-2 is associated with inflammatory conditions and that Cox-1 is mainly expressed as a constitutive enzyme have provided the rationale for the development of selective Cox-2 inhibitors in order to reduce the risk of gastric irritation and ulceration associated with the chronic use of NSAIDs (3, 4).

REGULATION AND ASSAY OF COX-2 ACTIVITY

Biological Roles of Cox-2 - The inducibility of Cox-2 by pro-inflammatory mediators, the detection of elevated Cox-2 in exudates and in the spinal cord in models of inflammation, and the demonstration that selective Cox-2 inhibitors possess anti-inflammatory, antipyretic and analgesic properties in animal models all provide convincing evidence for the role of Cox-2 in inflammation (2-4). Recent observations indicate that Cox-2 induction can also be triggered by a variety of stimuli including hypoxia (5), endothelin (6), synaptic excitation (7-9), peroxisome proliferators (10), laminar shear stress (11), injury (12) or incubation of tissues *in vitro* (13). Of particular interest is the detection of Cox-2 in colorectal carcinoma tissues (14-16) and the demonstration that selective Cox-2 inhibition reduces polyp number in the *Apc* $^{\Delta 716-/-}$ knockout mice (a model of human familial adenomatous polyposis) (17) and suppresses colonic aberrant crypt foci (18). This suggests that Cox-2 plays a key role in polyp formation and provides the basis for chemopreventive treatment of polyposis and cancer by selective Cox-2 inhibitors (19). Epidemiological studies also suggest that the use of NSAIDs may retard the progression of Alzheimher's disease (20) although the contribution of each of the Cox isoforms in the progression of this disease is unknown.

Cox-Deficient Mice - Cox-2 gene disruption in mice has been reported by two different groups and shown to result in reduced viability associated with abnormal kidney development (21, 22). No alteration of arachidonic acid-induced ear edema (mainly Cox-1-dependent) or carrageenan-induced inflammation were detected. Reduced sensitivity to endotoxin-induced hepatotoxicity (21) and increased susceptibility to peritonitis were observed (22). Cox-1 deficient mice had normal development, showed no spontaneous gastropathy and a decreased sensitivity to indomethacin-induced gastric ulceration (23). Introduction of the Cox-2 knockout mutation into the *Apc* $^{\Delta 716-/-}$ mice suppressed intestinal polyposis (17).

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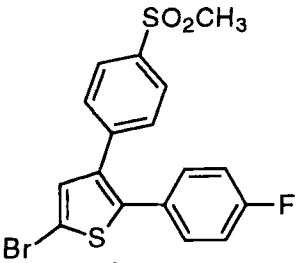
Cox-2 Structure - X-ray crystal structures of mouse (24) and human (25) Cox-2 have been determined. The structure of the Cox-2 active site is very similar to that of sheep Cox-1 with a larger binding site for NSAIDs in Cox-2 due to a difference in amino acid at position 509 (Val in Cox-2 vs Ile at the corresponding 523 position in Cox-1). Mutagenesis studies both on Cox-2 (26, 27) and Cox-1 (28) indicate that these amino acids are critical determinants for the interaction with selective Cox-2 inhibitors. Differences between the structures of the various enzyme-inhibitors complexes (24, 25) and in their sensitivity to limited proteolysis (29) suggest that conformational changes are induced by inhibitor binding. Ser 516 was confirmed as the acetylation site of aspirin by peptide mapping (30). The lipoxygenase activity of aspirin-acetylated Cox-2 (production of 15-HETE) was found to be inhibitable by NSAIDs (31) but with a decreased sensitivity to inhibition by fenamates (32).

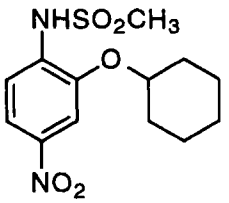
Evaluation of Inhibitor Selectivity - A major difficulty in comparing the properties of the various inhibitors has been the very large discrepancy in the selectivity ratios for the inhibition of Cox-2 and Cox-1 as measured using different assay systems and procedures. Assays have been reported using microsomal preparations of recombinant enzymes (33-36), mammalian cells and platelets (37-39), infected insect cells (40), transfected mammalian cells (41, 42) and whole blood (43, 44). Because selective inhibitors of Cox-2 are typically time-dependent inhibitors of Cox-2 and rapidly reversible competitive inhibitors of Cox-1, differences in preincubation times between the inhibitor and the enzyme or in arachidonic acid concentrations can markedly affect apparent selectivity. The initial report that several Cox-2 inhibitors were more potent inhibitors of cellular prostaglandin production than of Cox-2 enzymatic activity (45) has been confirmed with many cell types (37-39). As a result, a higher selectivity ratio for the inhibition of Cox-2 is observed in cell-based assays as compared to that determined with enzyme assays. It has been especially difficult to achieve selective Cox-2 inhibition in whole blood assays as measured from the coagulation-induced production of TXB_2 for Cox-1 and the LPS-induced PGE_2 production for Cox-2 (43). A sensitive assay that permitted the detection of inhibition of Cox-1 by selective Cox-2 inhibitors has been described using the production of PGE_2 by microsomes from U937 cells incubated with a low concentration of arachidonic acid (46).

SELECTIVE COX-2 INHIBITORS

Early efforts undertaken by several laboratories to develop selective Cox-2 inhibitors have focused mainly on modifying the two original leads: DuP 697 (**1**) and NS 398 (**2**). These studies have resulted in an impressive array of highly selective inhibitors. Since some of these new selective inhibitors have entered clinical trials, it will be interesting to see the long term clinical experience with these compounds, particularly with respect to the potential side effects of Cox-2 inhibition and to the level of selectivity required to prevent the attendant toxicity thought to be caused by concomitant inhibition of Cox-1 in the GI tract. All *in vivo* data reported here are from oral administration.

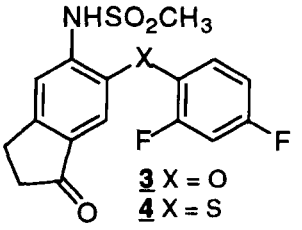


1

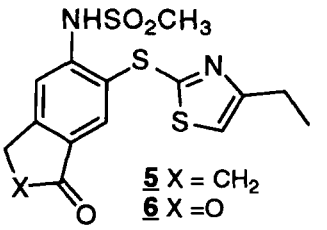


2

The Sulide Class - This class of compound continues to attract a lot of interest. A concise synthesis of flosulide **3** has been published (47) as well as the profile of a closely related analog L-745,337 **4** (48-50). The ethylthiazole analogs **5** and **6** had a superior inhibition profile (Cox-1 IC_{50} > 100 μM, Cox-2 IC_{50} ~ 0.005 μM) as compared to L-745,337. The lactone analog **6** was particularly potent in the carrageenan-induced foot pad edema model (CFE) (ED_{50} = 0.9 mg/kg) (51).



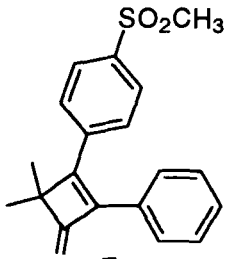
3 X = O
4 X = S



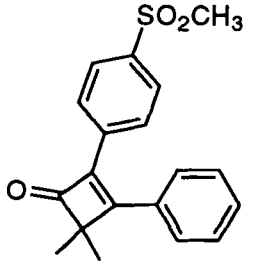
5 X = CH_2
6 X =O

Tricyclics - Because of the amount of activity in this area the subject is arbitrarily subdivided based on ring size. Unlike the sulide series which contains an acidic proton capable of salt formation, the main challenge for this class of compounds has been obtaining acceptable bioavailability. In general, the use of the -SO_2NH_2 in place of the -SO_2CH_3 group in DuP 697, for example, does provide an increase in absorption but this comes at the expense of an increased inhibition of Cox-1 activity.

4-Membered Ring - 2,3- Diarylcyclobutenone methylsulfones have been reported to be selective Cox-2 inhibitors (52). Compound **7** is highly potent on Cox-2 and displayed a selectivity ratio of approximately 700 (Cox-1 IC_{50} = 2 μM, Cox-2 IC_{50} ~ 0.003 μM). **7** was similar to indomethacin in the rat CFE model (ED_{50} = 2.4 mg/kg). Interestingly the regioisomer **8** was equally active in the rat CFE model, but with a lowered selectivity ratio. **7** caused a two fold increase over controls in the ^{51}Cr excretion model of ulcerogenicity in rats when dosed at 100 mg/kg, b.i.d. for 5 days as compared to an 8 fold increase with a single dose of indomethacin at 10 mg/kg.

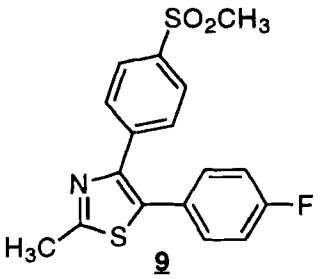


7



8

5-Membered Ring - A systematic study of the replacement of the thiophene ring of DuP 697 (**1**) by a variety of 5-membered ring heterocycles has been reported (53). Of note are the 1,3-thiazole **9**, 1,2-thiazole **10** and thiadiazole **11**. All three compounds are inactive as Cox-1 inhibitors at concentrations up to 100 μM with varying degree of potency in the Cox-2 assay. The thiadiazole **11** was extremely potent in the rat CFE (ED_{50} = 0.7 mg/kg) and exhibited no GI toxicity.



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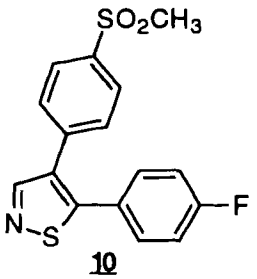

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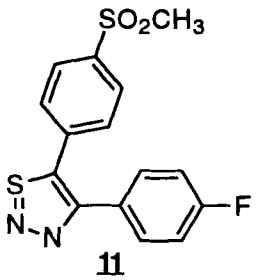
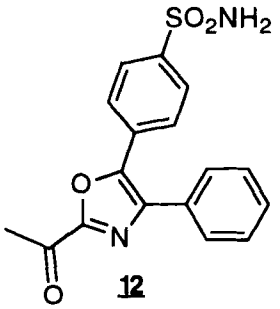


11

A number of oxazole based inhibitors have been investigated by various laboratories. The sulfonamides 12 displayed 175 fold selectivity (Cox-1 IC_{50} = 35 μM, Cox-2 IC_{50}~0.2 μM) and caused 61% inhibition at 20 mg/kg in the rat CFE assay (54). On the other hand, the hydroxymethyloxazole 13 exhibited a selectivity ratio of >500 fold and gave a 57% inhibition at 10 mg/kg in the rat CFE model (55). The 2-methyloxazoles 14 and 15 are reported to be orally active in the rat CFE model with an ED_{30} of 4.5 and 5.5 mg/kg respectively (56,57).



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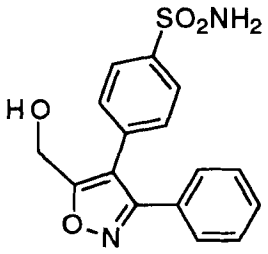

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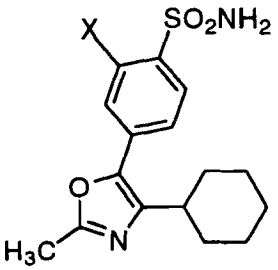
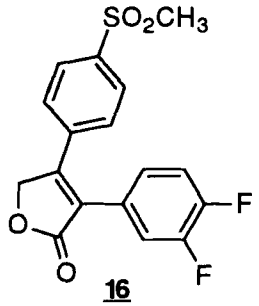


14 X = H
15 X = F

The finding that the furanone 16 (selectivity ratio > 3,000) reduced the number of polyps in *Apc* $^{\Delta 716-/-}$ knockout mice, a model of human familial adenomatous polyposis, more significantly than sulindac is particularly intriguing (17). It has been shown that the 5(H)-furanone based inhibitors can be further modified to enhance selectivity for Cox-2 by substituting at the 5,5'-positions with, for example, dialkyls and alkoxyalkyls moieties as in 17 and 18 respectively. Moreover some of these modifications provided additional metabolic stability (58,59).



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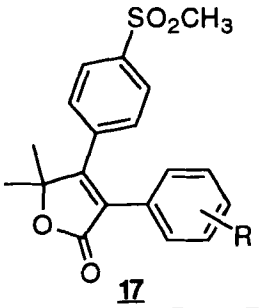
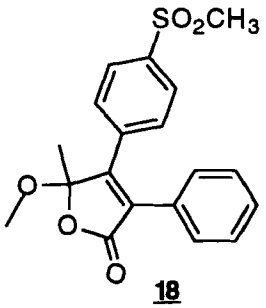


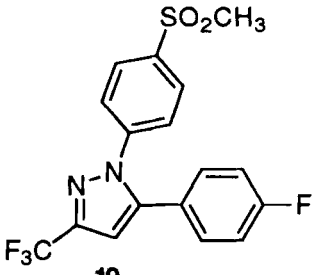
17
17a R = 3-F



18

Compound **17a** (DFU) has been reported to be orally active in all the relevant preclinical models of inflammation, pain and pyresis with ED_{50} ~1 mg/kg (46). Remarkably, although consistent with the lack of inhibition of Cox-1 activity, **17a** did not cause GI erosion in both rats and squirrel monkeys at 200 times the efficacious dose (i.e. 100 mg/kg b.i.d. over 5 days).

1,5-diaryl pyrazole-based inhibitors have continued to evolve (60-63). SC-58125 (**19**), the first well documented member of this series suffered from possessing a long terminal half-life in rats. Replacement of the 4-fluoro by a metabolically more labile methyl group and exchanging the methyl sulfone moiety for a sulfonamide provided SC 58635 (celecoxib, **20**) (64). The terminal half life of celecoxib in humans has been reported to be 10 hours. Its *in vitro* selectivity for Cox-2 is about 325 fold (Cox-1 IC_{50} = 13 μM, Cox-2 IC_{50} ~ 0.04 μM). The compound has an ED_{50} of 7 mg/kg, and of 34.5 mg/kg in the rat CFE and rat hyperalgesia models, respectively. It is very potent in the adjuvant arthritis model with an ED_{50} of 0.4 mg/kg.



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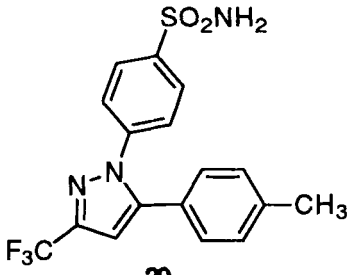
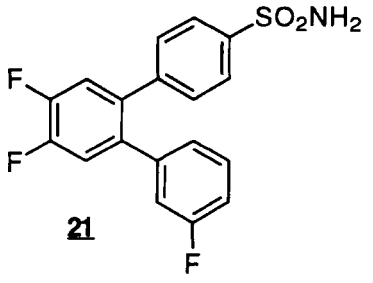


20

6-Membered Ring - Both benzene and pyridine rings have been used as the central templates in place of 5-membered rings (65). In both series, it appears that substitution on the central phenyl or pyridyl ring provided an additional potency enhancement (66). The sulfonamide **21** displayed a selectivity ratio of 2,700 fold (Cox-1 IC_{50} = 5.5 μM, Cox-2 IC_{50} ~ 0.002 μM). Although the compound was very potent in the adjuvant arthritis model (ED_{50} = 0.05 mg/kg), it proved to be less efficacious in the analgesic model (ED_{50} = 38.7 mg/kg). Another group demonstrated that a hetero atom such as oxygen atom can be inserted between the central phenyl ring and the ring that does not carry the methylsulfone as in **22**. This series of compounds has inhibitory effects in the adjuvant arthritis model in the range of 45-62% at a dose of 1 mg/kg (67). The pyridine derivatives **23** and **24** were reported to be highly selective but the potency did not appear to translate well *in vivo* (68,69).



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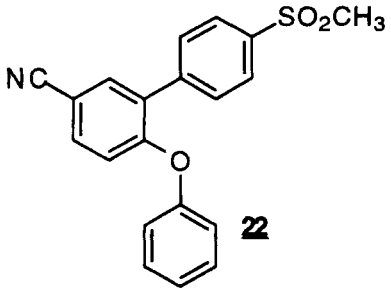

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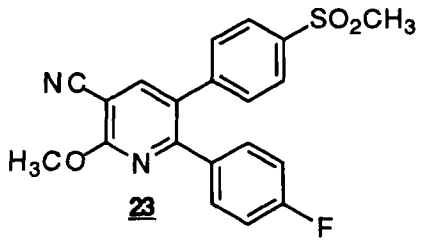

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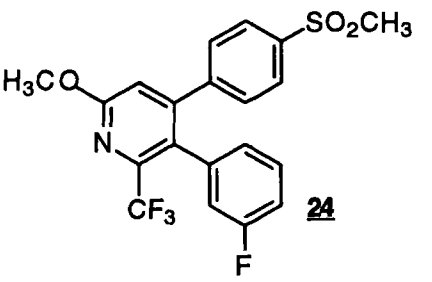
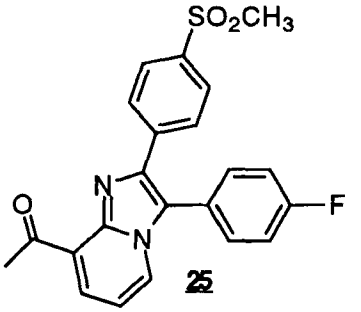


24

Fused-Ring System - Several series bearing fused-ring systems as the central template, such as indenes and benzofurans, have been reported (70). These compounds, however, suffer from poor pharmacokinetics. Imidazopyridines such as **25** have been described as Cox-2 selective inhibitors (Cox-1 IC_{50} = >100 μM, Cox-2 IC_{50} ~ 0.14 μM) (71) but were not further characterized in *in vivo* models. The orally active imidazothiazole **26** and thiazolotriazole **27** were reported to be potent and selective inhibitors (Cox-1 IC_{50} >50 μM, Cox-2 IC_{50} ~ 0.016 μM and Cox-1 IC_{50} = 43 μM, Cox-2 IC_{50} ~ 0.01 μM, respectively). No chromium leakage in GI integrity tests was observed for either of these compounds after administration for 5 days at 100 mg/kg b.i.d. (72,73) Another approach in attempting to enhance the bioavailability of this class of compounds was to administer them as prodrugs. The lactone functionality in compounds **28** and **29** can potentially be opened to the hydroxy-carboxylate which can serve as the prodrug (Cox-1 IC_{50} = 32 μM, Cox-2 IC_{50} ~ 0.051 μM and Cox-1 IC_{50} = 30 μM, Cox-2 IC_{50} ~ 0.03 μM, respectively for the closed forms) (74).



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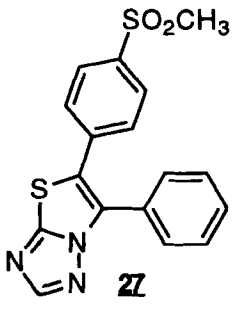

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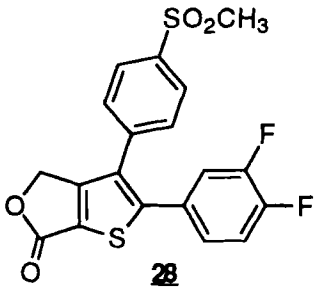

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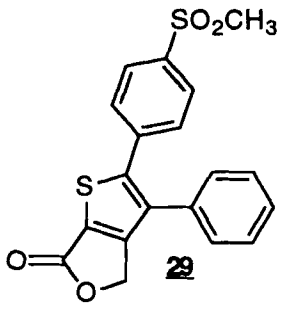
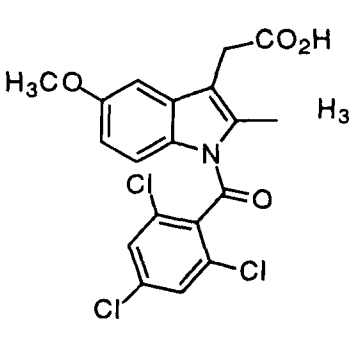


29

From Non-Selective NSAIDs - Two groups reported that highly selective Cox-2 inhibitors can be derived from dual Cox-1/2 inhibitors. It is interesting to note that the following two series of compounds do not carry the -SO_2R functionality as in previous classes.

Utilizing the fact that the active site of Cox-2 is slightly larger than that of Cox-1, the 4-chlorobenzoyl group of indomethacin was replaced with a larger 2,4,6-trichlorobenzoyl group, a change which forces the phenyl ring to adopt a sterically more demanding conformation perpendicular to the plane of the indole ring. This compound, (30), showed reasonable potency against Cox-2 (IC_{50} = 0.5 μM) but was virtually without effect on Cox-1 (75). Further optimization led to L-761,066 (31) which has an IC_{50} of 0.06 μM in Cox-2 and IC_{50} > 10 μM in Cox-1. The compound has an ED_{30} of 0.4 mg/kg in the rat CFE model, an ED_{50} of 1.9 mg/kg in the rat pyresis model and is non-ulcerogenic in rats when dosed at 100 mg/kg b.i.d. for 5 days (76).

The replacement of the carboxylic acid group of zomiperac by a pyridazinone moiety gave a highly selective series of inhibitors. 32 has an IC_{50} of 0.6 μM in Cox-2 and IC_{50} of 1000 μM in Cox-1. The compound has an ED_{30} of 3.3 mg/kg and an ED_{40} of 2.9 mg/kg in the rat CFE and adjuvant arthiritis models, respectively (77).



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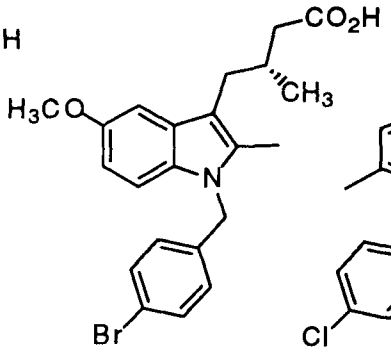

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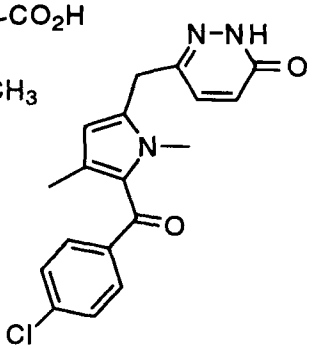

32

CLINICAL TRIALS WITH COX-2 INHIBITORS

A number of NSAIDs including meloxicam, etodolac, nimesulide and flosulide have shown some degree of selectivity for the inhibition of Cox-2 *in vitro* and appear to have an improved GI tolerability as compared to that of less selective NSAIDs. Celecoxib and MK-966 are selective Cox-2 inhibitors under clinical evaluation. Celecoxib has been reported to exhibit a 325-fold selectivity *in vitro* and to show efficacy in a post-dental pain model of analgesia (78) and in the treatment of the symptoms of osteoarthritis in a short-term pilot study (79). MK-966 shows >1,000-fold selectivity for Cox-2 in cell-based assays. The selective inhibition of Cox-2 after a single dose administration of MK-966 has been confirmed in humans using whole blood assays *ex vivo* (80). The compound has demonstrated an efficacy indistinguishable from that of ibuprofen in the treatment of postoperative dental pain (81). Further studies with these inhibitors should help to define the therapeutic properties and long-term safety of selective Cox-2 inhibitors.

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Chapter 22. Growth Hormone Secretagogues

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Introduction - In recent years, the growth hormone releasing peptides (GHRPs) and GHRP peptidomimetics have received considerable attention as potential alternatives to injectable GH replacement therapy (1, 2). These compounds can restore and enhance pulsatile GH release in humans (3). It is therefore anticipated that inducing physiologically relevant, pulsatile release of GH with GH-secretagogues (GHSs) may provide the beneficial effects of GH replacement therapy without the associated side effects that result from bolus GH administration. Furthermore, the GH-secretagogues may offer the potential advantages of oral dosing. Considerable progress has been made in identifying small molecule mimetics for the peptidyl secretagogues GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2; EC_{50}=10 nM) (4, 5), GHRP-2 (D-Ala-D-β-Nal-Ala-Trp-D-Phe-Lys-NH_2; EC_{50}=3 nM) (6), GHRP-1 (Ala-His-D-β-Nal-Ala-Trp-D-Phe-Lys-NH_2; EC_{50}=6 nM) (7) and hexarelin (His-D-2-methylTrp-Ala-Trp-D-Phe-Lys-NH_2; EC_{50}=1 nM) (8). Their design is of significant interest since these ligands are receptor agonists.

In 1993, a group at Merck disclosed benzolactam secretagogue **1** (L-692,429; EC_{50}=60 nM) (9, 10). Mechanism of action studies *in vitro* with cultured rat primary pituitary cells showed that its mode of action was identical to that of GHRP-6 and distinct from that of GHRH suggesting that its actions were mediated through the same putative GHRP receptor and not the GHRH receptor (11). In the clinic, **1** released GH after acute i.v. treatment in young and elderly subjects (12, 13). There were, like GHRP-6 (14), transient increases in cortisol and prolactin. The other measured hormones were in the normal range. Continous intravenous infusion of **1** to young healthy adults and healthy older subjects resulted in an upregulated GH secretory pattern that was pulsatile in nature (15).

The discovery of **1** showed that it was possible to design smaller peptidomimetics for GHRP-6. Furthermore, the clinical results with GHRP-6 and **1** validated the GHRP-mechanism for GH release as a viable alternative to GH replacement therapy. Encouraged by the these findings considerable effort has been spent to identify GH secretagogues that may have good oral bioavailability. To this end, significant progress has been made in the design of peptidomimetics for GHRP-6 within the past several years. Furthermore, breakthroughs have been attained in understanding their molecular basis and mechanisms of action. This is of interest since the GHRPs and peptidomimetics were discovered without knowledge of a molecular target or an endogenous counterpart This chapter will discuss developments in the design of new peptidomimetics of the GHRP pathway and in understanding their mechanism of action.

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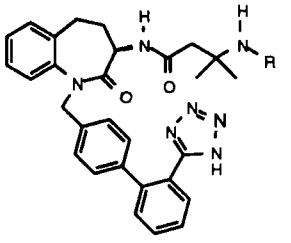
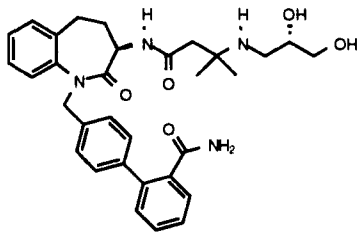
Benzolactams - Significantly more potent analogs of **1** include **2** (L-692,585; EC_{50}=3 nM) (16) and carboxamide **3** (EC_{50}=3 nM) (17). Acute i.v. administration of **2** to beagles caused significant increases in GH levels at doses as low as 0.005 mg/kg (18). Once-daily i.v. administration of 0.01 and 0.10 mg/kg of **2** to beagles did not lead to desensitization of the GH response (18). Futhermore, there were transient increases in levels of insulin-like growth factor-1 (IGF-1). A sustained amplification of episodic GH release resulted after a 12-h infusion of **2** to guinea pigs (19).

The oral bioavailability of **1** in dogs is less than 2%, which may be due its zwitterionic nature. Therefore, it is of interest that carboxamide **3** is orally active for releasing GH in beagles at 5.0 mg/kg (17). Urea-based replacements for the 2'-tetrazole of **1** have been disclosed in the patent literature (20). Recently, a group from Novo Nordisk disclosed naphtholactam-based secretagogues in a pending patent application (21). The effect of these structural changes on oral bioavailability is yet to be reported.



1 R = H

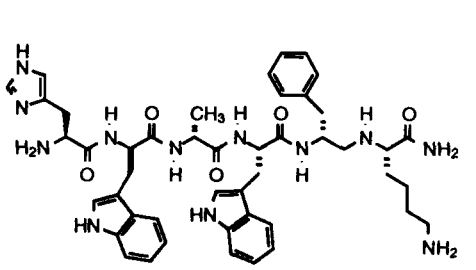


3

2 R = CH_2CHCH_3 (OH)

Development of GHRP Analogs - Considerable effort has been spent to identify analogs of GHRP-6 with improved metabolic stability. To this end, an analog of GHRP-6 that bears an aminomethylene substitution between D-Phe^5 and Lys^6 (**4**; EC_{50}=0.5 nM) displayed a twenty-fold increase in potency in an anesthetized rat model when compared with GHRP-6 (22).

Compound **5** (G-7203; EC_{50}=0.43 nM), a cyclic analog of the linear hexapeptide GHRP-2, was designed in an effort to understand the topographical requirements for the GH releasing activity of the GHRPs (23). McDowell and co-workers (23) showed that the D-2-Nal-Ala-Trp-D-Phe fragment adopts a compact conformation with nested hairpin turns initiated at D-Lys^1 and Ala^3. Other cyclic GHRP-2 analogs that did not readily adopt this conformation were considerably less active suggesting that a precise arrangement of the three aromatic side-chains was crucial for GH releasing activity.



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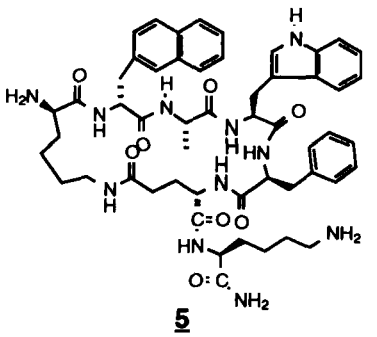

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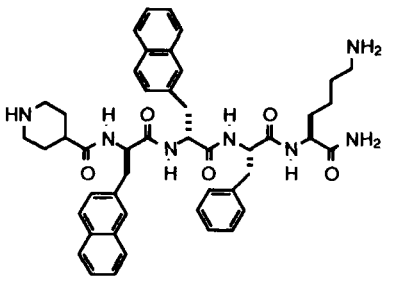
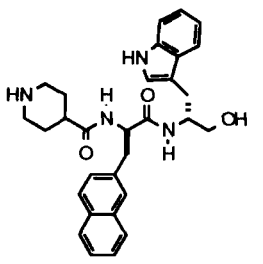


6

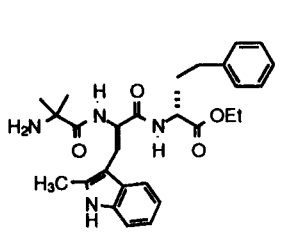


7

Extensive medicinal chemistry studies were carried out (23) around a tetrapeptide Tyr-D-Trp-D-Trp-Phe-NH_2 that was previously reported (24) to be weakly active for releasing GH. The D-Nal-Ala-Trp-D-Phe triaromatic core of GHRP-6 was converted to D-2Nal-D-2-Nal-Phe and an isonipecotic acid amino side-chain was utilized for the N-terminal histidine of GHRP-6 in the design of tetrapeptide secretagogue **6** (G-7039; EC_{50}=0.18 nM) (23). Further optimization of **6** by removal of the Phe and Lys residues and optimization of the C-terminus provided a series of small molecule GH secretagogues exemplified by **7** (G-7502; EC_{50}=10.6 nM). Compound **7** was active in rats with an i.v. ED_{50} of 0.80 mg/kg. The biochemical properties and specificity towards other hormones of **6** and **7** were similar to GHRP-6. These results suggest that these new peptidomimetics exert their action also through the GHRP pathway.

In 150-day old female rats **6** was significantly more effective for increasing body weight when it was administered by s.c. minipump 2X daily for 14 days as compared with the body weight gain after s.c. minipump infusion of **6** for 14 days at 100 μg per rat per day (25). In obese male ZDF rats serum fasting glucose levels over a 3 week period were considerably higher than those of lean controls when **6** 100 μg per rat per day by twice daily s.c. injection (25). However, giving the secretagogue+rhIGF-1 somewhat attenuated the effects on serum glucose elevation suggesting that IGF-1 is an important factor in moderating the effects of GH secretagogues on serum glucose and in maximizing the anabolic potential of these agents.

Tripeptides such as **8** (AIB-D-Trp-D-homoPhe-OEt; EC_{50}=3 nM; AIB = aminoisobutyric acid) and **9** (AIB-D-Trp-D-Trp-OEt; EC_{50}=6 nM) that are more potent than GHRP-6 (EC_{50}=10nM) have been described (26).



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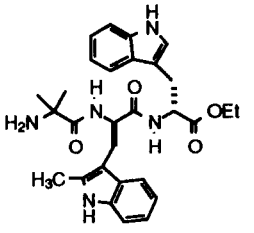
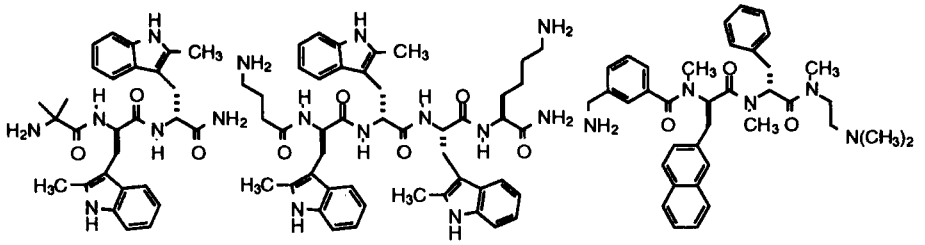


9

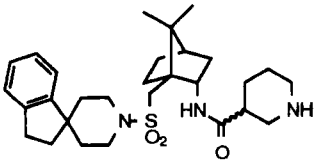
Compound **8** has been reported to be inactive for releasing GH in an infant rat model (27). Peptide analogs such as **10** (EP 51319; AIB-D-2-methylTrp-D-2-methylTrp-NH_2) and **11** (EP 51216; GAB-D-2-methylTrp-D-2-methylTrp-D-2-methylTrp-LysNH_2; GAB = γ-aminobutyric acid) elevated GH levels into the 160-200 ng/mL range in an infant rat assay (27-29). Both **10** and **11** are orally active in dogs and in man where they were reported to elevate GH levels without increasing cortisol (29).

Related to **7** are compounds with benzylamine side chains, as exemplified in **12**, that are claimed in pending patent applications by the Novo Nordisk company (30).



10 **11** **12**

Camphor-Based GH Secretagogues - Non-peptide camphorsulfonamide GH secretagogues were disclosed in 1996 (31). Structure-activity relationship studies around a weakly active screening lead **13** (L-368,112; GHS EC_{50}=300 nM), that came from the oxytocin (OT) receptor antagonist



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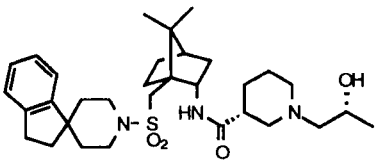
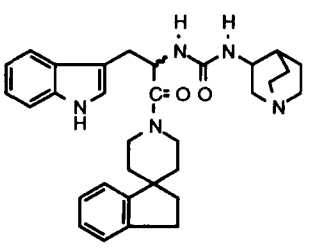


14

program (OT IC_{50}=68 nM), led to the discovery of **14** (GHS EC_{50}=90 nM; OT IC_{50}=130nM). It is interesting that **14** is an agonist at the GHS-R and an antagonist at the OT receptor.

Development of Spiropiperidine-based GH secretagogues (MK-0677) - A new structural class of GH secretagogues that has good oral bioavailability was disclosed in 1995 (32). The design of this spiropiperidine class of GH secretagogues originated in a project to derivativize so-called privileged stuctures as a strategy to identify leads for receptor projects (33). The spiroindanylpiperidine was chosen for derivatization since it was present in sigma receptor ligands (34) and in the oxytocin/GH secretagogue active **13** (31, 33, 34). From this type of approach, compound **15** (L-262,564; mixture of 4 diastereomers; EC_{50}=50 nM) was designed. Secretagogue **15** was active i.v. in dogs at 0.10 mg/kg but not active after an oral dose of 5 mg/kg. Extensive medicinal chemistry studies around **15** led to the discovery of **16** (L-163,191 (MK-0677); EC_{50}=1.3 nM).which was orally active in beagles at doses as low as 0.0675 mg/kg and i.v. down to 0.025 mg/kg (32, 35). The oral bioavailability of **16** in dogs was determined to be over 60%. Like GHRP-6 and **1**, transient increases in cortisol were observed after an 1 mg/kg oral dose to beagles (19). *In vitro* **16** was mechanistically indistinguishable from GHRP-6 and **1** (32).



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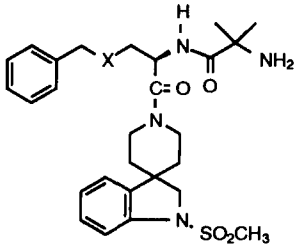
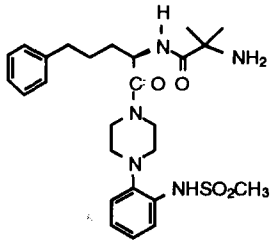


16 X = O

17 X = CH_2



18

MK-0677 is a long-acting GH secretagogue, since an oral dose of 1 mg/kg in beagles leads to a sustained elevation of GH for about 6 to 8 hours (35). Chronic once-daily oral administration of 0.50 or 1.0 mg/kg **16** to beagles leads to a marked desensitization of the GH response (36). Frequent sampling for GH on day 4 showed that the GH response was pulsatile in nature (1 36). Furthermore, there was a considerable increase in IGF-1 levels (~126%) that was also sustained throughout the experiment. Oral administration of 1.0 mg/kg **16** to dogs on alternate days did not result in desensitization of the GH response. The authors suggested that the desensitization observed on daily GHS administration results from the sustained IGF-1 elevation which may lead to IGF-1 mediated down regulation of GH secretion (36).

A 3-phenylpropyl-D-glycine derivative **17** (L-163,255; EC_{50}=1.5 nM) has been reported to be active in releasing GH and in increasing IGF-1 levels

in finisher pigs when it was administered for 72 hours at 360 ppm ad libitum in feed (37). These results suggest that GHSs may be useful as growth promotants in pigs. Of interest is a chronic study that investigated the anabolic and functional effects of **17** when administered in a dog hind limb immobilization protocol (38). At week 15, an 43% increase in muscle strength in the immobilized limbs, as measured by isometric torque, was seen in treated group *vs* 16% in the control group (38). The results of this study suggest that GHSs may be of value in rehabilitation therapy.

Other GHRP Peptidomimetics - By analogy with oxytocin receptor antagonists (39), use of piperazine-based replacements for the spiropiperidine of **16** provided analog **18** (EC_{50}=6.3 nM) with comparable GH releasing activity *in vitro* (40). As discussed above **16** is a long-acting GHS. It is unclear at this time what type of pharmacodynamic profile will result in optimal efficacy and, therefore, it is of interest that spiroindane esters and acids are potent, orally active and characterized by a much shorter duration of action than **16** have been reported (41). GH responses following once-a-day administration to dogs for several days with these short-acting secretagogues do not result in the same extent of desensitization as observed with a long-acting compound like **16**. As a result IGF-1 elevations are also less robust (41).

In pending patent applications, a group from Pfizer has claimed tetrahydroquinoline-based and 4-heterocyclic piperidine-based GH secretagogues (42). A group from Eli Lilly has claimed structures that include their NK1 clinical candidate LY 303870 (43) as well as 2-acylaminopropanamide derivatives as GH secretagogues (44). The biological activities and *in vivo* properties of these structurally distinct secretagogues is yet to be disclosed.

Identification, Characterization and Cloning of the GH Secretagogue Receptor (GHS-R) - As discussed above the GHRPs and GHRP peptidomimetics were discovered without knowledge of their molecular target or endogenous counterpart. Aided by tools like [^{35}S] MK-0677 (specific activity of 1000 Ci/mmol (16); GH release EC_{50} ~ 1.3 nM) (45), the growth hormone secretagogue receptor was identified in swine and rat anterior pituitary membranes (46). These binding sites exhibited the appropriate pharmacology in which the K_d and EC_{50} for GH release with diverse GH secretagogues had the predicted rank-order of potency. The binding of [^{35}S] MK-0677 was inhibited by GTP-γ-S suggesting that the GHS-R is most likely a G-protein coupled receptor (GPC-R) (46).

An expression cloning strategy was employed to isolate the first GHS-R cDNA from swine. The swine GHS-R clone was subsequently used to isolate human and rat GHS-R homologues (47). Determination of the nucleotide sequence of the full length human GHS-R mRNA (type 1a) showed that it encoded a GPC-R with seven transmembrane (TM) domains. The swine and human GHS-R encode a predicted protein of 366 amino acids, while the rat GHS-R encoded a 364 amino acid protein (47, 48). Human, rat and swine GHS-Rs are ~ 94 % identical at the amino acid level and share several GPC-R features, including an ERY GPC-R signature sequence, N-linked glycosylation sites and serine and threonine phosphorylation sites. A

second GHS-R mRNA (type 1b) encoded a shorter protein (265 amino acids) with only five predicted transmembrane domains. A comparison of GPC-Rs showed that the human GHS-R is most closely related to the neurotensin receptor (~35% identity) and the thyrotropin releasing hormone receptor (~29% identity). The GHS-R receptor exhibited the predicted pharmacology based on competition with MK-0677, GHRP-2 and GHRP-6. Type 1b cDNA transfection in COS-7 cells failed to express an MK-0677 binding site, even though type 1b protein was expressed on the COS-7 cell surface.

GHS-R mRNA Expression in Brain and Pituitary - In situ hybridization studies were carried out in rhesus brain (47), rat brain and pituitary (49) and GHS-R RNAse protection with human brain derived poly A^+ RNA (48) to determine the sites of type 1a and 1b GHS-R expression. Specific diffuse signals could be detected in the anterior rat pituitary, while the posterior pituitary failed to show specific signals (51). Analysis of rat brain gave the most complete overview of the pattern of GHS-R expression, and was consistent with the data obtained in both rhesus and human brain. First, as predicted from MK-0677 binding studies specific signals were detected in the arcuate nucleus, the ventral medial hypothalamus and the paraventricular nucleus (49). Interestingly, significant hybridization could be detected at several other brain regions including the dentate gyrus and CA2 and CA3 regions of the hippocampus and in dopaminergic neurons of the pars compacta of the substantia nigra and the ventral tegmental area. Other brain regions that showed GHS-R mRNA expression included the suprachiasmatic nuclei, preoptic nucleus, supraoptic nucleus, anterior hypothalamic area, the lateroanterior hypothalamic area (49) and tuberomammilary nuclei. Regions in the brain stem including the Edinger-Westphal, dorsal and median raphe nuclei also gave distinct hybridization signals (49). Finally, the latero dorsal tegmental area and the facial nerve revealed hybridization signals for GHS-R mRNA. Type 1a and 1b mRNA expression patterns were similar in the tissues examined. The role of GHS-R mRNA expression in other neurons in the hypothalamus, hippocampus and brain stem including, dopaminergic, serotonergic and NPY containing neurons (49-51) will require further investigation.

Clinical Evaluation of GH Secretagogues - The GHRPs are effective at releasing GH in healthy young and old human subjects and in numerous disease states (52). The GHRPs and peptidomimetics do show some degree of non-specificity since they also cause transient increases in cortisol and prolactin (14). An intact hypothalamic-pituitary connection is essential since GHSs dio not elicit a GH response in patients with a hypothalamic-pituitary disconnection (53). Co-administration of GHRP-6 and GHRH to young adults produced a synergistic GH reponse (14). As discussed above, i.v. infusion of short-acting GHRP-6 or 1 augments pulsatile GH secretion (3, 13). Likewise, once daily oral administration of long-acting MK-0677 to healthy elderly subjects daily for two weeks provided a prolonged upregulation of the GH secretory pattern (54). In this study, a down regulation of the initial cortisol response was also observed and no significant difference in cortisol release were observed on day 14 when compared to the placebo group (54). Furthermore, there was an approximately 60% increase in serum IGF-1 levels (54).

Long term intranasal administration of hexarelin (60 μg/kg, tid) to non-GH deficient short stature children enhanced growth velocity (55). MK-0677 treatment at 25 mg in short term diet-induced nitrogen wasting in healthy young men led to a sustained increase in serum GH, IGF-1 and IGFBP-3 levels (56). Treatment of catabolic conditions with MK-0677 may therefore prove beneficial. Eight-week treatment of obese individuals with once-daily 25 mg MK-0677 resulted in significant increases in basal metabolic rate and fat-free mass (57). But in contrast to GH studies, there were increases in body weight and body fat was unchanged. Longer term studies may be required to see the desired changes on body composition.

Therapeutic Potential - Growth hormone deficient children are candidates for treatment with GH secretagogues. Other conditions which potentially could be treated with GH secretagogues include a variety of catabolic conditions, including post-operative recovery, malnutrition from a variety of causes, and muscle wasting due to chronic exposure to corticosteroids, osteoporosis, Turner's syndrome and age-related deficiences in GH levels. Clinical studies are underway to evaluate the utility of secretagogues in several of these conditions. It is unknown whether they can overcome the GH resistance associated with catabolic states. Of considerable interest are potential clinical applications for GH secretagogues in reversing age-related loss in muscle strength and functional abilities. In this frail patient population GH secretagogues may show improved satey and tolerability since the GH profile is physiological in nature.

Summary - Within the past several years highly active GHRP peptidomimetics with short-acting and long-acting pharmacodynamic profiles have been identified. Furthermore, oral dosing is possible. The mechanisms by which pulsatile GH secretory pattern results from GH secretagogue treatment are being elucidated (11). GHRP-6 was shown to act directly on somatotrophs to cause GH release, to potentiate the actions of GH-stimulatory hormone, growth hormone-releasing hormone (GHRH; a hypothalamic peptide), and by being a functional antagonist of somatostatin, a hypothalamic peptide that inhibits GH release by several mechanisms (1). The relevance of this synergy with GHRH is exemplified by the loss of GHS sensitivity in hypothalamic/pituitary-stalk sectioned animals, which lack portal blood derived growth hormone-releasing hormone (GHRH) and which are no longer GHS responsive (58). The identification of the GHS-R that is specifically expressed in the hypothalamus, pituitary and several other discrete brain regions, provides significant evidence for the presence of a third neuroendocrine pathway, in addition to GHRH and somatostatin, with a dominant mode of action in the control of pulsatile GH release. Furthermore, it highlights the specificity of the action of the GH secretagogues. It is anticipated that the identification of the putative endogenous ligand of the GHS-R will shed further light on the normal physiological function of this receptor and its role in the control of pulsatile GH release. Finally, the small molecule GH secretagogues present important tools for evaluating their therapeutic potential in the clinic and in animal husbandry.

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Chapter 23. Novel Gene Switches for the Regulation of Gene Expression

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Introduction - DNA-based gene expression systems are finding broad application for the treatment of genetic and acquired disease through gene therapy. Most gene expression systems currently under development for gene therapy applications, whether viral or non-viral, produce therapeutic proteins constitutively and provide no means to regulate the dose of the therapeutic gene. Thus, there is a recognized need for gene expression systems that can be tightly controlled. Recently, a number of gene expression systems have been developed that can be regulated by the administration of specific small molecule drugs. These systems require the expression of genetically engineered regulatory proteins that function as molecular switches or gene switches. This chapter will describe a number of novel gene switches and discuss their potential use for *in vivo* human gene therapy. Much of the work in this area has been recently reviewed (1,2,3).

Background - The synthesis of intracellular and secreted proteins in normal cells is tightly regulated in response to numerous signals. Although protein production can be regulated at many levels, including the rate of transcription to produce mRNA, the stability of the mRNA and the rate of translation to produce the protein, the production of most proteins is controlled at the transcriptional level. Transcriptional regulation not only determines which cells are capable of producing a given protein but also when, in what amount, and for how long the protein is produced.

Gene transcription in eukaryotic cells is a coordinated, multi-step process. A key early step in this process is the binding of transcription factors (TFs) to specific DNA sequences (response elements) in the promoters and enhancers of expressed genes. Transcriptional activation typically requires the binding of multiple TFs, often 10 or more, to the promoter and/or enhancer of a gene and the interaction of the TFs with the general transcription complex which contains RNA polymerase, either directly or through the interaction of specific accessory proteins.

As our understanding of gene transcription has increased, it has become possible to design gene expression systems that regulate the expression of exogenously-administered target genes by controlling the interaction of TFs with specific response elements in the target gene's promoter or enhancer. Most approaches use similar strategies and involve the genetic engineering of chimeric transcription factors that contain 1) a domain capable of binding to a specific response element in the promoter/enhancer of the target gene (DNA-binding domain), 2) a domain capable of binding a specific ligand or small molecule drug to regulate the DNA binding activity (ligand-binding domain), and 3) a domain capable of stimulating or repressing the transcriptional process (transactivation or transrepression domain). These chimeric transcription factors function as gene switches since they are capable of switching the transcription of a target gene on or off in response to administration of the ligand or small molecule drug.

The primary functions of a gene switch are 1) to turn on (activate) or turn off (repress) the expression of an exogenously-administered target gene in a user-controlled manner, 2) to selectively activate or repress expression of the target gene without affecting the expression of endogenous cellular genes, and 3) to regulate the level of target gene expression in a manner dependent on the administered dose of small molecule drug. It is also important that the small molecule drug be easily administered, effective at low doses, non-toxic and have no deleterious side-effects.

Several inducible gene expression systems have been described. Some of the earliest systems include exposure to heat shock, heavy metals, and steroid hormones

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(reviewed in 4). These agents are generally toxic to cells, have pleiotropic cellular effects, and/or influence the expression of multiple endogenous genes. In addition, these systems tend to be very leaky in the uninduced state. Therefore, they may continue to be useful for transcriptional regulation in yeast, bacteria, and mammalian cell culture, but are not well-suited for use *in vivo* in animals or humans. A more recent publication describes the regulation of gene expression by modulation of intracellular cAMP concentrations (5). Other successful approaches to regulated target gene expression are based on the generation of novel, chimeric transcription factors derived from either bacterial repressor proteins or mammalian transcription factors. These two types of gene switches will be discussed in the remaining portion of this chapter.

GENE SWITCHES DERIVED FROM BACTERIAL PROTEINS

IPTG-regulated Gene Switch - One of the earliest gene switches was based on the bacterial lactose repressor protein (reviewed in 6). The lactose repressor protein (*lac* R) binds to a specific response element, the *lac* operator (*lac* O), located upstream of a cluster of genes involved in the metabolism of ß-galactosides such as lactose, and represses the expression of these genes. The binding of the *lac* R protein to *lac* O is controlled by allolactose, which is formed in the presence of lactose. When allolactose reaches a critical concentration within the cell, it induces a conformational change in the *lac* R protein, which is then released from the *lac* O sequence allowing transcription to proceed. As a result, the bacteria produce the enzymes needed for the metabolism of lactose only when lactose is present. As the lactose concentration falls below a threshold concentration in the cell, the *lac* R protein returns to its original conformation, rebinds to the *lac* O sequence and thereby turns off transcription of these genes. Thus, the *lac* repressor protein acts as a natural gene switch because it regulates the transcription of this cluster of genes so that expression occurs only when the gene products are needed.

It is possible to regulate the production of a specific eukaryotic gene product using lactose or the more active non-hydrolyzable galactose analog isopropyl ß-D-thiogalactoside (IPTG), by placing the *lac* O sequence in the promoter of a eukaryotic target gene and expressing the *lac* R protein in the cell (7,8,9). More recently, the repressor molecule was genetically engineered to act as a transcriptional activator instead of a repressor in mammalian cells by fusing the powerful transcriptional activation domain of the herpes simplex virus virion protein 16 (VP16) to the IPTG- and DNA-binding domains of the *lac* repressor protein, resulting in a 1200-fold increase in gene expression (10).

One advantage of the IPTG-regulated gene switch is that the *lac* O sequence is bacterial in origin and unlikely to occur in promoters or enhancers of endogenous eukaryotic genes. Thus, transcription of endogenous eukaryotic genes should not be affected by IPTG when the *lac* R protein is present in the cell. Only the target gene which has been engineered to respond to the gene switch should be affected. While this mechanism of action provides a tremendous level of control, the high concentrations of IPTG (5-50 mM) required for this switch and a temperature-sensitive variant (11) to function in mammalian cells can be cytotoxic, thereby limiting the *in vivo* applicability of this gene switch.

Tetracycline-regulated Gene Switch - A tetracycline-regulated gene switch has been developed that overcomes many of the problems of the IPTG-regulated switch (reviewed in 6). The bacterial tetracycline-resistance gene (*tet*) is similar to the *lac* system in that transcription of the *tet* gene is under control of a repressor protein, the *tet* repressor (*tet* R), that binds to the *tet* operator response element (*tet* O) in the absence of tetracycline and represses transcription of the *tet* gene. In early experiments, the *tet* R protein was engineered to function in eukaryotic cells and while sufficient concentrations of *tet* R could be produced in plant cells to cause transcriptional repression of a cauliflower mosaic virus promoter (12,13), this could not be duplicated in mammalian cells due to the inability to achieve sufficient levels of *tet* R (6). Repression-based systems have a number of inherent problems including the need

to express very high levels of the repressor protein in order to achieve significant repression of transcription (6).

More recently, a chimeric tetracycline-activated gene switch, the tetracycline-regulated transactivator (tTA), was engineered by fusing the *tet* O-binding domain of the *tet* R protein with the transactivation domain of VP16 (14). When the tTA protein is bound to *tet* O sites in the promoter of the target gene, the gene is transcriptionally active. Following tetracycline administration, the tTA protein undergoes a conformational change that causes it to be released from the *tet* O sites and transcription is terminated. Thus, the tTA gene switch differs from the natural *tet* R protein in that tTA activates rather that represses transcription of *tet* O-containing promoters in the absence of tetracycline, and tetracycline administration results in transcriptional repression.

As with the IPTG-regulated gene switch, there is a low probability of the bacterial *tet* O sequence occurring in the promoters or enhancers of eukaryotic genes. Therefore, tetracycline administration should only affect the transcriptional activity of the exogenously-administered target gene whose promoter has been engineered to contain *tet* O sequences. However, the tetracycline gene switch must be expressed in the same cells that contain the target gene. This can be achieved using stably-transfected cell lines or double-transgenic animals (animals transgenic for both the tTA gene and a tetracycline-responsive target gene). In double-transgenic mice, target gene expression was constitutive in the absence of tetracycline and repressed after tetracycline administration (15). No toxicity was observed in these experiments using slow release pellets that delivered a dose of 0.7 mg of tetracycline per day over 7 days. The delivery of two genes to the same cells has also been addressed using retroviral constructs carrying both gene expression cassettes (16,17). In this case, administration of tetracycline resulted in a 340-fold repression of target gene expression. Thus, the tetracycline-regulated gene switch can function when both genes are administered in one expression vector. This modification of the original tetracycline system allows for much broader applications, including application to areas of *in vivo* gene therapy where viral administration is acceptable.

The tTA gene switch was improved by engineering an autoregulatory tetracycline-responsive system with the intention of eliminating the potential toxic effects of constitutive production of high tTA concentrations (18). The tTA gene was put under its own transcriptional control by placing *tet* O sequences in the tTA gene promoter. In this system, transcription of both the tetracycline-responsive target gene and the tTA gene are repressed in the presence of tetracycline. Although tTA expression is repressed in the presence of tetracycline, a low level of tTA protein is still produced due to the leakiness of the tetracycline-responsive promoter. When tetracycline is withdrawn, the small amount of tTA present binds to the *tet* O sequences in the promoters of both the tTA and target genes and activates their transcription. As the concentration of tTA increases, both genes are further activated. Thus, in the absence of tetracycline, tTA drives the expression of itself and the tetracycline-responsive target gene, eliminating the problem of constitutive tTA overexpression. It was found that tTA protein was expressed at immunologically-detectable levels only in the absence of tetracycline and induction of target gene expression was higher than observed using the previous system, where tTA was constitutively expressed. Furthermore, the autoregulatory system was shown to function in both stably-transfected cell lines and transgenic mice. It remains to be seen if this system will be suitable for *in vivo* expression in animals or for human gene therapy.

One fundamental problem with all tTA-based gene switches is that target gene transcription is constitutive in the absence of tetracycline. Tetracycline must be continuously administered to keep the target gene repressed. While long-term administration of tetracycline at doses necessary to maintain the transcriptionally-repressed state in animal studies does not seem to have dramatic side-effects, this system may be unacceptable for many *in vivo* applications. For example, it would be inconvenient and undesirable to require tetracycline administration for extended periods of time to maintain the therapeutic gene in a repressed state in animals or humans until

expression is desired or to terminate expression if it became necessary to do so for safety reasons. In addition, induction of therapeutic gene expression would require the withdrawal of tetracycline which is a relatively slow process *in vivo*. Only about 20% of the steady-state expression level is achieved within 12 hours of tetracycline withdrawal (14). This is especially problematic in studies where tight control of temporal gene expression is important.

Improved tetracycline-regulated gene switches were engineered that maintain the target gene in a repressed state in the absence of drug and activate gene expression in the presence of drug (19). This was achieved by mutating the natural *tet* R protein and screening for tetracycline-dependent transcriptional repression. A tTA protein with four amino acid changes was identified that produced the desired result. The mutated region was transferred to the tTA protein generating a reverse tetracycline-regulated transactivator protein (rtTA). The rtTA protein binds to the *tet* O sequence and activates gene expression only in the presence of tetracycline. In an attempt to increase the level of transcriptional activation, a nuclear localization signal (nls) was added to the rtTA protein to generate rtTA-nls. Addition of a nuclear localization signal to cytoplasmic or secreted proteins often results in their accumulation in the nuclei of transfected cells. Since rtTA acts as a transcription factor it must be present in the nucleus to function. Stably-transfected cell lines were generated that contained both the rtTA-nls and a *tet*-responsive target gene. More than 1000-fold induction of target gene activity was achieved following administration of doxycycline, a derivative of tetracycline. At a concentration of 1 μg/mL, doxycycline produced a 100-fold increase in transcriptional activity after 5.5 hours and maximal induction (approximately 1000-fold) after 24 hours. One problem with this system is that, like the original tTA-based gene switch, the rtTA and rtTA-nls proteins must be constitutively expressed at very high levels to be effective. This can be deleterious to eukaryotic cells and, since these proteins are bacterial in origin, they may be immunogenic *in vivo*. However, it should be possible to combine this system with the autoregulatory system described above such that both the rtTA or rtTA-nls gene and the target gene are maintained in a repressed state until induced by administration of tetracycline.

All of the studies described thus far have been conducted in either stably-transfected cell lines or double-transgenic mice. As discussed above, in order for these gene switches to function after direct *in vivo* administration, both the gene switch and the target gene must be delivered to the same cells within the body. With conventional non-viral gene delivery technologies, this means that either a single DNA plasmid (circular DNA molecule) containing both genes must be engineered or two DNA plasmids, each containing one of the two genes, must be delivered to and taken up by the same cells within the target tissue. The first approach requires production of relatively large plasmids which tend to be unstable and have the potential for transcriptional read-through from one gene to the other. The problem with the second approach is that the ability of cells to take up exogenous DNA is limited, and the likelihood with which the same cell will take up two plasmids is even more limited. However, it has been demonstrated that two plasmids can be delivered to cells following direct injection into muscle tissue *in vivo* (20). Two plasmids, one carrying the tTA gene switch and the other carrying a tetracycline-responsive target gene, were injected directly into the tibialis anterior muscles of mice. Tetracycline was administered by intraperitoneal injection or in the drinking water. Target gene expression was low but detectable in the presence of tetracycline and induced approximately 100-fold with tetracycline withdrawal. Repression of target gene expression was dependent on both the dose of tetracycline and duration of treatment. While the repression of target gene expression was dramatic, it was not complete. The level of basal target gene expression in injected muscle was approximately 10-fold higher than that observed in non-injected naive muscles. Therefore, this gene switch would not be appropriate in situations where tightly-controlled target gene activity is necessary. However, these experiments were performed with one of the original tetracycline-repressed gene switches. It would be interesting to test one of the tetracycline-activated gene switches, either rtTA or rtTA-nls in this system. Nevertheless, these findings are important in that they demonstrate that a gene switch can be used to regulate the expression of a desired target gene after direct *in vivo* administration to animals. It remains to be determined if even short- term

expression of proteins derived from bacterial sequences will generate a significant immune response in humans or other mammalian systems.

GENE SWITCHES DERIVED FROM EUKARYOTIC RECEPTORS

A number of recent reports describe novel gene switches derived from eukaryotic receptor proteins. Three types of gene switches will be discussed here including 1) the "chemical inducer of dimerization" or CID-regulated gene switch developed in the laboratories of Stuart Schreiber and Gerald Crabtree (2,21-23), 2) the ecdysone-regulated switch developed in the laboratory of Evans (3,24) and 3) the progesterone antagonist-regulated gene switch developed in the laboratory of Bert O'Malley (25).

CID-regulated Gene Switch - The CID-regulated gene switch is based on the ability of certain immunosuppressive drugs, including cyclosporin A (CsA), FK506 and rapamycin, to promote the intracellular association of two otherwise independent cellular proteins (2). CsA, FK506 and rapamycin are natural small molecule drugs produced by soil microorganisms and are capable of binding to specific eukaryotic intracellular proteins known as immunophilins. They are active only when bound to their cognate immunophilin. CsA binds to cyclophilin while FK506 and rapamycin bind to FK506 binding protein 12 (FKBP12). Subsequently, CsA/cyclophilin, FK506/FKBP12 and rapamycin/FKBP12 complexes bind to and inhibit the activity of calcineurin A, a protein phosphatase involved in many signal transduction pathways. Thus, trimeric complexes are formed whereby CsA, FK506 and rapamycin induce the dimerization of two proteins, an immunophilin and calcineurin A. For this reason, CsA, FK506 and rapamycin are often referred to as "chemical inducers of dimerization" (CID).

The dimerization activities of CIDs have recently been exploited to create novel eukaryotic gene switches (21-23), referred to here as CID-regulated gene switches. In one case, a homodimeric CID containing two copies of FK506, referred to as FK1012, is used to induce the dimerization of two inactive proteins to form a functional transcriptional activator (21). The two inactive proteins were 1) a chimeric protein containing three copies of FKBP12 and the DNA-binding domain of either GAL4, a galactose-dependent yeast transcription activator, or HNF1, a mammalian transcription activator, and 2) a chimeric protein containing three copies of FKBP12 and the transactivation domain of VP16. The individual proteins are inactive because each has only a DNA-binding domain or a transactivation domain. Plasmids containing genes that encode the two inactive proteins were cotransfected into cultured Jurkat cells along with a plasmid that contains either a GAL4- or HNF1-responsive target gene. FK1012 was capable of inducing target gene expression, demonstrating dimerization of the inactive proteins to form a functional complex containing both DNA-binding and transactivation domains. However, the level of transcriptional activation achieved with the FKBP12-GAL4 and FKBP12-HNF1 chimeric proteins was only about 35% and 13% of that achieved with the constitutively active GAL4-VP16 or HNF1-VP16 chimeric proteins, respectively. The low level of expression observed may reflect formation of non-functional FKBP12-GAL4 or FKBP12-VP16 homodimers in addition to functional FKB12-GAL4/FKBP12-VP16 heterodimers.

This problem was effectively addressed by the development of a bifunctional, heterodimeric CID (FKCsA) that contains the FKBP12-binding activity of FK506 and the cyclophilin-binding activity of CsA (22). FKCsA is capable of selectively inducing the heterodimerization of two proteins that contain either a FK506- or CsA-binding site. Multiple copies of FKBP12 were fused to the GAL4 DNA-binding domain and multiple copies of the CsA-binding domain from cyclophilin were fused to the VP16 transactivation domain. Three FK506-binding sites and two CsA-binding sites gave the maximum response after treatment with different doses of FKCsA. A dramatic increase in target gene expression was detected 40 hours after FKCsA treatment. Importantly, the level of basal target gene expression in the absence of FKCsA was comparable to that seen with untransfected cells. This demonstrates that a synthetic bifunctional CID is capable of efficiently bringing two inactive chimeric proteins together to create a functional heterodimer capable of DNA sequence-specific transcriptional activation.

More recently, a CID-regulated gene switch was engineered exclusively from human proteins that is activated by rapamycin (23). This switch is based on the ability of the natural monomeric form of rapamycin to induce the dimerization of two cellular proteins, FKBP12 and FRAP. One protein contained a composite DNA binding domain (ZFHD1) that interacts specifically with a novel DNA response element not recognized by endogenous transcription factors, and three copies of FKBP12. The other protein contained the C-terminal transactivation domain of NK-kB (p65) and the region of FRAP that interacts with the FKBP12/ rapamycin complex. Dose-dependent expression of a target gene containing 12 ZFHD1 binding sites was demonstrated in both transiently and stably transfected cells after rapamycin treatment. In *ex vivo* experiments, cultured cells were stably transfected with three genes (one for each of the two chimeric rapamycin-binding proteins and a ZFHD1-responsive hGH target gene) and implanted into skeletal muscles of nude mice. Human growth hormone was detected in serum from these animals 17 hours after a single injection of rapamycin. The level of hGH expression was dose-dependent, with maximal response at a dose of 10 mg/kg. A major advantage of this rapamycin-regulated gene switch is that the chimeric proteins are constructed entirely of human proteins and should be less immunogenic that the gene switches previously discussed. However, these proteins are novel chimeric proteins and, therefore, may still induce an immune response.

The authors do not fully discuss the time course of gene expression after drug treatment and do not address the issue of terminating transcription of the target gene if it became necessary to do so. The limitations of these systems are 1) the requirement for the uptake of three plasmids, two encoding the individual components of the activator and the target gene, into the same cell in order to achieve biological activity, and 2) the known property of these drugs to block cell cycle progression, leading to immunosuppression *in vivo*. The effects on immune function likely will restrict the application of this system for human gene therapy. However, generation of non-immunosuppresive rapamycin analogues may eliminate this limitation.

Ecdysone-regulated Gene Switch - Ecdysone-regulated gene switches have been described in which ecdysone, a steroid hormone that regulates metamorphosis in the fruit fly Drosophila, is used to regulate target gene expression in mammalian cells (24). In Drosophila, ecdysone regulates the transcription of a set of genes during development by first binding to and activating the ecdysone receptor (EcR), a cytoplasmic receptor and member of the steroid receptor family of transcription factors. The activated receptor then translocates to the nucleus and activates transcription by binding to a specific DNA sequence in the promoters of ecdysone-responsive genes.

Steroid hormone receptors are intracellular proteins that belong to a large family of transcription factors. They have been extensively studied and their mechanism of action is well-characterized. Classically, hormone/receptor complexes bind to specific response elements in the promoters or enhancers of hormone-responsive genes and either activate or repress the expression of these genes. Some steroid hormone receptors bind to their cognate response elements as homodimers. Others bind only as heterodimers formed with other receptors or proteins. The EcR acts as a heterodimer, interacting with the product of the ultraspiracle gene (USP) in Drosophila.

In early studies, the wild-type EcR and USP genes along with an ecdysone-responsive target gene were transfected into mammalian cell lines. A 3-fold increase in the level of target gene activity was observed following administration of muristerone A, an ecdysone analog or ecdysteroid (26,27). In more recent experiments, the modular nature of the receptors was exploited to develop an improved ecdysone gene switch (24). Steroid hormone receptors contain well-defined functional domains including 1) a transcriptional activation domain, 2) a specific DNA-binding domain, and 1) a hormone- or ligand-binding domain. Previous work showed that these functional domains could be recombined to produce chimeric receptors with mixed specificity or higher activity (28,29). Although chimeric receptors remain hormone-inducible, their function reflects the specificity dictated by the individual functional domains. To increase functional activity of the EcR, the natural transactivation domain was replaced by the transactivation domain of the GR (glucocorticoid receptor). These chimeric receptors

contain the ligand- and DNA-binding domains of EcR and are, therefore, still activated by ecdysteroids and selectively bind to ecdysone response elements inserted into the promoters of target genes. In transient transfection assays with the EcR-GR chimeric receptor and USP, expression of an ecdysone-responsive target gene increased from 3- to 11-fold after treatment with muristerone. However, a 34-fold activation of target gene expression was observed when USP was replaced by the retinoid X receptor (RXR), the mammalian homolog of USP. An even more potent chimeric receptor was generated by replacing the relatively weak EcR or GR transactivation domain with the much stronger transactivation domain of VP16. This VP16-EcR chimeric receptor and RXR was able to induce expression of the ecdysone-responsive target gene more than 200-fold in transiently and stably transfected cells. Kinetic studies showed that target gene expression could be induced approximately 100-fold after 3 hours of muristerone treatment (1 μM) and 1000- to 20,000-fold after 8 and 20 hours, respectively. Drug-dependent gene expression was confirmed in transgenic mice harboring an ecdysone-responsive target gene, and both the VP16-EcR chimeric receptor and RXR genes under control of a T-cell promoter (24). Target gene expression was muristerone-dependent and appeared restricted to cells in the thymus, as expected when using a T cell-specific promoter.

Transcriptional activity of the ecdysone-regulated gene switch (VP16-EcR/RXR) was also compared with the tTA and rtTA tetracycline-regulated gene switches in transient transfection experiments (24). Interestingly, target gene expression was induced to a significantly greater extent with the ecdysone-regulated gene switch (1000-fold) compared with the rtTA tetracycline-regulated gene switch (2.5-fold). This dramatic difference is partly due to the higher basal activity of rtTA, which is 500 times more than that observed with the ecdysone-regulated switch. The authors comment that ecdysteroids do not seem to be toxic or teratogenic, and are not known to affect mammalian physiology. They suggest that this system is suitable for many types of studies in mammalian cells and transgenic mice, but do not comment on its suitability for human gene therapy. Considering that the activity of the EcR-derived gene switch depends on the recruitment of other members of the steroid receptor family, including the retinoid X receptor which influences biological responses to retinoid and thyroid hormones as well as Vitamin D, it seems likely that this system may yield pleiotropic effects in mammalian cells, making it unsuitable for *in vivo* therapeutic use.

Antiprogestin-regulated Gene Switch - The antiprogestin-regulated gene switch also exploits the modular nature of steroid hormone receptors and is derived from the progesterone receptor (PR) (25,30). Specifically, this switch is derived from a mutated PR that contains a 42 amino acid truncation of the C-terminal ligand binding domain (31). This mutated PR no longer binds or is activated by progesterone (agonist) but still binds and is now activated by drugs that normally antagonize the activity of progesterone (antiprogestins). To construct the antiprogestin-regulated gene switch, the DNA-binding domain of the mutated PR was replaced with the DNA-binding domain from GAL4 and the relatively weak transactivation domain was replaced with the stronger transactivation domain from VP16 (25). The yeast GAL4 protein binds to a specific 17 bp DNA response element, the GAL4 binding site, that is contained in the promoters of yeast genes coding for galactose-metabolizing enzymes. The resulting chimeric steroid receptor is capable of selectively activating transcription of a target gene that has one or more GAL4-binding sites in its promoter without affecting the expression of endogenous genes, including progesterone-responsive genes. Furthermore, activation of the target gene occurs only in the presence of the activator antiprogestin. Utilization of the mutated antiprogestin-dependent ligand-binding domain was key to the success of this gene switch because the switch is not activated by endogenous hormones and will remain inactive until the activating drug is administered. Furthermore, the antiprogestin, now acting as an agonist for the chimeric receptor, is active when administered at extremely low doses (5 μg/kg) relative to the therapeutic dose needed for functional antagonism of endogenous hormone function (10 mg/kg). This represents a dose reduction of more than three orders of magnitude. Incorporation of the GAL4 DNA binding domain provides specificity for target gene activation. Since the GAL4 binding site is derived from promoters of yeast genes and has not been detected in the regulatory regions of any mammalian genes, only the target gene which is engineered to

contain one or more copies of the GAL4 recognition sequence in its promoter will be transcriptionally activated by the gene switch.

Preliminary experiments with the antiprogestin-regulated gene switch were based on transfection of plasmid DNA into cells in culture and an *ex vivo* animal model (25). In these experiments, target genes were engineered with multiple GAL4 binding sites in the promoters. Target genes were selectively expressed in cells expressing the gene switch and only after administration of synthetic antiprogestins. The effect on transcriptional activation was dependent on both the dose and duration of antiprogestin administration. Expression of the target gene was observed within 2 hours of antiprogestin administration, and was maximal within 8 hours of administration. Importantly, activation of the gene switch was detectable at an antiprogestin concentration of 0.1 nM and maximal at 1 nM. This is well below the affinity of the natural, unmodified progesterone receptor for antiprogestins, and approximately 3 orders of magnitude below the concentration required for functional antagonism of the progesterone agonist.

In subsequent work, double transgenic mice were created that harbor the gene switch under transcriptional control of the liver-specific transthyretin promoter/enhancer and a GAL4-responsive human growth hormone (hGH) target gene (32). In these animals, expression of the gene switch was constitutive and liver-specific. Furthermore, hGH was barely detectable in serum of untreated mice and induced up to 33,000-fold after a single intraperitoneal injection of 250 μg/kg of the antiprogestin. This demonstrates that the antiprogestin-regulated gene switch is not activated by endogenous hormones or other natural agents. Expression of hGH was detected in serum within 5 hours of antiprogestin administration and maximum by 12 hours post-injection. Dose-dependent activation was demonstrated after oral administration of either 250 μg/kg (1500-fold activation) or 500 μg/kg (3500-fold activation) antiprogestin. Also, as expected, the animals demonstrated significant weight gain (50-60%) following antiprogestin treatment compared with untreated transgenic littermates. Importantly, the antiprogestin-regulated gene switch demonstrated cyclic or pulsatile target gene expression when antiprogestin administration was withdrawn and treatment repeated.

These studies demonstrate that the antiprogestin-regulated gene switch can be used to control the expression of specific target genes in a tissue-restricted manner with extremely low levels of basal activity in the absence of antiprogestin (activator). The onset of target gene expression is rapid and the expression kinetics mimic the physiological kinetics observed with endogenous hormones without interfering with other signal transduction pathways in the cell and without the need for other receptor family members. It is, therefore, possible to achieve tightly-regulated expression of an exogenously-administered target or therapeutic gene without deleterious side-effects.

Summary - There has been tremendous progress in the development of systems for the control of gene expression in eukaryotic cells over the past decade. Recently, gene expression systems have been devised that allow exogenous target genes to be selectively regulated by the administration of small molecule drugs. These systems are particularly amenable to studies of the effect of a particular protein on differentiation or developmental processes. Molecular switches also are likely to have a crucial role in development of controllable human gene therapies for the treatment of genetic and acquired diseases since it will be possible to regulate expression of the therapeutic protein to achieve maximum therapeutic benefit and to terminate therapy, if desired.

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Chater 24. Agents that Block TNF-α Synthesis or Activity

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Introduction - Tumor necrosis factor-α (TNF-α) is a cytokine that appears rapidly in response to infection and trauma (see refs. 1 and 2, for recent reviews). Its main physiological function appears to be mobilizing host defense. TNF-α-deficient mice are abnormally susceptible to certain infectious agents and show impaired hypersensitivity responses; their only other apparent defects are in formation of primary B cell follicles, follicular dendritic cell networks, and germinal centers (3). However, when produced inappropriately (for example, due to an autoimmune response or to tissue injury), TNF-α can cause severe damage. High TNF-α levels correlate with various pathological states (*e.g.*, cachexia and sepsis), and transgenic mice overexpressing TNF-α develop pathological inflammatory conditions, including polyarthritis and CNS demyelination (4). Moreover, neutralization of TNF activity leads to improvement in models of inflammatory diseases and in arthritis patients in clinical trials. A structurally related cytokine called lymphotoxin-α (or TNF-β) is more restricted in its expression and appears to be involved primarily in development of the immune system (5).

TNF-α production has been studied most intensively in macrophages, but it is also produced by many other cell types (1). Transcription is induced by a variety of agents, including bacterial products [*e.g.*, lipopolysaccharide (LPS)] and other cytokines, and is downregulated by cAMP. Several transcription factors, including NF-κB and AP-1, act on its promoter. TNF-α messenger RNA stability and susceptibility to translation are influenced by factors that bind to its 3'-noncoding region. The message encodes a 26 kD transmembrane protein, the C-terminal two-thirds of which protrude into the extracellular space. A metalloproteinase-disintegrin called the TNF-α converting enzyme (TACE) releases the extracellular domain from cells (6,7). Both the membrane-bound and soluble forms are functional in an assay for cytolytic activity, but in some situations they cause different effects. For example, cells of a human colon carcinoma line are killed by membrane-bound but not by soluble TNF-α (8).

The two TNF-α receptors (TNFRs), designated p55 and p75, are expressed on most cell types (1). Although both receptors are capable of transducing signals, the majority of TNF-α's effects (*e.g.*, expression of adhesion proteins, secretion of proteases, production of other cytokines and intercellular mediators, and apoptosis) can be induced via the p55 receptor alone. Binding of TNF to this receptor initiates a series of parallel protein serine/threonine kinase cascades which lead to the activation of members of the mitogen-activated protein kinase superfamily including extracellular signal-regulated kinases (ERKs) (9), *jun* terminal kinase JNK-1 (also called stress-activated protein kinase-1) (10), and p38/stress-activated protein kinase-2 (11). The stress-activated protein kinases directly phosphorylate and activate transcription factors including Elk-1, ATF2 and c-jun. Distinct pathways lead to the activation of NF-κB. The putative second messenger ceramide is involved in some p55 receptor-mediated signaling events (12,13). Several proteins that interact with the cytoplasmic domain of the p55 receptor, directly or indirectly, appear to determine the nature of the downstream response (14).

A previous review covers inhibitors of phosphodiesterases and TACE particularly well (15), and provides brief commentary on a variety of other compounds that block

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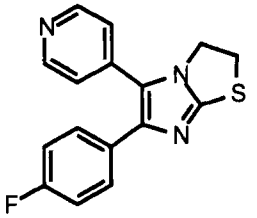
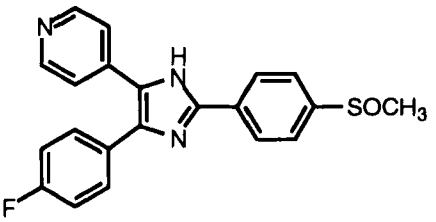
TNF-α production. The discussion below focuses on more recent inhibitors of TNF-α production and signaling, on the structure and function of TACE, and on proteins that prevent TNF-α from binding to cell-surface receptors.

INHIBITORS OF TNF-α PRODUCTION OR TNF RECEPTOR-MEDIATED EVENTS

Cytokine-Suppressing Anti-Inflammatory Drugs (CSAIDS) - A relatively new class of compounds that inhibit cytokine production, including that of TNF-α, are the CSAIDS typified by the bicyclic imidazole **1** (SKF86002). Although this compound is an inhibitor of both cyclooxygenase and 5-lipoxygenase, its cytokine inhibitory activity is unrelated to its effects on arachidonic acid metabolism (16). CSAID binding proteins have been cloned (17) and shown to correspond to isoforms of the stress-activated protein kinase p38/SAPK-2. In LPS-stimulated THP-1 cells (a human monocytic cell line), **1** apparently blocked the initiation of TNF-α translation (18).



1

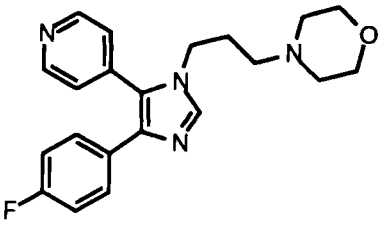


2

Because p38 kinase is a potential mediator of some of the downstream signals transduced by TNFRs, it might be expected that CSAIDS would also control some of the inflammatory effects induced by TNF-α. Indeed, TNF-α-induced IL-6 synthesis and activation of p38/SAPK in L929 cells are completely inhibited by the CSAID SB203580 **2** at micromolar concentrations (19,20). Neither NF-κB DNA-binding activity, nor phosphorylation of its cytosolic inhibitor, IκB, were affected by **2**, indicating that NF-κB activation lies on a separate pathway from the p38 kinase cascade. The treated cells were still sensitive to TNF-α-induced cytotoxicity.

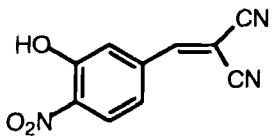
The *in vivo* efficacy of **1** was shown in an experimental model of neonatal sepsis, in which neonatal rats were given a lethal dose of heat killed group B streptococci (21). 10 mg/kg **1** given 2 h prior to the challenge reduced plasma TNF-α levels at 2.5 h post-challenge by 45%. This treatment also reduced mortality at 72 h to 54% from 85%.

The prototype CSAIDS were characterized by varying degrees of inhibitory activity against cyclooxygenase and 5-lipoxygenase, prompting an effort to synthesize analogues which bind p38/SAPK but do not affect enzymes involved in arachidonate metabolism. 1-alkyl- or 1-aryl-4-aryl-5-pyridinylimidazoles were found to have the desired properties *in vitro* (22), and one of the most potent of these, SB 210313 **3**, was tested for activity in the rat adjuvant arthritis model. Daily injection of **3** at 80 mg/kg surpassed the effect of indomethacin treatment, resulting in a 42% suppression of hindpaw edema, and a more marked increase in bone mineral density.



3

Other Kinase Inhibitors - A large group of compounds called tyrphostins inhibit tyrosine kinases (reviewed in 23), often with strikingly distinct specificities. In a recent study with LPS-challenged mice, injection of 4 (tyrphostin AG 126) at 400 μg per mouse 2h prior to challenge protected 90% of the mice from lethality. 4 inhibited TNF-α production in the LPS-challenged mice by up to 80% at the 400 μg dose and also inhibited TNF-α production in LPS-stimulated peritoneal macrophages. The mechanism of action of 4 is not known, but could involve inhibition of kinases upstream of MAP kinase, since LPS-induced tyrosine phosphorylation of p42 MAPK was blocked by the inhibitor (24). Recently, some benzamidine derivatives (JM34, 5; JM42, 6) which had previously shown benefit in experimental inflammatory edema were found to inhibit TNF-α production in zymosan or LPS-activated macrophages, probably through their inhibition of PKC (25).



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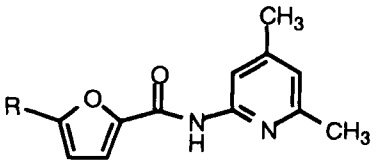
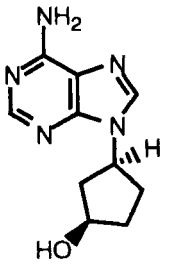


5: R = H
6: R = Br

Adenosine Receptor Agonists - Adenosine (Ado) or 7, a related carbocyclic nucleoside (MDL201112), inhibited the production of TNF-α but not IL-1 from activated mouse peritoneal macrophages and from macrophage cell lines (26). 7 inhibited the expression of steady-state TNF-α mRNA in the cell lines, but Ado did not, suggesting that the latter blocked TNF-α synthesis at a post-transcriptional level. A single injection of 100 mg/kg 7, 1 h prior to challenge, or at the time of challenge, protected 90% of mice lethally challenged with a combination of LPS and D-galactosamine. Ado itself failed to protect the mice against LPS-induced lethality, perhaps due to its rapid metabolism. Indeed, blocking the metabolism of endogenous Ado with an inhibitor of adenosine kinase, 8, protected against both LPS-induced shock in mice and bacterial peritonitis in a rat model (27).



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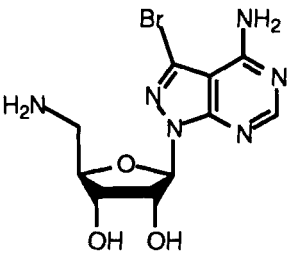
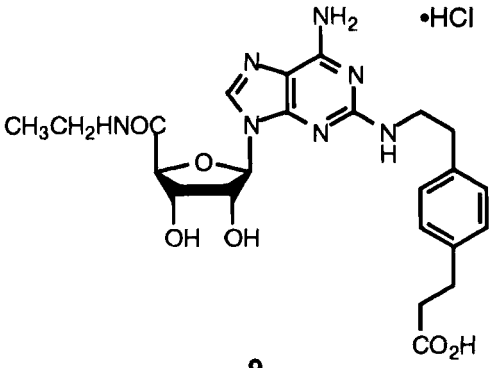
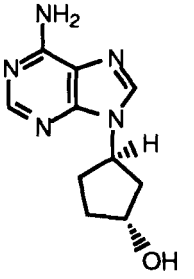


8

It is not clear which Ado receptor mediates these effects on TNF production. 9, an agonist highly selective for A_2 receptors, inhibited the production of TNF-α by LPS-stimulated human monocytes by 75%, whereas an A_1-selective agonist reduced TNF-α production by only 25% (28). The A_2 Ado receptor is expressed on mononuclear phagocytes, and it is coupled to activation of adenylyl cyclase and hence a rise in the cellular levels of cAMP. Despite evidence implicating involvement of the A_2 receptor, more recent studies indicate the A_3 receptor mediates the Ado inhibitory effect on TNF-α production in two monocytic cell lines (29). A striking property of many Ado receptor agonists, in contrast to CSAIDS and antioxidants (see below), is their selectivity for TNF-α; carbocyclic nucleotides such as 10 have little or no effect on the production of IL-1β or IL-6 (30,31).



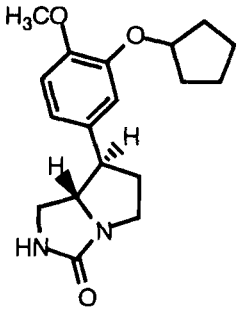
9



10

Phosphodiesterase Inhibitors and Related Compounds - Specific inhibitors of PDE IV, the major phosphodiesterase in monocytes and neutrophils, decrease TNF-α production in human peripheral blood monocytes (see 15 for review). In addition to pentoxifylline analogues, rolipram (and rolipram analogues such as Ro 20-1724 (32)) and nitrogen heterocycles such as CP77059 (33) specifically inhibit PDE IV and reduce TNF-α production. 11, a conformationally constrained analogue of Ro 20-1724, was found to inhibit PDE IV with a Ki of 27 nM and TNF-α secretion with an IC50 of 290 nM; in parallel experiments, Ro 20-1724 gave a Ki of 1930 nM and an IC50 in the TNF-α secretion assay of 1800 nM (32). Another recent PDE IV inhibitor reported to block TNF-α production is the rolipram analogue 12, with an IC50 against LPS-stimulated human monocytes of 219 nM (34). Both rolipram and the more potent CP-77059 reduce serum TNF-α levels *in vivo* and have potent anti-inflammatory effects (33). Surprisingly, analogues of pentoxifylline metabolites have been made with little

activity against PDE IV that still block TNF-α production by peripheral blood mononuclear cells, with an IC50 of 5-10 μM (35).



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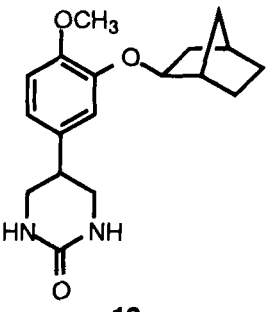
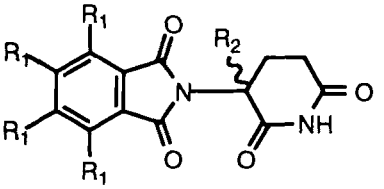


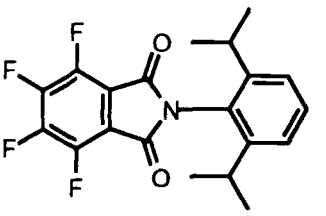
12

Raising cAMP levels by activating adenylate cyclase also reduces TNF-α expression. The β_2-adrenergic receptor agonist salmeterol inhibits TNF-α production by THP-1 cells without affecting IL-1β levels. This compound was also highly efficacious in blocking the LPS-induced serum TNF-α level in mice and was partially protective in the galactosamine/LPS model of endotoxic shock (36).

Thalidomide - The efficacy of thalidomide, **13**, in blocking TNF-α production in cell systems is modest (with an IC50 of 200 μM in one recent report), but tetrafluorothalidomide, **14**, inhibits TNF-α production in a human monocytic cell line with an IC50 of 400 nM (37). A tetrafluorophthalimide analogue, **15**, was 4-fold more potent. The (R) form of 3-methyl-thalidomide, **16**, is about 10-fold more active against TNF-α production in human monocytic cell lines than the (S) form or thalidomide itself (38).

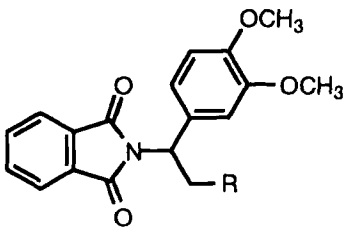


13: $R_1 = H$, $R_2 = H$
14: $R_1 = F$, $R_2 = H$
16: $R_1 = H$, $R_2 = CH_3$



15

Replacement of the glutarimide moiety of thalidomide with substituted phenyl rings has led to substantially more potent compounds, **17** and **18**, with IC50s against LPS-stimulated human monocytes of 12.5 and 2.7 μM, respectively; thalidomide in these experiments had an IC50 of 194 μM (39,40). Adding an amino group to the phthalimide ring of **18** further lowered the IC50 to 0.5 μM.

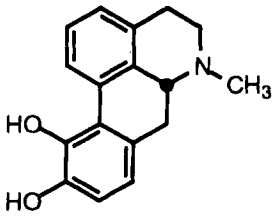


17: R = $CONH_2$
18: R = CO_2CH_3

Unlike thalidomide, which at best has a partial effect, these analogues completely inhibit TNF-α release (and reduce GM-CSF levels). As with the parent compound, they reduce TNF-α mRNA but do not affect release or mRNA levels of IL-1β or IL-6. The compounds reduce serum TNF-α in LPS-challenged mice; **17** is the most effective (nearly 90% reduction at 100 mg/kg), perhaps because it is more stable in serum than **18**. **17** protected mice from a lethal dose of LPS (80% survival).

Tetravalent Guanylhydrazone - This compound, developed as an inhibitor of arginine transport and nitric oxide production, reduces the production of TNF-α and other inflammatory cytokines (IL-1β, IL-6, MIP-1α, and MIP-1β) by LPS-stimulated human monocytes with an IC50 of less than 1 μM (41). Studies with a mouse monocytic cell line showed that the compound must be added prior to the LPS. It suppresses serum TNF-α levels in LPS-challenged mice with an IC50 of about 1 mg/kg, and it protects mice against carrageenan-induced inflammation and a lethal dose of endotoxin. Unlike glucocorticoids, the compound is effective in the presence of interferon-γ. It does not prevent NF-κB activation or nuclear translocation and hence does not significantly reduce TNF-α mRNA levels (42). Rather, it suppresses the translation of the message. The target has not been identified but could lie in the p38 MAP kinase pathway.

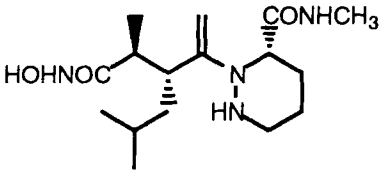
Antioxidants - Antioxidants have long been known to inhibit the activation of transcription factors, including AP-1 and NF-κB, required for the inducible expression of TNF-α and other inflammatory cytokines. A systematic survey of effects on TNF-α production revealed a wide range of potency among antioxidants with differing structures, from the lipophilic tetrahydropapaveroline (which inhibited TNF-α production from LPS-activated PBM cultures at 1-10 μM), to more hydrophilic compounds, such as ascorbic acid, which had little effect at 200 μM (43,44). In general, those antioxidants which inhibited TNF-α production were also effective inhibitors of IL-1 production, but had little effect on the induction of IL-6 synthesis. Treatment of lethally LPS-challenged mice with 100 mg/kg 10,11-dihydroxyaporphine, **19**, one of the more potent antioxidants tested in this survey, inhibited TNF-α production by 95% if administered 30 minutes prior to challenge.



19

INHIBITORS OF TNF-α RELEASE

The enzyme that releases TNF-α from cells is in the ADAM family of metalloproteinases, which is closely related to the matrix metalloproteinases (MMPs) (6,7). The role of TACE in generating soluble TNF-α has been confirmed by genetic inactivation (6). Broad-spectrum, peptide-hydroxamate inhibitors of the matrix metalloproteinases inhibit TACE (see 15 for review), and compounds utilizing an α-mercaptoamide group for chelation may also be effective (45). One nonpeptidic hydroxamate, **20**, has been reported to inhibit TNF-α production by human whole blood, with an IC50 of 10.5 μM (46). This compound is orally available, with an ED50 in LPS-challenged mice of 30 mg/kg. Like the peptide-based compounds, **20** is a potent inhibitor of several MMPs. No selective inhibitors of TACE have been reported, but its substrate specificity, though not completely defined, is different from those of the MMPs with which it has been compared (47).



20

The validity and feasibility of targeting TACE therapeutically remain to be determined. One potential pitfall is that several of the hydroxamate-based inhibitors described to date block the release of both TNF-α and various soluble receptors, including the p75 TNFR and L-selectin (48). Indeed, no compounds have been reported to inhibit TNF-α release and not these receptor shedding events, suggesting that the same enzyme may be involved. The physiological significance of receptor shedding, and hence the possible effects of blocking it, are not clear. It remains unclear if compounds that selectively inhibit TACE but not MMPs can be developed. Although broad-spectrum inhibitors may be desirable in treating some inflammatory diseases, it is also likely that they will cause unnecessary side-effects. Finally, inhibitors of TACE will not eliminate membrane-bound TNF-α, the physiological role of which remains uncertain. Indeed, these compounds cause a modest increase in cell-associated TNF-α *in vitro* (49,50).

INHIBITION OF TNF RECEPTOR OCCUPANCY

Monoclonal antibodies to TNF-α and soluble forms of the TNF receptors have been used to block binding of TNF-α to cell-surface receptors (for a recent review, see 51). In pre-clinical studies, monoclonal antibodies demonstrated a therapeutic effect in experimental allergic encephalomyelitis (52) and reversed the cachexia that afflicts transgenic mice constitutively expressing human TNF-α (53). Multimeric fusion proteins consisting of two TNFRs, either p55 or p75, linked by the Fc region of human IgG or by a bifunctional PEG molecule, have shown benefit in various pre-clinical disease models, including endotoxemia (54-56), collagen-induced arthritis (57), and bone resorption in ovariectomized mice (58).

In clinical trials with patients suffering from sepsis or septic shock, the monoclonal antibody MAK 195F showed benefit in the subpopulation with baseline serum IL-6 concentrations of > 1000 pg/ml (59), and a p55 TNFR-IgG1 fusion protein improved the outcome for patients with severe sepsis (60). A third study in sepsis, utilizing a different soluble receptor construct, showed no benefit (61). In a small study involving patients with active Crohn's disease treated with the monoclonal antibody cA2, eight of the 10 participants showed normalization of Crohn's Disease Activity Index scores and healing of ulcerations (62). Clinical trials utilizing four different protein antagonists, two monoclonal antibodies (63,64) and two TNFR-fusion constructs (65,66), have demonstrated significant clinical benefit in rheumatoid arthritis patients. Treatment was restricted to the most severely affected patients who had failed conventional therapy with DMARDs (Disease Modifying AntiRheumatic Drugs). In this population, the TNF-α binding proteins caused an improvement in several clinical parameters, including the number of swollen joints, tender joints, morning stiffness, erythrocyte sedimentation rate and C-reactive protein.

There are significant differences among the various protein antagonists of TNF-α binding. The antibodies must be humanized to avoid antigenicity. Most of the anti-human TNF-α monoclonal antibodies bind only TNF-α, whereas the TNFR fusion proteins neutralize both TNF-α and lymphotoxin-α. The equilibrium binding affinities of the various protein antagonists for TNF-α are similar ($K_i = \sim 1 \times 10^{10}$) (67). However, the kinetic stability of the complexes of TNF-α and the soluble receptor constructs varies. Thus, the on and off rates are faster with the p75 TNFR than with the p55 TNFR (68).

CONCLUSION

The therapeutic utility of TNF-neutralizing agents has been proven in studies with specific TNF-α-binding proteins. It remains to be seen if small molecule antagonists, which thus far are less selective, can offer therapeutic advantages. Many of the compounds discussed in this review, including the CSAIDs, affect the expression or signaling of multiple cytokines. Blocking IL-1 and IL-6 as well as TNF-α may increase a compound's anti-inflammatory potential, or it may generate deleterious side-effects. Other blockers of TNF-α production, such as the adenosine receptor agonists, the phosphodiesterase inhibitors, and thalidomide derivatives, do not affect other inflammatory cytokines. Inhibitors of TACE should also be relatively specific in terms of cytokines affected but may alter the levels of surface and soluble receptors and possibly the activity of matrix metalloproteinases. Only *in vivo* experiments will indicate which spectrum of effects is most desirable for the treatment of inflammatory diseases.

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Chapter 25. Nuclear Orphan Receptors: Scientific Progress and Therapeutic Opportunities*

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Introduction - The nuclear receptors are a superfamily of ligand-gated transcription factors that are regulated, in many cases by the binding of small lipid-soluble ligands (1-4). This superfamily of proteins includes the known receptors for steroid and thyroid hormones, vitamin A, and vitamin D, as well as a large number of newly discovered orphan receptors for which ligand activators are initially unknown. Since their first identification in 1988, over thirty different vertebrate orphan receptors have been characterized (5). These discoveries have revealed the existence of previously unknown hormone signaling pathways and created new paradigms to study transcriptional regulation of gene expression (6). Because many of these orphan receptors are also associated with metabolic and inherited disorders, they are logical targets for development of improved medicines to treat a variety of endocrine and metabolic diseases.

Steroid receptors, including receptors for estrogens, androgens, mineralocorticoids, glucocorticoids, and progestins, bind to ligands that are biosynthetically derived from pregnenolone and share similar mechanisms of action (7-9). In the absence of ligand, steroid receptors form quaternary complexes with heat shock proteins that preclude their interaction with DNA. Binding of a hormone or drug induces a conformational change in the receptor that, following liberation of heat shock proteins, permits the ligand-receptor complex to bind to its cognate response element and interact with a variety of co-activation proteins; this completes formation of multiprotein complexes that regulate gene transcription (10).

The most widely studied nonsteroid nuclear receptors include vitamin D receptor (VDR), thyroid hormone receptors (TRs), and retinoic acid receptors (RARs). In contrast to the steroid receptors, the non-steroid nuclear receptors do not associate with heat shock proteins and appear to bind their cognate DNA response elements in the absence of ligand, and cause transcriptional repression or activation in the basal, un-liganded state (11,12). In absence of ligand, the nuclear receptor is often associated with co-repressor proteins that effectively inhibit basal transcription of target genes (13,14). Formation of the ligand-receptor complex triggers a conformational change that inhibits interaction of the receptor with co-repressor proteins and restores basal transactivation. Moreover, this ligand-altered conformation is poised to interact with co-activator proteins that facilitate transcription of specific target genes (10).

Major scientific advances in how non-peptide hormones exert their powerful effects have significantly increased the molecular understanding of hormone signal transduction, and provide important opportunities to create novel medicines which

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mimic or block specific hormones. These opportunities utilizing "classical" or non-orphan nuclear receptors have been reviewed recently in this series (15-18) and elsewhere (4). Recent discoveries involving the classical nuclear receptors, including hitherto unknown subtypes such as ERβ (19, 20), differentially modulatable transactivation domains (21, 22) and co-activator/co-repressor proteins (13, 14, 23) suggest that design of novel medicines using these proven drug targets is still in its infancy. However, the objective of this chapter will be to highlight recent biological discoveries in the field of nuclear orphan receptors, with particular emphasis on examples of novel pharmacologically active compounds that regulate these transcription factors.

Discovery of Orphan Receptors - Nuclear receptors for lipid-soluble hormones such as sex steroids, thyroid hormone, vitamin D, mineralocorticoids and glucocorticoids were discovered by classical biochemical means using radiolabeled hormones to identify their cognate receptors. Application of molecular biology techniques led to molecular definition of these nuclear receptors, and the realization that they represented a superfamily of proteins that transduced signals for a structurally diverse array of nonpeptide hormones (24-26). Thereafter, screening of cDNA libraries *via* low stringency hybridization revealed a panoply of structurally related proteins, but with no known signaling hormone or ligand; this absence of a ligand led to the term nuclear orphan receptors. Almost 60 nuclear orphan receptors have been discovered, to date, and almost half of these have been found in vertebrates (5,6). Delineation of the physiologic function of these receptors has proven to be a daunting challenge, but pharmacologic exploitation of several nuclear orphan receptors (*vide infra*) has revealed novel strategies for drug discovery.

Table 1 lists a representative subset of nuclear orphan receptors which are found in vertebrates, known subtypes of these receptors, and lead references. Virtually all these nuclear orphan receptors have been identified in human tissues, underscoring their physiologic significance and possible therapeutic importance. Many of these receptors exist as multiple subtypes which represent the product of discrete individual genes. These receptor subtypes often manifest distinct but overlapping patterns of expression, and because the family of target genes they regulate are often different, these receptor subtypes represent logical avenues for design of medicines with selective therapeutic effects. The multiplicity of retinoid receptor subtypes, and the impact of receptor subtypes on drug discovery in this superfamily has been reviewed (18).

Structural and Mechanistic Biology - All the nuclear receptors are structurally similar and share certain functional similarities (1). As depicted in Figure 1, their common molecular architecture has a trio of motifs that enable: 1) ligand binding; 2) interaction with DNA control regions in promoters of hormone regulated genes; and 3) transcriptional activation. The C-terminal portion is responsible for ligand binding and receptor dimerization. In most nuclear receptors, this region contains a transactivation domain (AF-2) which is ligand-dependent and highly conserved. The central DNA binding domain is highly conserved and contains two zinc fingers that make critical contacts with specific nucleotide sequences typically termed hormone response elements. The N-terminal region typically contains an activation function (AF-1) that modulates transcription independently of ligand binding; this region, in part because of the diversity of genes regulated by these receptors, is not well conserved (6).

Table 1. Representative Nuclear Orphan Receptors

Receptor	Subtypes	References
COUP-TF	α,β,γ	27
DAX-1	none	28
ERR	α,β	29
FXR	none	30
GCNF	none	31
HNF-4	none	32
LXR	α,β	11, 33
MB 67	none	34
NGFI-B	α,β	35, 36
ONR1	none	37
PPAR	α,β,γ	38, 39
REV-ERB	α,β	40, 41
ROR	α,β,γ	42-44
RXR	α,β,γ	45
SF-1	none	46
SHP	none	47
TLX	none	48
TR2	α,β	49, 50

Most nuclear orphan receptors contain the same functional domains as the classical nuclear receptors (6). Steroid receptors bind to DNA exclusively as homodimers on response elements arranged as palindromic repeats of two consensus hexanucleotide half-sites separated by three spacer nucleotides (Figure 2). The classical nonsteroid receptors modulate transcription by binding as heterodimers with RXRs to response elements that are direct repeats (DRs) or inverted repeats of two consensus half-sites separated by a variable number of nucleotides (51,52). Most orphan receptors bind as hetero- or homo-dimers to direct repeats of the consensus sequence AGGTCA (6). Response element specificity, and thus patterns of differentially regulated gene expression, is determined largely by the number of nucleotides (n) between each repeat, and for most orphan receptors and non-steroid receptors, n = 0 - 5 (53).

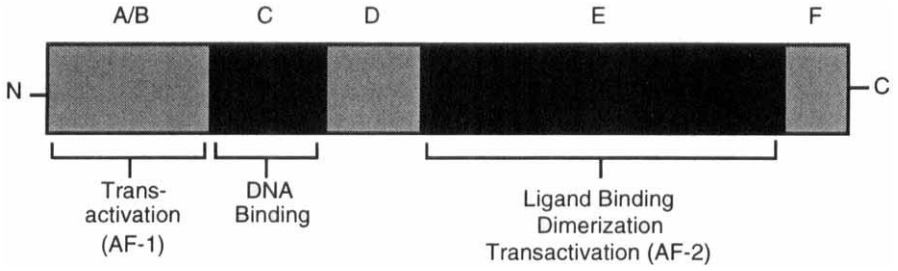


Figure 1. Structure of nuclear orphan receptors. See text for description of different receptor domains.

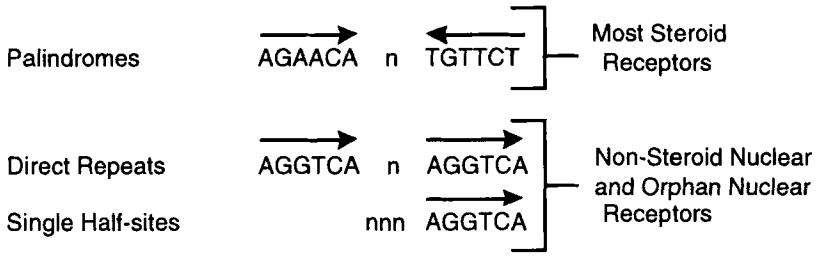
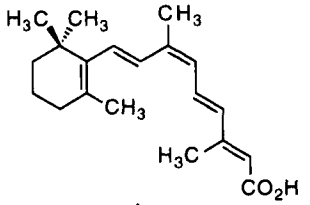
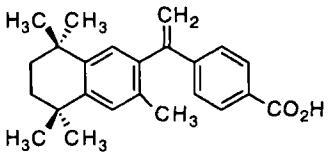


Figure 2. Sequences of consensus DNA binding sites (response elements) for nuclear orphan receptors compared to steroid and nonsteroid nuclear receptors. Response elements for most steroid receptors are palindromic whereas most orphan receptors, like nonsteroid nuclear receptors, bind to direct repeats.

Ligands have been disclosed recently for several nuclear receptor families that were originally described as orphans: RXRs, PPARs, LXR, FXR, and SF-1. Some of the most recent discoveries, and ligands, will be summarized herein to delineate the avenues used in discovery of nuclear orphan receptor ligands as potential therapeutic agents; more exhaustive reviews on nuclear orphan receptors are available (5,6).



1



2

Retinoid X Receptors (RXR) - In mammals, the RXR family of receptors contains three subtypes, RXRα, RXRβ, and RXRγ, and these are present in most cell and tissue types, but have distinct patterns of expression (45,54,55). Although RXRs can be activated by retinoids and share limited identity with RARs, they do not bind the endogenous RAR ligand, all-*trans*-retinoic acid. Instead, RXRs bind with high affinity the unique retinoid metabolite, 9-*cis*-retinoic acid (56, 57). This discovery produced an important probe for understanding the complex mechanism of retinoid action in physiology. Moreover, it spawned use of 9-*cis*-retinoic acid (**1**) as a drug candidate: This compound was active in several preclinical tumor models, and enhanced tumor sensitivity to *cis*-platin in a human oral squamous cell carcinoma xenograft model (58). Recently reported clinical studies suggest 9-*cis*-retinoic acid is effective as topical therapy in treating Kaposi's sarcoma lesions in about one-third of AIDS patients, and induces complete remission after oral administration to patients with acute promyelocytic leukemia; the drug is being studied in a variety of additional indications (59-62).

Since 9-*cis*-retinoic acid can also bind to and activate the RARs (56), a significant effort has been mounted to identify agents that are selective ligands for the RXRs. This resulted in synthesis and characterization of LGD1069 (**2**, Targretin™) which is a potent RXR ligand that displays over 50-fold selectivity for RXRs over RARs (63). 9-*cis*-Retinoic acid and **2** have been used as pharmacological tools to demonstrate the differences in biological effects mediated *via* RARs versus RXRs, and to elucidate the crucial role of RXRs in modulation of apoptosis (64, 65). Administration of **2** to rats bearing NMU-induced mammary tumors produced a 90% reduction in tumor

burden and tumor incidence compared to control animals; and the efficacy was similar to that achieved with tamoxifen (66). Compound **2** is the first RXR selective compound to reach clinical studies and the compound appears to have efficacy in treating mycosis fungoides (cutaneous T-cell lymphoma, CTCL) following topical administration (67-69).

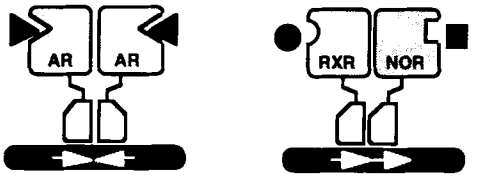
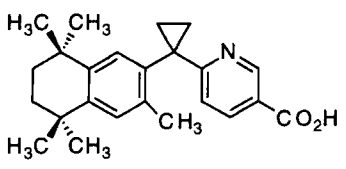
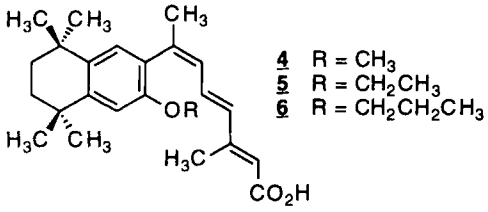


Figure 3. Representation of nuclear receptor dimers and their interaction with DNA. Steroid receptors (*e.g.*, androgen receptor, AR) form homodimers and bind to specific response elements. Most nonsteroid (e.g., TR, VDR, RAR) nuclear receptors, and nuclear orphan receptors (NOR) bind to their cognate response elements as heterodimers with RXR. Arrows in DNA represent directionality of response element sequences.

In addition to being key mediators of retinoid signaling, RXRs also serve as obligate heterodimeric partners to the nonsteroid receptors (see Figure 3) and many of the nuclear orphan receptors (9,70). To more fully understand these signaling pathways, it was important to have compounds with greater specificity than **2** (which activates RARs at high concentrations.) This led to identification of specific RXR ligands that can activate certain RXR heterodimer pairs. A congener of **2**, LG100268 (**3**), displays >1000-fold selectivity for the RXRs, and is useful for investigating RXR dependent pathways (71). LG100754 (**6**) was reported to be the first RXR homodimer antagonist and the SAR was highly specific; the methyl and ethyl analogs, **4** and **5**,



3



4 R = CH_3
5 R = CH_2CH_3
6 R = $CH_2CH_2CH_3$

were agonists in this RXR context (72). This dimer selective ligand **6** confers differential interactions on the transcription machinery and promotes association with TAF110 TATA-binding protein (TBP) associated factor 110 and the co-repressor SMRT (silencing mediator for retinoid and thyroid-hormone receptors), but not with TBP, and these properties are distinct from **3** and other pure RXR agonists (73). This unique class of RXR ligands appear to provide a means to control distinct nuclear orphan receptor target genes at the level of transcription. As can be seen from these studies, the discovery of RXRs, 9-*cis*-retinoic acid, and several highly selective RXR ligands has helped elucidate the critical involvement of RXRs in a myriad of developmental and physiological processes controlled by retinoid and other endocrine signaling pathways.

Peroxisome Proliferation Activated Receptor (PPAR) - The PPAR family of receptors consists of three known gene products that were named because of activation of PPARα by several classes of chemicals that cause a form of hepatomegaly that is

etiologically derived from peroxisomal proliferation. These chemicals include medicinally important compounds such as gemfibrizol and other fibrates (74). Although these clinically important medicines are relatively impotent activators of PPARα, this receptor does appear to mediate both the pharmacological effects of these drugs and their toxic effects on the liver; administration of fibrates to mice rendered devoid of PPARα via targeted gene disruption produces no peroxisome proliferation or hepatomegaly (75). Subsequently, it was discovered that arachidonic acid and other long-chain fatty acids can activate PPARα, and that the transcription of several key enzymes involved in fatty acid metabolism is regulated by PPARα (76). More recently, it was found that 8-*S*-HETE, LTB_4 and carbacyclin can activate PPARα, albeit at high concentrations, and it has been suggested that PPARα, via catabolism of these important mediators of inflammation, may serve as a negative feedback control in regulation of inflammation (77).

PPARγ is expressed highly in adipocytes and is believed to play roles in adipocyte differentiation and fat deposition. Recently this orphan receptor was found to be the transcriptional target for the thiazolidinediones (78,79), an important class of antidiabetic compounds which includes troglitazone and BRL49653 (7); troglitazone was launched recently in the United States for treatment of Type II diabetes. While these compounds have been optimized and developed using classic whole animal models of diabetes, discovery of the molecular target for these compounds should aid in design of more potent congeners, perhaps with altered pharmacological profiles.

Orphan Receptor Crosstalk: Application to Diabetes - In their regulation of gene expression, PPARs must form heterodimers with RXRs (*vide supra*). The crosstalk, or interaction between orphan receptor heterodimeric partners and response elements (Figure 3), is complex and ligand dependent. In some heterodimeric complexes, RXRs are silent partners, and while they facilitate the function of the other subunit, retinoid binding to the RXR partner is not obligatory, and in fact, may be precluded; this is the case, for example, in RXR/TR heterodimeric complexes wherein binding of thyroid hormone to the TR ligand-binding domain precludes 9-*cis*-retinoic acid binding to the RXR ligand-binding domain (80,81). In the case of RXR/RAR heterodimers, the RXR becomes responsive to 9-*cis*-retinoic acid only when RAR is in a ligand-activated state and addition of both RXR and RAR ligand may result in additive or synergistic effects 82,83). However, these RXR/RAR interactions are still being elucidated and other investigators have different observations (84,85). Finally, in some cases such as LXR, interaction of the heterodimeric partner converts RXR into a fully active receptor for RXR ligands. In summary, the heterodimeric complex can sometimes be activated by the RXR ligand; by a ligand for the heterodimeric partner; or by both subunits' ligands acting together in an additive or synergistic fashion (6,86).

Cells transfected with RXR:PPARγ respond to 7 and other PPARγ ligands (78,87), and cells transfected with RXR:LXR respond to LXR activators such as 22(*R*)-hydroxy cholesterol (86). Moreover, both RXR:PPARγ and RXR:LXR heterodimers are activated by 3, an RXR-specific ligand, in the absence of PPARγ or LXR ligands, respectively. In dramatic contradistinction, 3 does not activate RXR:RAR or RXR:TR heterodimers, although these complexes are activated by RAR-selective ligands or triiodothyronine (T_3), respectively (81,84,88). These results suggest that RXR:LXR and RXR:PPARγ heterodimeric complexes can be activated by ligands acting on either side of the heterodimeric partner, i.e. dual-ligand responsiveness, whereas in RXR:TR and RXR:RAR heterodimeric complexes, RXR is a silent partner, and these complexes can be activated only *via* the non-RXR receptor.

A recent study was conducted to more fully investigate the functional interactions between RXR and PPARγ. In CV-1 cells cotransfected with RXR:PPARγ, activation of the heterodimer complex with either **3** or **7** produced a concentration-dependent increase in activation, and the magnitude of activation was comparable regardless of which subunit of the heterodimeric complex was stimulated with ligand. At submaximal concentrations of either ligand, there was a synergistic response when the other ligand was added (88). These interactions were then evaluated in *db/db* and *ob/ob* mice, strains of genetically diabetic animals which respond to thiazolidinediones such as **7** or troglitizone (89). Daily treatment of these diabetic mice with **3** or **7** produced a coordinated decrease in both glucose and insulin concentrations in plasma, suggesting an overall improvement in glucose homeostasis. Combination treatment with **3** and **7** was more efficacious than either compound alone in improving glucose homeostosis or reducing serum triglycerides. These studies demonstrated that RXR agonists, like PPARγ agonists, can function as insulin sensitizers in two animal models of Type II diabetes. These data have obvious therapeutic implications, but in terms of the scientific ramifications of nuclear orphan receptor signaling, they demonstrate that RXR:PPARγ heterodimers respond well to ligands that bind avidly to either receptor subunit, and that the response to both ligands is synergistic. Moreover, the data suggest that the role of RXR, and its ability to respond to RXR-specific ligands is very dependent upon its biological (i.e., heterodimeric partner) context, and underscores the central role of RXR in regulation of many hormonal signaling pathways (3).

Additional Nuclear Orphan Receptors - Most known nuclear orphan are expressed in humans. Because of their recent identification, many of which were discovered simultaneously in multiple laboratories, nomenclature remains confusint and these orphan receptors often have multiple names in the literature. Discussion of each of these nuclear orphan receptors, their physiological role(s) and utilities as a potential vehicles for drug discovery is beyond the scope of this chapter. However, a few are worth noting briefly. FXR (farnesoid X-activated receptor) was cloned from a rat liver cDNA library and is most homologous with the *Drosophila* ecdysone receptor (90). Although the specific function of this receptor has not been delineated, it is activated by several metabolites in the mammalian mevalonate pathway, including farnesol. Several lines of evidence suggest that FXR, and other nuclear orphan receptors, may modulate gene expression under the regulation of intermediary metabolites in the mevalonate/cholesterol biosynthetic pathway.

LXRα and LXRβ form heterodimers with RXR and regulate transcription via DR-4 type response elements (11,91). The receptors are activated by oxysterols that are key intermediates in cholesterol metabolism (86,92). Recent studies demonstrated that 24(*S*),25-epoxycholesterol, 22(*R*)-hydroxycholesterol and 24(*S*)-hydroxy cholesterol were potent stereospecific activators of LXRα and LRBβ (86,92). A variety of data, taken together, suggest that these cholesterol metabolites function as LXR ligands and that LXR may regulate transcription of genes encoding enzymes in cholesterol signaling pathways.

HNF-4 (hepatocyte nuclear factor-4) binds to DR-1 type response elements and is highly expressed in liver where it appears to regulate lipid transport and metabolism (93), blood coagulation (94) and several other liver-centered homeostatic processes. HNF-4 is a positive regulator of transcription and because it is constitutively active, it may be activated *via* a ligand-independent pathway or by an endogenous ligand.

However, even if activation occurs *via* a ligand-independent pathway, it may be possible to modulate this receptor, and the processes it controls, *via* small-molecule drugs; analogous examples of pharmacology mediated through unanticipated protein-protein interactions would include cyclosporin A and its regulation of immune function *via* the immunophilins (95).

The NGFI-B (nerve growth factor induced) family of receptors appear to contain three distinct gene products which have been independently discovered and named by several laboratories (6). These nuclear orphan receptors are immediate-early response genes that are highly expressed in the CNS (96). NGFI-B may influence adrenocortical steroidogenesis (97), T-cell apoptosis (98,99), regulation of growth of certain tumors such as extraskeletal myxoid chondrosarcoma (100), and retinoid signaling.

SF-1 (steroidogenic factor-1) is a mammalian homolog of the *Drosophila* transcription factor FTZ-F1 (101), and this family of receptors appears to contain at least two distinct gene products in mammals. Like the LXR orphan receptors, SF-1 has recently been shown to be activated by oxysterols (46). The SF-1 activators show a different rank order of potency than those that activate the LXRs, suggesting an entire family of nuclear orphan receptors may exist for different oxysterols, each with its own unique regulatory pathway. Similar to the LXRs, which may regulate cholesterol conversion to bile acids, SF-1 is believed to play a major regulatory role in conversion of cholesterol to steroid hormones. Indeed, SF-1 binding sites have been discovered in the promoter regions of genes encoding many of the enzymes and proteins involved in development and maintenance of the hypothalamic-pituitary-adrenal axis (102). Identification of oxysterols as potential ligands for SF-1 should prompt further insight into the physiology of this orphan receptor and may permit discovery of medicines modulating steroid hormone synthesis.

Conclusions and Future Directions - In the last decade, nuclear orphan receptor research has had a major impact on the fields of molecular and cell biology, endocrine physiology, and pharmacology. The proven efficacy of drugs that modulate nuclear receptors; the increasingly large number of orphan receptors that are structurally and mechanistically related to known nuclear hormone receptors; and, finally, the successful exploitation of receptors previously known as orphans, augurs well for the future of this superfamily of receptors in drug discovery and development initiatives.

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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

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Chapter 26. Discovery and Identification of Lead Compounds from Combinatorial Mixtures

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Introduction - The search for new compounds with therapeutic activity often begins with the serendipitous discovery of an active lead compound in a large random compound library. Maximization of the number of compounds (and chemical variety or diversity) tested increases the probability of successful drug discovery. Advances in screening technology have led to a dramatic increase in throughput. The ultimate limitation is often the availability of new compounds that can be efficiently screened, particularly when the goal is to find the kinds of non-biooligomeric, low molecular weight organic compounds that are most desirable from a medicinal chemistry and pharmacological standpoint. The term "combinatorial chemistry" describes a relatively new collection of techniques and ideas, all generally aimed at producing very large sets of compounds for use in drug discovery. Several recent publications have reviewed a variety of methods in combinatorial chemistry and the screening of compounds synthesized by combinatorial chemistry (1-4). This chapter will review the more specific issues relating to screening mixtures of compounds that are produced by combinatorial synthesis.

Two particular synthetic methods in combinatorial chemistry produce mixtures of compounds. The now routine technique of randomly incorporating monomers into oligomeric libraries was first demonstrated nearly one decade ago for the synthesis of peptide (5) and oligonucleotide (6) libraries. This method, which will be referred to here as simply "random incorporation" produces mixtures in which a vast number of different compounds are synthesized in nearly equimolar amounts. When the synthesis is done on solid support, such as polystyrene beads, each product bead can contain millions to billions of different molecular species. The library mixtures are typically cleaved and released from the resin so that they can be analyzed or tested in solution. Split synthesis, on the other hand, results in "one compound per bead" because each bead is subjected to only one reaction pathway without mixed reactants. The split synthesis method, which has also been referred to as "mix and split" or "pool and divide" synthesis was also first published nearly one decade ago (7,8). Though each bead has a unique reaction history and ideally one synthetic product, split synthesis results in the production of large mixtures of beads, so that the identity of the product on each particular bead is unknown. The synthesis of mixtures of compounds by either of these methods is non-ideal from a variety of synthetic and biological testing perspectives but no other synthetic method can compare to these in the sheer numbers of compounds that can be produced quickly.

An ideal compound screening method, in theory, would quickly provide robust information on the activity of every compound tested while being able to handle very large numbers of compounds. Parallel screening of individual compounds in high density arrays (typically in 96- or 384-well microplate format) is commonly used with this ideal in mind. Combinatorial chemistry, however, has enabled chemists to synthesize many more compounds than have been traditionally screened with these methods, and these compounds are produced in mixtures. This has resulted in a compromise in which the vast number of compounds that can be synthesized as combinatorial mixtures are

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subjected to screening techniques that require deconvolution, decoding, or other specialized methods to identify those compounds that have a desired biological activity. The quality of the method of deconvolution or identification, in general, will determine whether this compromise is successful in increasing the rate of discovery of useful compounds. Screening small mixtures of compounds which are pooled post-synthesis to decrease the cost or increase the throughput of screening is fundamentally different from screening compounds that are synthesized as combinatorial mixtures. Combinatorial mixtures or pools can be very large, and one cannot simply use a pooling strategy to get back to the original discrete compounds.

There are three underlying challenges that can dramatically affect the screening of combinatorial mixtures. First, synthesizing orders of magnitude higher numbers of compounds cannot be readily accomplished without synthesizing correspondingly less quantity of each compound. Otherwise, the total mass of chemicals produced would be unwieldy. This results in very small quantities of each compound produced. For example, there is typically less than 1 nmol of compound synthesized per bead in split synthesis. Second, high combined mixture concentrations can lead to problems with solubility and nonspecific activity during screening. These problems effectively provide an upper limit to the total concentration that can be reasonably used in screening, which in turn sets an upper limit to the concentration of each compound screened. So, while there is very little of each compound synthesized, even less may be actually screened if the screening method relies on using vast mixtures in solution. Third, the signal from a few compounds that are very active in a particular assay can be overwhelmed by the signal from a high combined concentration of more weakly active compounds. An ideal screen for combinatorial mixtures, therefore, should consist of methods that can distinguish between the activity of highly active compounds and the combined activity of more abundant less active compounds, provide a measure of activity even though each compound may be at low concentration, and enable identification of the active compounds even though they are in mixtures in lower quantities than would normally be required for many analytical methods.

DISCOVERING ACTIVITY IN COMBINATORIAL MIXTURES

The first steps in screening combinatorial mixtures are similar to those in any biological screen. The compounds need to be tested in such a way as to eliminate the vast majority of them from further study, while providing information on the relatively few compounds that exhibit a desirable activity or affinity. Once a subset of compounds has been determined to have activity, then the challenge of identifying the structure of these compounds remains. In a screen of individual compounds from a classical archival library, the identification can be as trivial as noting which compound was supposed to be in a particular well of a particular microplate, retesting that compound for confirmation and better quantitation of activity, and verifying the compound structure with analytical methods. With combinatorial mixtures, the method used in the initial discovery of activity must take into account the subsequent challenge in identifying the molecular origins of that activity. For example, knowing that a mixture of 10,000 compounds can inhibit a particular enzyme with an apparent IC50 $< 1\ \mu M$ is intriguing, but this information alone does not simply or necessarily result in a researcher identifying an active compound for further development.

Microplate Activity Assays - Screening combinatorial libraries in 96- or 384-well microplate format activity assays still has the particular advantage that a variety of assays are amenable to this format, so that the biochemical, cellular, or ligand binding assays that are now routinely screened this way can have access to combinatorial libraries. Combinatorial library mixtures have been tested in microplates as mixtures of compounds as the first step toward identifying active individual library members. Many examples of successful use of this strategy have been reviewed (9). Perhaps the most important factor in this kind of screening is the concentration of each individual compound, which can be very low if there are many compounds in a mixture. This

concentration must be high enough to ensure that rare active compounds can be discovered in the screen. If the discovery of a compound with an IC50 = 1 μM would be considered important in the search for a new inhibitor, for example, then each compound should be present at a minimum concentration of 1 μM. Operationally, the total mixture concentration can only be set to a certain maximal level without having nonspecific activity and/or solubility problems, so it can be difficult to achieve adequately high concentrations of each compound if the mixtures are large. There are other potential pitfalls to using this method because the mixtures that have the highest activity may not always be the ones that contain the most highly active single compound in a library. Though these are important issues, the fact remains that screening a large number of combinatorial mixtures in high-throughput activity assays of this kind is a proven method for determining whether different mixtures contain any compounds with a measurable level of activity. And, if most mixtures tested do not exhibit activity, this can be a very cost and time effective method for narrowing the screening focus to a subset of a library that contains active compounds.

Combinatorial libraries made by split synthesis are mixtures that can be divided simply by separating individual beads, because each library bead contains the products of a unique reaction pathway. By dispensing single beads into the wells of a microplate and then releasing those compounds so that they can diffuse into solution, compounds can be screened in a microplate format high throughput screen, similar to any collection of unmixed compounds. This avoids some of the challenges of screening mixtures altogether, though the quantity of each compound can be low and the identification of compounds more difficult than in a traditional screening of a noncombinatorial library. In one published example, the enzyme carbonic anhydrase was screened by this method (10). In general, the first goal in screening libraries made by split synthesis is to isolate individual beads that contain active compounds. While the discovery of activity in split synthesis libraries by this method is straightforward, there are a few complications. First, to statistically ensure that at least one bead is tested for 95% of the compounds in a mixture, one must test at least 3 beads per compound. Given that this would provide a situation in which a significant number of compounds are tested on only one bead with no repeats and the fact that split synthesis will result in variable yields of products per bead, testing approximately 10 beads per compound is more robust but very costly compared to screens with non-combinatorial compounds (11). Second, many wells will exhibit activity in the primary screen due to various screening artifacts, as in any screen, and one cannot trivially assess which are more likely to be truly active because repeats are randomly spread throughout the screen and structure-activity information is unknown until the hits are all identified. Therefore, it is important to have methods for retesting the hits before identification to narrow the focus to those that are at least reproducibly active. This has been accomplished by chemistry that allows repetitive partial release of compounds for retesting (12). Another advantage of partial release and repetitive testing is that it enables a screen of 10-20 beads per well as mixtures to offset the high numbers of beads per compound that need to be tested. The individual beads in any active well can be retested to confirm the activity and to enable the isolation of active beads. However, partial release, or the alternative of full release followed by aliquoting for repeat testing, does have the unfortunate effect of further lowering the attainable concentration of each compound in the screen, which can already be very low from the limited load on each synthesis bead. Regardless of the precise methods chosen, mixtures of split synthesis compounds can be screened as individuals provided the amount of compound on each bead is sufficient to show activity in the assay (volume of standard 96-well assays can be too large to detect μM hits if smaller beads are used in the synthesis), and hits can be confirmed for activity prior to their identification.

Gel Diffusion Assays - Gel diffusion or gel permeation assays provide an interesting alternative to screening libraries made by split synthesis. The general method involves configuring an assay so that the components are in an agarose gel. Active compounds

released from a particular bead will cause a zone of activity or inhibition in the gel. A very large number of beads can be tested in one petri dish, for example, with each bead serving as a point source for a particular compound being tested. Beads can be spread in random or ordered arrays on top of or inside of the agar containing the other assay components. This has been demonstrated with cell based screens for G-protein coupled receptors (13,14), cytotoxicity (15), and enzyme assays using a fluorescence read-out (16,17). Using controlled partial release, once an active compound is observed, all of the beads in that area of the gel can be collected and retested at a lower spreading density to isolate the precise bead that released the active compound. For certain assays, the gel diffusion format has been shown to be an efficient alternative to microplate based screening, though in concept it is essentially the same in that the goal is to physically isolate individual beads from the combinatorial mixture that contain active compounds that can then be identified.

Affinity Selection - Affinity selection is a completely different approach to screening combinatorial mixtures that is useful for mixtures from split synthesis or random incorporation synthesis (18-24). In affinity selection, protein targets are used to purify those library compounds that have a sufficiently high affinity for the target away from the rest of the library mixture. The advantages of affinity selection as compared to activity assays (microplate or gel diffusion) are particularly relevant for combinatorial libraries. First, unlike activity assays, the stringency of the assay is determined by the target protein concentration, so that screening for weaker micromolar leads is possible even with very small quantities of each combinatorial compound. Second, by separating the highest affinity compounds from all the compounds with lower affinity, the procedure inherently addresses the problems of screening mixtures. Abundant more weakly binding compounds will not mask the more rare high affinity compounds the way they can in an activity assay with mixtures, particularly if the separation is achieved in a noncompetitive manner with the target protein in vast excess over each library compound (23,24). Affinity selection, therefore, can be an unusually robust procedure for finding high affinity individual compounds in very large mixtures. For random incorporation libraries, the only other option is the more complicated testing of activity of mixtures. However, affinity selection has its own unique challenges. First, compounds must be detected after the affinity purification, necessitating either high enough concentrations of compound to be detected by analytical methods, or labeling of every library compound to facilitate detection (25). Also, one needs to have purified targets available along with a method of separating the bound compounds from the remainder of the library. Once a mixture is known to include a compound with high affinity, the challenge of identifying that compound is still significant.

One other alternative screening method that has been demonstrated with smaller mixtures of compounds is to directly use electrospray ionization mass spectrometry to measure relative binding affinities to a target protein under competitive equilibrium conditions (26). Extending this to other kinds of small molecules and increasing the sensitivity to allow the use of larger mixtures would make this an interesting alternative because it is the only method in which the screening and identification of the compounds is completed in a single step. Currently, however, the requirements of high target purity and small mixtures of easily ionizable compounds is a major limitation.

IDENTIFYING ACTIVE COMPOUNDS IN COMBINATORIAL MIXTURES

Any of the aforementioned methods can result in the discovery of combinatorial mixtures or individual combinatorial synthesis beads that contain compounds of unknown identity that have apparent activity or affinity in an assay. The next issue to be dealt with is how to identify such compounds so that they can be examined further in a drug discovery research program.

Identification By Pooling Strategies - Regardless of the format of a screen (microplate, gel diffusion, or affinity selection), the one method that is always an option for identifying discrete active compounds from combinatorial mixtures is indirect identification by screening pools or subsets. By dividing a library into a number of nonoverlapping subsets, one can narrow the possibilities down to facilitate identification of active compounds. It is common to synthesize library subsets that differ in the choice of one monomer or chemical subunit incorporated into one chemical position. The precise pooling strategy can vary as libraries can be divided into subsets in a variety of useful ways. Recent publications have compared the probabilities of successful deconvolution using different pooling strategies with a theoretical model (27) and with experimental data (28). The two most common strategies are the use of iterative fixed position subpools (29) and positional scanning libraries (9).

Iterative subpooling involves successively choosing the best chemical subunit at each position by comparing the activity of subpools differing only at that position. After each subunit is chosen in this manner, a new set of subpools is synthesized with all previously chosen subunits fixed, until one is finally comparing individual compounds. Therefore, this method of deconvolution requires iterations of chemical synthesis and testing. Positional scanning is an alternative method that allows all of the chemical synthesis to be completed before all of the testing. Positional scanning is essentially a deductive method in which each chemical subunit at each variable chemical position is specified in a separate subpool. For example, if a 1000 member library has 3 positions that each have 10 incorporated subunits, the positional scanning library would consist of 3 sets of 10 separate subpools, each having one subunit in only one position fixed. By screening each of these subpools, one can deduce that the most active compounds are likely to be those that have all 3 subunits specified by the most active subpools for each of the 3 sets. When subpools are compared in microplate activity assays, these methods can fail to identify the most active compounds if more abundant weakly active compounds mask the activity from the less abundant more strongly active compounds. Even successful examples in which active compounds have been discovered and reported clearly demonstrate this problem in that the compounds ultimately reported do not reside in the mixtures that initially show strongest activity. A clear example of this was demonstrated in an iterative deconvolution of a peptide ligand for an opioid receptor from a hexameric peptide library (29). Though this is unfortunate, there is no other method of identification or deconvolution possible for random incorporation libraries screened in activity assays.

Mixtures that are shown to contain novel ligands by affinity selection can be solved with either a positional scanning or iterative fixed position strategy without this problem, because the selection itself eliminates the effects of the more weakly binding compounds (22). The combination of affinity selection and positional scanning can provide structure-activity relationship data that is not obtained in some of the other identification methods described below. In an example using a positional scanning approach in combination with a solution-phase membrane filtration affinity selection procedure, a full structure-activity relationship was elucidated for each position of pentameric peptide binding to an anti-β-endorphin antibody (23). Though costly in terms of synthesis, these indirect identification methods require no technology other than that necessary for the initial library synthesis and screen since the identification problem is essentially solved by the collection of additional screening data. If, instead, a library is to be synthesized with minimal cost and screened without further need for synthesis, then pooling strategies, in general, are not appropriate methods of identification. For screening random incorporation libraries, the only alternative to deconvolution by a pooling strategy is direct analytical identification of affinity selection purified compounds. Mass spectrometry has been used for this purpose, and this procedure, called Affinity Selection/Mass Spectrometry (AS/MS) has been shown to be an efficient method for the identification of novel ligands from combinatorial libraries (21,24,30). Fortunately, other options exist for identifying active compounds from split synthesis

libraries. However, as was recently noted (31), a common method in screening split synthesis libraries is to keep the pools used in the final variable step of synthesis separate, so that the final chemical subunit is identified merely by the identify of the pool in which it resides (31). This is, of course, a use of subpool screening that happens to arise naturally from the split synthesis method.

Identification From Split Synthesis Beads - The observation of activity in an initial screen of a split synthesis library can lead to the isolation of individual library beads, and this enables a variety of options for identifying the active compounds that were synthesized on the isolated beads. First, the compound on a particular library bead can be identified by direct analytical methods, provided there is enough compound remaining on the bead after the assay for these methods and that the quality of the synthesis results in a pure enough product on the bead. Mass spectrometry has been widely used recently to analyze the products on combinatorial beads (32,33). Advances in spectroscopic techniques such as nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR) have also been published recently in pursuit of additional methods to identify compounds on single split synthesis beads (34,35). Another option available to split synthesis libraries is the coding of each library bead (36). The reaction history of every bead can be coded during synthesis and then decoded after the compound on a particular bead is shown to be active. This has been implemented with several different chemical methods, recently reviewed in detail (12). Early coding methods were demonstrated with oligonucleotides (37) or peptides (38) being used as codes for combinatorial libraries so that sequencing the oligonucleotide or peptide on a bead would unambiguously identify a corresponding library compound. An interesting recent publication describes an enzyme-mediated spatial separation of peptide codes, which are confined to the interior of the library beads, from the peptide library members, which are on the surface of the beads (39). Peptides and oligonucleotides, however, are not ideal codes from a chemical perspective, as compatibility between the library synthesis and code synthesis is required. Examples have been published for codes that are more chemically inert and therefore more useful for the widening range of chemistries that are being used on solid support. In one case, haloaromatic codes are attached to library beads using a binary coding scheme (40,41). Once a library bead is chosen for identification, the codes are read by electron capture gas chromatography. An alternative approach uses secondary amines as codes (42,43). These codes are read by removal of the amines from the chosen beads, dansylation, and HPLC analysis. Coding, as opposed to direct analytical identification, has the significant advantage of not requiring that any compound be left on a bead subsequent to the initial screening. Codes also identify a particular reaction history, so that even unforeseen chemical products that are always a potential threat to deconvolution can eventually be tracked down.

Summary - Choosing from different methods of screening and identifying active compounds from combinatorial mixtures depends on a comparison of the costs and benefits of resynthesizing, retesting, coding, and using direct analytical chemistry. Relying on resynthesis and retesting of library subsets and eventually individual compounds can take considerable time between the initial observation of activity in a mixture and the identification of the compounds responsible for the activity, but the process is relatively simple and does not require specialized technology for compound identification. Relying on coding of split synthesis libraries raises issues of chemical compatibility between the code and library in the library design and increases the cost of the initial library synthesis, but once these issues are addressed, lead compounds can be identified rapidly after their initial discovery. Finally, relying on direct analytical identification may be the very cost and time effective for the initial synthesis and ultimate identification, but these methods are limited by the sensitivity and accuracy of the analytical methods. These limitations make this particular approach more useful for smaller libraries with fewer compounds but higher quantity and purity, and the success will vary depending on the chemistry of each particular library. Given the growing interest in the use of combinatorial chemistry, it is likely that each of these methods will

continue to be successfully used in the future, enabling the use of large combinatorial mixtures in the search for new therapeutic lead compounds.

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Chapter 27. Electrospray Mass Spectrometric Characterization of Adducts Between Therapeutic Agents and Proteins

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Introduction - The formation of covalent adducts between proteins and therapeutic agents can provide a basis for toxicity, inactivation, and drug resistance *in vivo*; and often constitutes the central phenomenon in mechanism-based inhibition. Reversible adduct formation with proteins can play an important role in drug distribution, can form the basis for new drug delivery systems, and will contribute to pharmacokinetic profiles. The synthesis of drug-protein adducts is an important step in the development of antibodies and imaging agents. In view of the multiple roles of covalent bonding, it is important to develop appropriate analytical techniques to allow researchers to understand the chemistries of bond formation, including the roles of specific and nonspecific binding. With the recent rapid development of electrospray (ES) and matrix assisted laser desorption ionization (MALDI), mass spectrometry (MS) can now be applied readily to analysis of proteins and covalent adducts of proteins with xenobiotics, including drugs. In electrospray mass spectrometry the sample is introduced in solution, sprayed across an electrical field in such a way that protonated or deprotonated molecules are freed from solvent and steered into the mass analyzer (1-3). These solutions are aqueous or mixtures of water and organic solvents, contain volatile electrolytes, and may be used at any pH (4). Electrospray is the technique of choice to interface HPLC (for example, 5) and capillary electrophoresis (for example, 6) to mass spectrometers and it has been implemented on all analyzer configurations, including magnetic sector and Fourier transform instruments.

Multiple protonation or deprotonation occurs in larger molecules, and usually an envelope of ions with a range of charge states (m/z values) is recorded in the mass spectrum. The technique does not usually generate fragment ions unless special conditions are used. This is advantageous when molecular masses are to be determined for novel drug-protein adducts or stochiometric distributions. Samples are ionized in this way with minimal manipulation, little degradation, and few artifacts. It should also be pointed out that proteins as a class are particularly favorable samples for electrospray, generally being detected with sensitivity in the picomole range and mass accuracy in the range of 0.01 %. No theoretical limit exists on the mass range of the ionization technique, however most applications thus far have been to proteins under 100,000 daltons.

MOLECULAR MASS DETERMINATIONS

Molecular mass determinations by electrospray mass spectrometry can reveal the stochiometries of drug-protein reactions (7-10). Good examples of analyses where this is important include the synthesis of hapten-protein conjugates and of drug-protein adducts intended for drug delivery (11-14). In studies of haptenconjugates with bovine serum albumin, for example, Adamczyk et al. found that up to twenty-five hapten molecules could be detected per protein molecule (13). They argue that superior resolution of the stochiometric distribution of adducts, including

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minor components, is provided by the combination of HPLC and electrospray, and point out the serious limitations of SDS-polyacrylamide electrophoresis.

Molecular mass determinations also provide information about what chemical elements of a drug are retained and eliminated in reacting with a protein (15-17). For example, isobutyrylation of thrombin was deduced from the increase in molecular weight of α-thrombin incubated with the inhibitor methyl 3-(2-methyl-1-oxopropoxy)[1]benzothieno[3,2-b]furan-2-carboxylate (18), and phosphonamidates were shown to phosphonylate the active site serine of β-lactamase with cleavage of the phosphonamidate bond (19). Inhibition of leucocyte elastase by the trans-4-(ethoxycarbonyl)-3-ethyl-1-[(4-nitrophenyl)sulfonyl]-azetidin-3-one, on the other hand, was found to occur with addition of the mass of the entire β-lactam molecule (20). In a toxicologic application, the mass shift or mass addition for each molecule of 4-hydroxy-nonenal covalently bonded to hemoglobin or β-lactoglobulin revealed that Michael addition is the major mechanism rather than Schiff base formation (21, 22). Other changes in the protein structure, e.g., oxidation of thiol groups (15), disulfide bond formation (23,24), or the loss of metal ions from metalloproteins, will also be reflected in the molecular mass.

One important outcome of the capability to sample solutions directly is the detection of unstable reaction intermediates. Early workers used electrospray to confirm the expected acyl enzyme intermediates in β-lactamase catalysis (25) and serine protease catalysis (26), based on increments in the molecular masses of the proteins. A covalent enzyme-substrate complex has been reported for nitrilase from Rhodococcus (27). An imine intermediate has been observed in the conversion of 3-dehydroquinate to 3-dehydroshikimate by dehydroquinase (28), confirming earlier trapping studies. Four intermediates were detected in the inhibition reaction of the therapeutic agent clavulinic acid with TEM-2B-lactamase (29). The relative abundances of the molecular masses of these intermediates were recorded as the reaction proceeded, plotted as a function of time and interpreted to support a mechanism whereby the reaction is initiated by acylation at serine-70 (29). Electrospray was also able to provide rate constants for some deacylation reactions by TEM-1 (30).

Oxidation of the zinc-binding nucleocapsid protein from HIV leads to final protein products in which zinc cations are lost and internal disulfide bonds are formed (23,24,31). Studies with electrospray ionization indicate that these reactions proceed through several intermediates (23,24). Figure 1 records the molecular ions of the protein species present in the reaction at pH 7.4 of dipyridinyl disulfide (as a prototype drug) with the nucleocapsid zinc finger at t = 2.5 and t = 26 min (24). Charge states were detected ranging from +4 to +6. These peaks are identified based on their molecular masses, as A , the starting material coordinating two zinc dications; B, the nucleocapsid protein bonded to one molecule of thiopyridine and missing one zinc dication; C, the protein bonded to two molecules of thiopyridine and fully demetallated; D, the apoprotein containing three disulfide bonds. Methylation of zinc fingers like this can occur without loss of the metal cation (32).

It should be pointed out that electrospray mass spectrometry can also characterize the masses of some noncovalent complexes. Although such measurements need to be made carefully with appropriate control experiments, one compelling application is to the analysis of ligand interactions of combinatorial libraries (33-37).

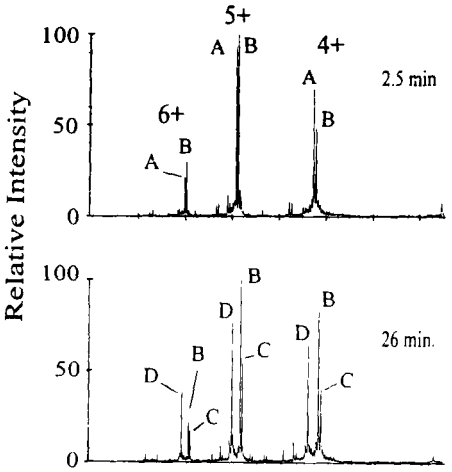

Figure 1.Electrospray mass spectra obtained by monitoring the reaction between 2,2'-dithiodipyridine with a nucleocapsid zinc finger protein on line, a0 t=2.5 min. b) t= 26 min. Protein ions with three charge states +4 to +6 were recorded and correspond to starting material, intermediates and products as explained in the text. Reprinted with permission from The American Society for Pharmacology and Experimental Therapeutics

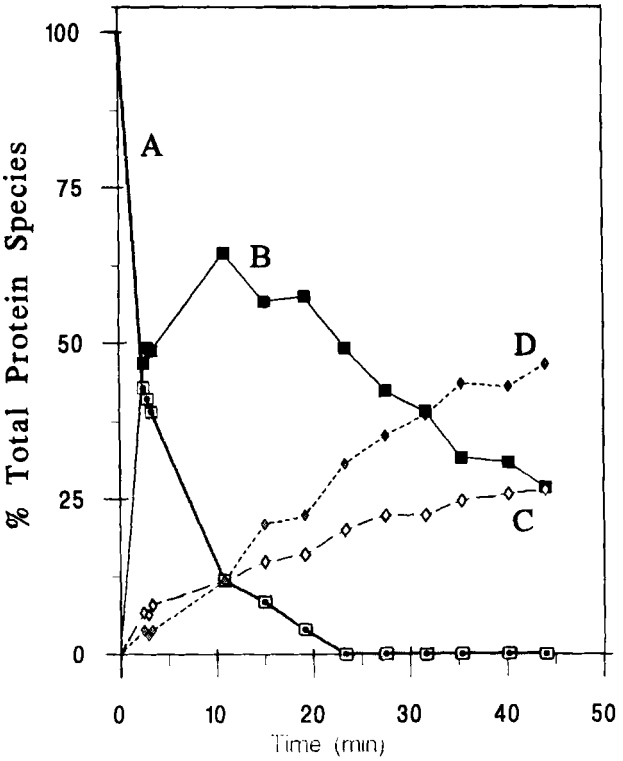

Figure 2. Abundances of Protein ions during the reaction of 2,2'-dithiodipyridine with a nucleocapsid zinc finger protein monitored on-line by electrospray ionization mass spectrometry. Reprinted with permission from The American Society for Pharmacology and Experimental Therapeutics.

REACTION MONITORING

As pointed out above for the case of clavulinic acid, electrospray also offers the possibility of monitoring reactions continuously. Figure 2 shows reaction profiles constructed from ion intensities in spectra obtained continuously as the reaction took place in a syringe attached to the electrospray ionization chamber on a double focusing sector mass spectrometer (24). Curves A to D represent the same protein species characterized in Figure 1. It can be seen that the starting material (holo protein) decreases quickly as a percentage of total protein species. The intermediate with one zinc center oxidized (B) rises and then falls as it is converted to the final oxidized protein D. Species C appears to be an oxidized and demetallated secondary reaction product that is not converted to D. This reaction profile tells us that the two zinc centers are oxidized one at a time, and that each zinc coordination center is disrupted by attachment of a thiopyridine moiety. Additional experiments revealed that the C-terminus zinc finger is oxidized first, apparently on the basis of kinetic accessibility (24).

Metallothionein provides a contrasting example, in which covalent bonds are formed to deactivate one and two equivalents of antineoplastic mustards without loss of metal ions from the two protein clusters (38). Figure 3 shows electrospray spectra (4+ charge state) of the protein species in the in vitro reaction (pH 7.4) between chlorambucil and rabbit liver metallothionein IIA recorded at t = 3 and t = 60 min. The families of molecular ions in the spectra derive from the presence of different combinations of zinc and cadmium ions among the seven divalent metals complexed by this sample of metallothionein. In the spectrum of the reaction mixture after 60 minutes, increases in mass show attachment of both one and two equivalents of drug without loss of metal ions. Figure 4 shows a kinetic curve constructed from protein ion intensities in spectra obtained continuously as the reaction proceeds (38). The apparent first order kinetic behavior is consistent with other evidence that alkylation occurs within a drug/protein complex formed at a selective binding site on the protein.

DEFINING REACTION SITES

Most researchers have also been interested in defining the sites of covalent drug attachment in proteins and other biopolymers. This is usually approached by hydrolyzing the protein using site-selective enzymes and/or chemical reagents, mapping the resulting peptides to detect modifications, and sequencing the peptides of interest. This general strategy has been as effective for drug-modified proteins, as it has been historically for proteins and post-translationally modified proteins. Several variations of LC-ES-MS have been used to compare peptide maps from a drug-protein adducts with those from unreacted protein. The protein sequence usually is already known in these studies. In analysis of the covalent bonding of α-thrombin by a selective inhibitor, mass maps were reported to identify peptides not resolved in maps based on chromatographic retention times (18). If the structure of the attached drug and a reliable fragmentation pattern are already known, overlapping chromatographic peaks can also be interrogated by tandem mass spectrometry techniques such as constant neutral loss scans (39,40) and precursor ion scans (41). If the masses of both the attached drug and the proteolytic peptides can be predicted, selected mass chromatograms may be reconstructed which trace the intensities of expected masses throughout the chromatographic separation (42,43).

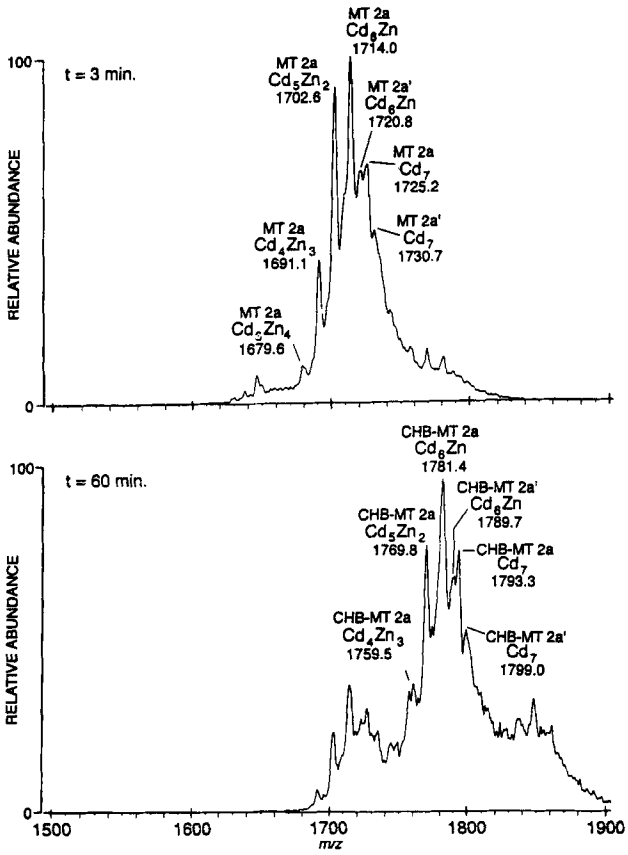

FIGURE 3. ESI MS spectra obtained by monitoring the reaction between MT and CHB on line: (a) t = 3 min; (b) t = 60 min. All ions are in the 4+ charge state and correspond to rabbit metallothionein IIA or IIA' with various cadmium/zinc metal ion compositions. Reprinted with permission from The American Chemical Society.

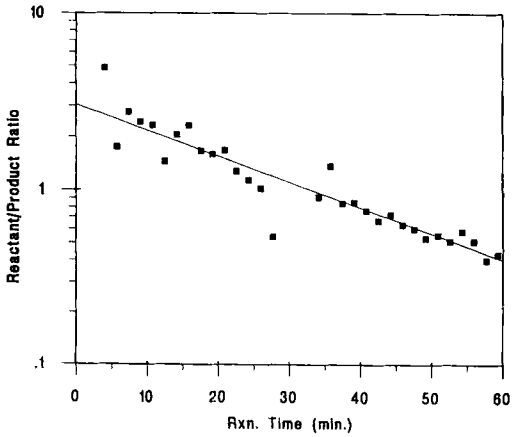

FIGURE 4. Product formation for the reaction between rabbit metallothionein and chlorambucil monitored on-line by electrospray ionization mass spectrometry. Each point is the quotient of the reactant and product ion abundances at a given time. Reprinted with permission from The American Chemical Society.

In most cases the modified peptide(s) will have to be sequenced to locate the actual site(s) of alkylation. This is usually facilitated by prior knowledge of the protein and peptide sequence, the unknown information being the identity and location in the sequence of the modified amino acid. Considerable experience indicates that conditions used for Edman sequencing can hydrolyze modifications, for example, adducts linked through an ester bond (18). Furthermore, modified amino acids are not readily assigned in Edman sequencing. Consequently tandem mass spectrometry using collisional activation is often the method of choice. Occasionally Edman sequencing and tandem mass spectrometry are used together (44).

Mechanism-based inhibition usually leads to the modification of a limited number of nucleophilic amino acids within the active site of the enzyme (e.g. 45-47). In the study of the inactivation of human α-thrombin by methyl 3-(2-methyl-1-oxypropoxy)[1]benzothieno[3,2-b]furan-2-carboxylate, it was determined from the molecular mass that the protein had been isobutyrylated (18). Care was taken to eliminate the possibility that the observed complex was noncovalent. Following proteolytic cleavage, reduction and alkylation, HPLC and mass mapping by LC-ES-MS indicated that an amino acid in peptide [198-212] was modified. Tandem mass spectrometry provided fragment ions from the peptide doubly charged by electrospray, which allowed Ser-205 to be identified as the unique locus of alkylation. This serine is known to be involved in the active site, and the investigators concluded that its isobutyrylation results from hydrolysis of the inhibitor tightly bound in the active site, They propose to use this information in conjunction with X-ray crystallography to design new inhibitors.

Affinity labeling studies can also reveal selective binding sites that are not catalytic (38,48). Covalent bonding of chlorambucil with metallothionein, discussed above, was further investigated by comparing maps of peptide products from tryptic cleavage of metallothionein with those from drug-modified metallothionein. Both chromatographic maps and mass maps were obtained using HPLC interfaced with electrospray mass spectrometry (49). These maps revealed that the majority of covalent modification occurred in peptides [32-43] and [44-51]. Cysteine sulfhydryl groups were expected to be the reaction sites, however each of these tryptic products contains several cysteine residues. Consequently, the modified peptides were sequenced in tandem mass spectrometry experiments using collisional activation. Ninety percent of the alkylation of metallothionein by chlorambucil and melphalan was found to occur at Cys-33 and Cys-48, whose sulfhydryl groups co-chelate the same metal ion in the folded protein (38,50). Molecular modeling and docking studies defined a favorable binding site that placed the reactive aziridinium moieties of these drugs adjacent to the two sulfhydryl groups. As yet no natural ligand has been recognized for metallothionein, however a variety of structures containing nitrogen mustards have been shown by this kind of affinity labeling study to form complexes (38,50,51).

Adducts that are formed without selective binding usually occur under kinetic control at multiple solvent accessible sites. Examples include polyethylene glycol attachment to bioactive proteins (52), conjugation of drugs to proteins for drug delivery or production of haptens (6, 11-14), the oxidation (vide supra) of the nucleocapsid protein by potential antiviral compounds (23, 24, 31), and adduction of β-lactoglobulin B by 4-hydroxy-2-nonenal (21). In addition to providing information about the range and average loading values, ES-MS can identify sites of alkylation, which can be important for patents, quality control and regulatory issues. Information about the extent of modification at each site is also important. One strategy has been

presented (53), in which the remaining unmodified sites are reacted with an isotope-labeled analog of the original modifier. The mixture of proteins carrying labeled and unlabeled modifiers is then hydrolyzed into peptides, and the ratio of unlabeled and isotope-labeled drug attached to each peptide is determined by mass spectrometry. This ratio corresponds to the ratio of modified and unmodified peptides. Quantification of the extent of modification at each amino acid can be obtained from sequence ions produced in tandem mass spectrometry experiments.

PROSPECTUS

The report on covalent inhibition of human α-thrombin concludes that the trilevel strategy of molecular mass determination, proteolytic peptide mapping, and sequencing "can be used to probe the site of covalent modifications without significant effort" (18). The widespread availability of reliable electrospray mass spectrometers suggests that such inquiry will also become widespread. The resulting information provides a powerful foundation for the development of new therapeutic agents, used in combination with three-dimensional structure elucidation and molecular modeling. Improvements in the sensitivity of electrospray (54) suggest that drug-protein adducts can now be isolated for analysis from cells in culture and from living organisms. Drug adducts with nucleic acids also have important implications for therapeutics, and strategies analogous to those developed for proteins would appear to make these structures susceptible to study by electrospray mass spectrometry (for example, 55-57).

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Chapter 28. Nonpeptide Agonists for Peptide Receptors: Lessons from Ligands

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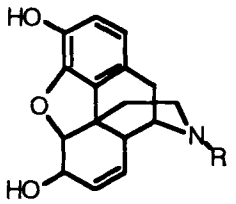
Introduction - The diverse biological processes mediated by peptide hormones and neurotransmitters suggest a tremendous potential for the development of new therapeutic agents with improved specificity and selectivity. Since peptides generally have limited oral bioavailability, lack receptor subtype selectivity and are metabolically unstable, the identification of nonpeptidyl structural mimics with improved pharmacokinetic and pharmacodynamic profiles has been the primary goal of medicinal chemists in this field. While numerous nonpeptidyl antagonists have been reported in the last ten years (1-4), until recently, the only examples of nonpeptide agonists for peptide receptors were morphine and related opioids. The structure-activity relationships of the opioid analgesics were actually characterized before their endogenous receptors or peptide ligands were discovered. However, it is the opioids that first demonstrated the feasibility of developing nonpeptide agonists for peptide receptors and the feasibility of interconverting agonist and antagonist ligands. In the last two years, additional examples of nonpeptide agonists have appeared, discovered through serendipity (angiotensin II) or directed functional screening (growth hormone secretagogues, cholecystokinin). This chapter will summarize strategies and recent successes in the discovery and optimization of nonpeptidyl agonists for seven transmembrane (7-TM) G-protein coupled peptide receptors and discuss the implications for future drug discovery efforts.

Enkephalins - The prototypical nonpeptidyl agonist is morphine **1**. Numerous SAR studies have helped define many series of opioid analgesics using *in vivo* models of pain (5,6). Replacement of the N-methyl substituent with N-allyl (**2**) provides the potent opioid antagonist nalorphine. This interconversion is extremely sensitive to the size of the N-substituent. Substitution of the nitrogen with a three carbon side chain with or



1 R = CH_3
2 R = CH_2-CH=CH_2

without branching provides potent antagonists, while the N-ethyl or N-butyl analogs are essentially inactive. Analgesic activity is restored as the side chain increases in bulk and the N-amyl and N-hexyl derivatives are almost equipotent to morphine. The N-allyl substitution has been extensively explored in a variety of opioid templates. Interestingly, the interconversion between agonist and antagonist is only possible when the starting N-methyl derivative is a potent analgesic (7), suggesting similar determinants for receptor recognition of opioid agonists and antagonists.

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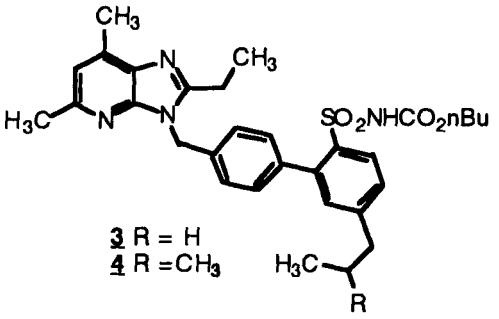
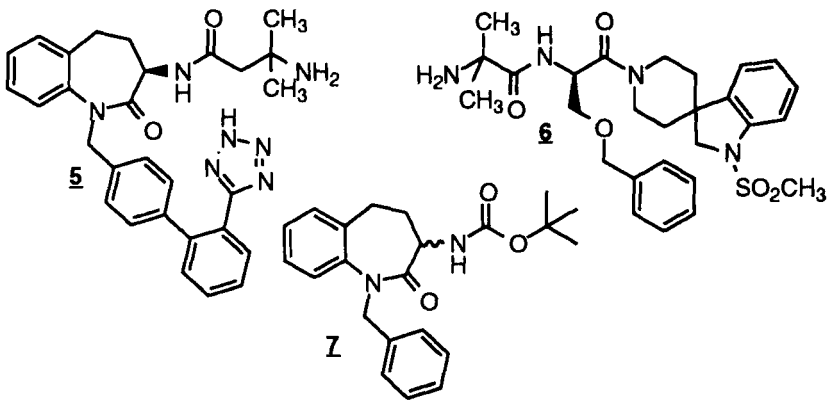
Angiotensin II (AII) - Analogs of the potent AT_1-selective angiotensin II antagonist losartan have been reported with balanced AT_1/AT_2 receptor affinity, potent *in vitro* antagonist activity and *in vivo* agonist efficacy (8, 9). A single methyl group converts the potent antagonist **3** into agonist **4** (10). Analog **4** is a mixed agonist/antagonist on



transiently transfected human AT_1 receptors (10). Both *in vivo* and cellular agonist responses are blocked with AT_1 antagonists.

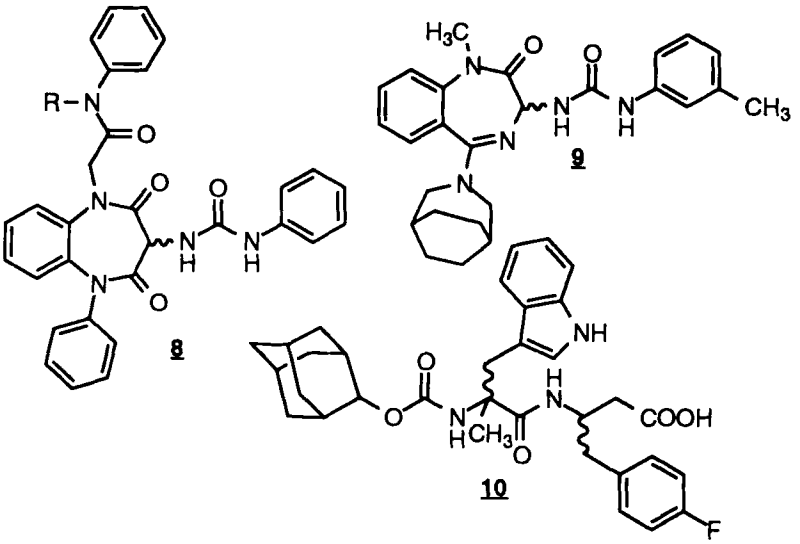
Growth Hormone Secretagogues (GHS) - A new series of compounds were recently reported as growth hormone secretagogues. The original benzolactam **5** was identified in a functional screen (growth hormone release from a rat pituitary cell assay) (11). Compounds were selected for screening based on structural elements thought to be critical for the bioactivity of the parent hexapeptide GHRP (11). Poor



pharmacokinetic parameters inspired the search for an alternative series. A second screening lead was subsequently optimized to the dipeptide derivative **6** (12). In parallel with the morphine story, both **5** and **6** were optimized before the endogenous receptor was identified (13). The endogenous ligand for this receptor remains unknown. Within this series of compounds, only benzolactam **7** has been reported as a weak GHS antagonist (11). Now that the receptor for these compounds has been cloned, more potent antagonists may be identified in receptor binding assays.

Cholecystokinin (CCK) - A series of 1,5-benzodiazepine CCK-A agonists, exemplified by **8**, were recently reported. These compounds were identified through directed screening for functional activity (contraction of the isolated GPGB) and are full agonists

in both *in vitro* and *in vivo* models of CCK activity (14). Compounds in which R = H or CH_3 are CCK-A antagonists, while compounds with R = ethyl, n-propyl or i-propyl are CCK-A agonists. As seen with the opioid analgesics, there is a limit to steric bulk and the n-butyl and cyclohexyl derivatives are less efficacious. Structural modifications previously shown to improve CCK-A receptor affinity and selectivity of the 1,4-benzodiazepine CCK antagonists produced similar changes in the affinity and selectivity of these 1,5-benzodiazepines (15-17). Some analogs within this series are mixed CCK-A agonists and CCK-B antagonists (17).



A recent abstract reported that *S*-**9** was a potent CCK-A antagonist while *R*-**9** was an inverse agonist (18). Stereochemistry also influences the functional activity of peptoid **10**. While the *RS*-diastereomer was a full CCK-A agonist, the *SR*-diastereomer was a potent CCK-A antagonist (19).

RECEPTOR LIGAND INTERATIONS

Early efforts in peptide analog design focused on identifying "the message" within the amino acid sequence responsible for bioactivity, potency and selectivity and stabilizing the peptide to enzymatic degradation. These early analogs usually retained the linear topography of the parent peptide. Following the discovery of the enkephalins and subsequent demonstration that these pentapeptides bound and activated the same receptors as morphine (20), the focus switched to the synthesis of hybrid peptide mimics which retained the global topography but contained more rigid "drug like" templates (21, 22). The underlying assumption of this strategy was that peptide and peptidomimetic ligands would interact with a common receptor binding site.

As advances in molecular biology and screening technology made it feasible to screen large numbers of compounds in receptor binding assays, the number and diversity of nonpeptide ligands for peptide receptors increased dramatically (1-4). Despite the absence of obvious structural homology between these nonpeptide antagonists and the endogenous peptide agonist, it was still possible to invoke a single binding site by rationalizing that these nonpeptide antagonists would not need to completely occupy the site in order to block signal transduction. However it is more

difficult to rationalize a single binding site which accommodates both peptide and nonpeptide *agonists*.

For the three peptide families for which both nonpeptide agonists and antagonists are known, the agonist/antagonist ligand pairs are structurally more similar to each other than to the endogenous peptide they mimic. Minor chemical modifications (alkyl substituents; stereochemistry) provide the "trigger" for interconversion between agonist and antagonist. The structure activity relationships of opioid (7) and CCK (14-17) antagonists and agonists parallel in rank order, if not in potency. This close structural similarity suggests that nonpeptide agonists and antagonists may share structural determinants for receptor recognition that are distinct from those for peptide ligands - a concept that is difficult to reconcile with the theory of a single binding site.

Site Directed Mutagenesis Studies - 7TM receptors are integral membrane proteins which do not retain biological activity when dissociated from the membrane, making it difficult to study ligand-receptor interactions directly through standard physico-chemical techniques (X-ray diffraction or NMR spectroscopy). An indirect approach which has been exploited extensively over the last ten years is alteration of amino acid residues through genetic engineering and transient or stable transfection of this novel mRNA into an appropriate cell line. The receptor binding affinity, and, more recently, functional activity of diverse ligands are then compared on native and mutant receptors. This strategy has permitted exploration of the amino acid residues involved in receptor recognition of the monoamine neurotransmitters and more recently peptide hormones and neurotransmitters (23-28). While the monoamine agonists and antagonists appeared to interact with similar amino acid residues within the transmembrane domains of the 7TM receptor, peptide agonists appear to interact primarily with amino acid residues in the extracellular domains of these receptors. More importantly, nonpeptide antagonists appear to interact with amino acid residues that are distinct from those for the peptide agonists. In a recent report, site directed mutagenesis of the AII receptor was used to evaluate affinity and efficacy of AII, **3** and **4** (10). Only one mutation modestly reduced the affinity of both **3** and **4**. Multiple mutations were found which reduced the efficacy of **4**, but had no affect on receptor affinity or antagonist potency. None of these mutations reduced the affinity or efficacy of AII. Thus, site directed mutagenesis data also discounts the theory of a single, common binding site for both peptide and nonpeptide ligands for 7TM receptors.

These results are best understood by recognizing the dynamic nature of receptor ligand interactions (27, 29). The receptor protein can exist in multiple conformational states, depending upon G-protein stoichiometry and the presence of agonist or antagonist ligands. Receptor proteins can bind ligands in the absence or presence of G-proteins, and interact with multiple G-proteins. Antagonist or agonist activity simply depends upon the ability of a ligand to stabilize or induce a particular receptor conformation. Considering the size of the receptor protein, it is not difficult to envision multiple regions of the receptor which might function as "binding sites". The concept of multiple modes for receptor ligand interactions is therefore supported both by evidence for the the diversity of receptor states (29,30) as well as the diversity of ligands which can activate or block 7TM receptors.

STRATEGIES FOR THE DISCOVERY OF NONPEPTIDE AGONISTS

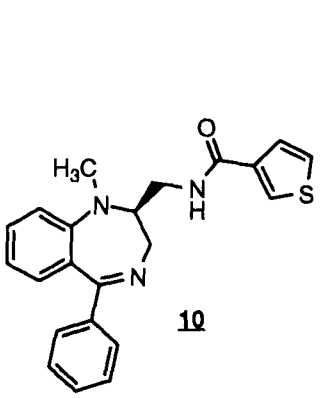
1. Screen in functional assays - Improvements in technology (instrumentation) and advances in molecular biology and biochemistry have made high throughput functional screening feasible and, in some cases, comparable to high throughput receptor binding assays. There are now multiple ways to evaluate 7TM receptor signal transduction (Table 1). In addition to providing a method for identifying ligands for orphan 7TM receptors and allowing pharmacological discrimination (agonist vs antagonist), functional assays provide increased sensitivity and real time analysis of receptor ligand interactions. The disadvantages of functional screening include the detection of "false positives" which interact with the biochemical cascade beyond the receptor and complications due to the presence of endogenous receptors and the altered stoichiometry of 7TM receptor and G-proteins in artificial cell lines. However, with appropriate assay design and pharmacological tools, it is now possible to routinely screen for functional response in a high throughput mode.

Table 1: Functional Assays for 7TM Receptors

Assay Mode	Examples
Direct Measurements	GTPase activity
	$[^{35}S]$-GTPγS binding
	cAMP
	IP(s)
	Ca^{2+}
	DAG
	Arachadonic Acid
Indirect Measurements	Luciferase
	secreted placental alkaline phosphatase (sPAP)
	β-galactosidase
	Aequorin
	Green Fluorescent Protein
	Fluorescent Dyes
	Microphysiometry
	Melanophores

2. Screen known 7TM receptor ligands - Nonpeptide agonists and antagonists reported to date are structurally more similar to each other than the endogenous peptide they mimic or block. Although it appears that the previously reported high throughput receptor binding assays have only identified nonpeptide antagonists (1-4), it must be recognized that many of these ligands have not been evaluated for functional activity. Where secondary functional assays have been employed, the tendency of medicinal chemists to prioritize leads based on potency may have excluded compounds which were weak (μM) agonist leads.

Some of the nonpeptide antagonists reported to date are known to bind more than one 7TM receptor family (31,32). This structural promiscuity appears to extend to nonpeptide agonists, as tifluadom **10** is both a potent κ-opioid agonist and CCK-A antagonist (33). Benzodiazepine **11** is both a CCK-A agonist and a CCK-B antagonist (17).



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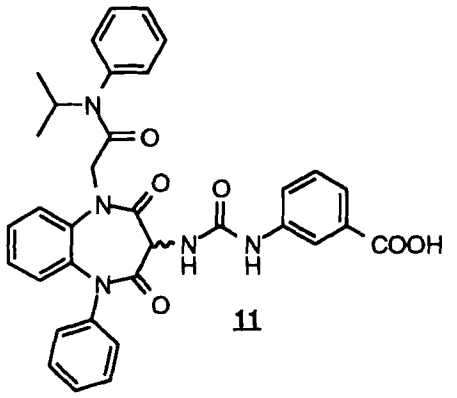

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3. Use known 7TM receptor ligands, or structural elements of known 7TM receptor ligands, as the starting point for combinatorial and/or medicinal chemistry programs - Antagonist ligands share common structural elements (34) and minor chemical modifications provide the "trigger" for interconversion between the nonpeptide agonists and antagonists for the opioid, AII and CCK receptors. While the precise molecular mechanisms by which these modifications induce or stabilize antagonist or agonist 7TM receptor conformations are unknown, and it is not obvious where modifications should be incorporated into known antagonist templates, random chemical modification of existing 7TM receptor ligands or templates may be a successful approach for the identification of new nonpeptide agonists.

SUMMARY

With the recent successes in the discovery of nonpeptide agonists for the AII, GHS and CCK receptor families, morphine and related opioids no longer represent an isolated example. The structural similarities of the nonpeptide agonists and antagonists for opioid, AII and CCK 7TM receptors suggest that previous attempts to identify peptidomimetics through iterative structural modifications of the parent peptide may not be the preferred approach. Functional screening of existing databases, coupled with chemical modification of known 7TM receptor ligands should provide additional examples of nonpeptide agonists.

Acknowledgements - I want to thank the participants at the 1996 Medicinal Chemistry Gordon conference for helpful comments and discussion.

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Chapter 29. Natural Products Research and Pharmaceuticals in the 1990's

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Introduction - Natural products have proved to be a useful source of successful drugs with diverse activities. The nature of natural products research in the pharmaceutical industry, however, has embarked on a process of change which may turn out to be both rapid and radical. Examination of lists of top-selling drugs for 1994 and 1995 (1, 2) reveals a number of natural products and natural product derivatives. It is two decades, however, since compounds such as cyclosporin A, clavulanic acid, mevinolin and ivermectin were discovered by natural product screening approaches. These discoveries stimulated an expansion of natural products-based drug discovery efforts in the pharmaceutical industry in the 1980's. Natural products continue to be important in terms of compounds introduced for human therapeutic use in the 1990's. Listed in the "To Market, To Market" chapters in this series, covering the period 1990-1995 (3-8), are a number of natural products and their derivatives. Detailed analyses for anticancer and anti-infective drugs can be found in a recent review (9). The 1990's have also seen many pharmaceutical companies turning to other sources of chemical diversity to satisfy their high throughput screening capacities and requirements for short discovery project timelines. Natural product screening appears to be viewed in some quarters as a slow and costly way of finding lead candidates and combinatorial synthetic approaches are seen as an attractive and rapid alternative for accessing chemical diversity. There are, however, exciting developments in the field of natural products, particularly in terms of new technologies providing faster routes to compound purification and identification, increasing awareness of biodiversity and the limited extent to which it has so far been explored for useful compounds, and the prospects offered by molecular biology to produce new, "unnatural", natural products. The following is a brief survey of natural product drugs and development compounds in the 1990's and the factors influencing the direction of natural products research in the pharmaceutical industry.

NATURAL PRODUCTS PHARMACEUTICALS IN THE 1990'S

Cancer - Nature is a rich source of compounds which regulate cellular proliferation by binding to specific proteins required for cell division, as reviewed recently (10). Some of these compounds have been, and continue to be, developed as antineoplastic agents or immunosuppressants. In terms of antineoplastic agents, the 1990's have been particularly significant for plant products. Taxol (Paclitaxel) and its derivatives prevent cell division by accelerating the polymerisation of tubulin and stabilising microtubules (11-13). The development of taxol has been reviewed previously in this series (11). Taxotere (Docetaxel) is a taxoid derivative synthesised during the search for a semi-synthetic production route to taxol from 10-deacetylbaccatin III, a precursor extracted from the needles of the European Yew, *Taxus baccata* (12, 13). Docetaxel has been launched for the treatment of ovarian, breast and non-small cell lung cancers and is being investigated for wider application (8). Further synthetic modification of taxol is an active research area and the synthesis and SAR of a second generation of antitumour taxoids was recently reported (14). These new 3′-(2-methyl-1-propenyl) and 3′-(2-

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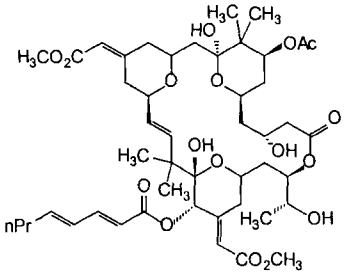
methylpropyl) taxoids with modifications at C-10 show improved cytotoxic potency compared to taxol and taxotere against a range of cell lines including drug-resistant lines and are postulated to exhibit reduced binding to P-glycoprotein. A second class of semi-synthetic antineoplastic agents from plants consists of the camptothecin derivatives irinotecan, topotecan and 9-aminocamptothecin. These compounds were developed after identification of the mechanism of action of camptothecin, an alkaloid from the Chinese tree *Camptotheca acuminata*, as topoisomerase 1 inhibition (15, 16). Irinotecan and topotecan are water soluble camptothecin derivatives. Irinotecan is a prodrug which undergoes de-esterification *in vivo* to yield a more potent metabolite (7). Topotecan does not require metabolic activation (17). 9-Aminocamptothecin exhibits strong activity against solid tumour xenografts (16, 18).

Marine organisms are proving to be an abundant source of potentially useful antineoplastic substances (19), several of which are at various stages of preclinical and clinical evaluation (19, 20). Evaluation is most advanced for bryostatin 1 (**1**), a metabolite of the colonial bryozoan, *Bugula neritina*. Bryostatin 1 is a PKC activator which exhibits potent and selective activity against leukaemia cell lines and excellent *in vivo* activity (20). As is the case for other promising marine organism metabolites, material supply is a concern, not only in respect of enabling efficient and timely evaluation but also from the point of view of marine habitat conservation. Large scale collections of *B. neritina* have been processed to provide sufficient bryostatin 1 for completion of the phase II clinical trials currently in progress. Marine aquaculture of *B. neritina* is currently being studied to develop a production route for bryostatin 1 should it progress to marketable status (20). Other marine products at earlier stages of development include ecteinascidin 743, dolastatin 10 and halichondrin B. Ecteinascidin 743 (**2**) is one of a series of alkaloids isolated from the Caribbean tunicate *Ecteinascidia turbinata* and shows potent activity in a number of *in vitro* and *in vivo* systems (21, 22). It has recently been reported to be a DNA minor groove, guanine-specific alkylating agent (22). Dolastatin 10 from *Dolabella auricularia* and the sponge metabolite halichondrin B are inhibitors of glutamate- and microtubule-associated protein-dependent polymerisation of tubulin (23). Members of another series of marine products, the spongistatins from a *Spongia* sp., also inhibit tubulin polymerisation by binding to the *Vinca* alkaloid domain of tubulin and have exceptional cytotoxic properties which are ascribed to highly efficient cellular uptake (24). The extremely low natural abundance and the structural complexity of the spongistatins, however, present challenging barriers to development (25).



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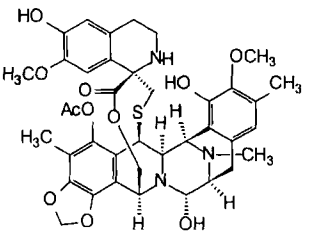

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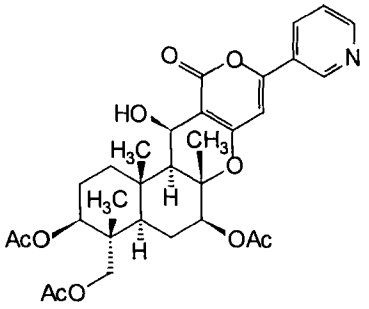
A number of microbial metabolites are also being investigated for use in the treatment of cancer. Pentostatin A, a purine nucleoside found in fermentations of *Streptomyces antibioticus* is an adenine deaminase inhibitor which has been introduced for the treatment of hairy cell leukaemia (5). Side effects observed in

clinical trials include immunosuppression and myelosuppression (26). Pentostatin is also being studied for other potential applications (27). Echinomycin A from *Streptomyces echinatus* is a bifunctional DNA intercalating agent which has been evaluated clinically for use as an antitumour agent (28). Cyclosporin A has been found to reverse multidrug resistance (MDR) (29). A semi-synthetic cyclosporin A derivative, PSC 833, has improved potency without increased toxicity and has shown promising results for restoring sensitivity to certain anticancer drugs in cases where drug resistance is due to MDR (29). XR9051, a synthetic derivative of a *Streptomyces* sp. metabolite which is able to restore sensitivity to multi-drug resistant cancers is currently in phase 1 clinical trials (30).

Immunosuppressants - A number of microbial products have been introduced or are being evaluated as immunosuppressants, as reviewed recently (31). Cyclosporin A remains the main drug in this area and together with FK506 from *Streptomyces tsukübaensis* and rapamycin from *Streptomyces hygroscopicus* forms the immunophilin class of immunosuppressants, which are linked by similar but non-identical mechanisms of action (32, 33). FK506 (Tacrolimus) has been introduced for the treatment of transplant rejection (6) but, although more potent *in vitro*, appears to have a lower therapeutic window than cyclosporin A (31). Rapamycin is currently in development for use in combination with cyclosporin A (31). Mycophenolate mofetil is the morphylinoethyl ester of the *Penicillium* sp. metabolite mycophenolic acid. It acts as a prodrug for mycophenolic acid and has been introduced for the prevention of acute kidney transplant rejection in conjunction with other immunosuppressive therapy and for treatment of refractory acute kidney graft rejection (8, 31). Mycophenolic acid is a selective, reversible, non-competitive inhibitor of inosine monophosphate dehydrogenase (34, 35). 15-Deoxyspergualin (Gusperimus) is a synthetic analogue of the polyamine fermentation product spergualin, originally isolated from *Bacillus laterosporus* (36). It has strong immunosuppressive properties and has been introduced for use in the treatment of kidney transplant rejection and is being evaluated for use in combination with other immunosuppressive drugs (7, 31). The mechanism of action of 15-deoxyspergualin is thought to involve binding to a member of the hsp70 family of heat shock proteins, leading to blocking of nuclear translocation of some key proteins, including the transcription factor NFκB (37).

Cardiovascular Agents - Microbial screening of two enzymes involved in cholesterol and lipid biosynthesis has proved to be successful in terms of new and potentially useful metabolites identified. A number of inhibitors of squalene synthase have been reported, the most interesting to date being a family of metabolites isolated from a *Phoma* sp. as the squalestatins and from various fungi including *Sporormiella intermedia* as the zaragozic acids (38). These compounds are potent *in vitro* inhibitors of squalene synthase, exhibit potent activity in *in vivo* models of cholesterol synthesis and have stimulated much interest in terms of chemical synthesis (38). Acyl-CoA:cholesterol acyltransferase (ACAT) has proved to be a productive target (39). The most potent inhibitors found by microbial screening were the pyripyropenes from *Aspergillus fumigatus* (40,41). Pyripyropene A (**3**) reduces cholesterol absorption in hamsters when administered orally (40, 41). Synthetic modification of the pyripyropenes has resulted in analogues with improved properties (42, 43). Microbial screening has also been used in the search for low molecular weight inhibitors of plasminogen activator inhibitor-1 (PAI-1). Two diketopiperazines (**4** and **5**) isolated from a *Streptomyces* sp. were found to inhibit PAI-1 activity in an amidolytic assay for tissue plasminogen activator-mediated plasmin generation, to enhance fibrinolysis *ex vivo* and to protect against thrombus formation in rats (44, 45). Subsequent

development has generated derivatives with improved properties (46).



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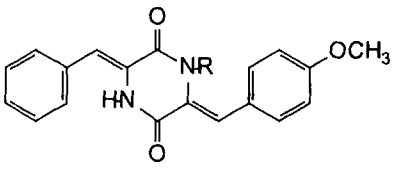
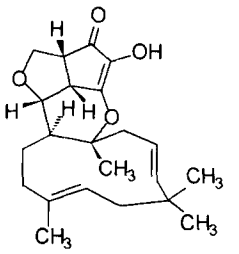


4 R=H
5 R=Me

Anti-infective Agents - The status of natural product leads in the antifungal area has been reviewed recently (47). These leads include the cyclic lipopeptide echinocandins from *Aspergillus* spp., pneumocandins produced by directed biosynthesis using the fungus *Zalerion arboricola* (48), the cyclopeptide aureobasidins from the fungus *Aureobasidium pullulans* (47), the pradimicins from *Actinomadura hibisca* (49) and the nikkomycins from *Streptomyces tendae* (47). L-743,872, a derivative of pneumocandin Bo with improved solubility, potency and pharmacokinetics, is currently undergoing phase II clinical trials (50). The need for new antibacterial agents has also been subject to recent review (51), clearly outlining an important role for several classes of known antibiotics in providing templates for chemical modification and listing novel targets against which natural products screening might be expected to be productive. Promising developments include semi-synthetic glycopeptide antibiotics with greatly improved potency against vancomycin-resistant enterococci (52, 53) and ziracin, a novel oligosaccharide from *Micromonospora carbonacea* var. *africana* which has good activity against drug-resistant bacteria and which is undergoing phase I clinical studies (54). Screening plants for antiviral activities has resulted in the discovery of agents such as michellamine B and SP-303. The michellamines were isolated as the *in vitro* anti-HIV agents from the liana *Ancistrocladus korupensis* and are novel naphthylisoquinoline alkaloid dimers (55). Michellamine B inhibits HIV-induced cell killing by at least two distinct mechanisms (56) and is currently in preclinical development (20). SP-303 is a plant flavonoid discovered by an ethnobotanical approach which is currently being evaluated for use against influenza viruses (57, 58).

CNS-Active Agents - One noteworthy example in this area is the $GABA_A$-benzodiazepine receptor agonist xenovulene A (**6**). This was the main metabolite of a series produced in fermentations of *Acremonium strictum* and was a potent *in vitro* inhibitor of the binding of flunitrazepam to $GABA_A$-receptor containing membrane preparations (59). The xenovulenes are of mixed biosynthetic origin, consisting of a sesquiterpenoid humulene ring fused to oxygenated, polyketide-derived, tricyclic systems. They are structurally distinct from the benzodiazepines and other $GABA_A$-receptor binding compounds (59, 60). Xenovulene A and an analogue with increased potency were found to enhance $GABA_A$-mediated responses and to bind selectively to Type 1 $GABA_A$ receptors, with subtype selectivity (60). Preliminary structural modifications have identified features important for functional properties at the $GABA_A$ receptor (60).



6

NATURAL PRODUCTS RESEARCH IN THE 1990'S

Changing Approaches - Traditional natural product screening programmes involve testing crude extracts of microbial fermentation broths, plants or marine organisms in drug discovery assays. Where activity of sufficient interest is detected, the active sample components are isolated by a process of bioassay-guided purification. The physico-chemical characteristics of the active components are determined at the earliest possible stage so that known compound identification (dereplication) can be facilitated by database interrogation (61). For novel compounds, obtaining sufficient pure material for structure elucidation can involve large scale refermentation of microorganisms or large scale resupply of plant or marine organism extracts, steps which can be uncertain and time-consuming. These traditional screening programmes can focus on one source of chemical diversity, such as microorganisms (62) or encompass complementary sources of diversity, such as microorganisms and plants (63). Short discovery project timeline expectations are driving such natural products screening operations to apply process reengineering methods to maximise efficiency in order to compete with other chemical library approaches (64, 65). A new variant of the traditional phytochemical screening approach involves screening plant tissue culture samples, keeping scale-up functions under in-house control (66). A focused alternative can be provided by an ethnomedically-driven approach (67). This utilises traditional medical knowledge of plants used for specific purposes by humans and then testing extracts in animals prior to isolating and evaluating the active components (67).

A completely different approach is provided by chemical screening for novel metabolites. This involves searching for compounds with particular chemical characteristics using procedures such as thin layer chromatography combined with specific staining methods (68). A significant example of a compound discovered by chemical screening is the PKC inhibitor staurosporine (69). Recently reported examples include compounds discovered by a TLC/staining approach (70) and by physico-chemical screening using HPLC and photodiode array UV-visible detection (71). Advances in HPLC-mass spectrometry (HPLC-MS) are set to make a significant contribution in this area, since HPLC-MS has now been reduced towards routine practice by the availability of reliable commercial interfaces (72). Physico-chemical screening of natural product extracts with photodiode array and mass spectrometric detection enables sample component characterisation by UV-visible and mass spectra and known compound identification by automated spectral library searching (73). This screening method provides the basis for establishing a library of purified natural

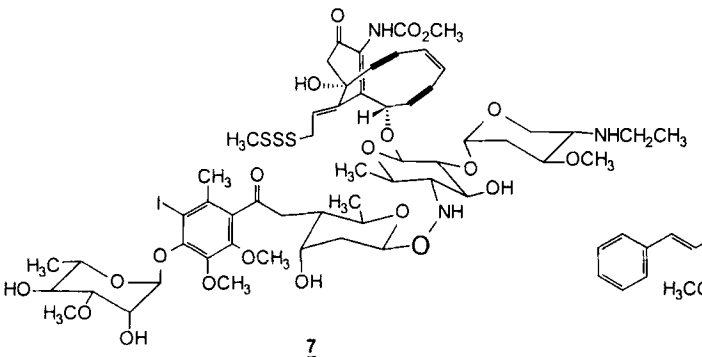
products selected for potential novelty or rarity and avoiding known, non-selective or "nuisance" compounds (73). Compounds are purified on a multi-milligramme scale to permit both extensive screening at known assay concentrations and full spectroscopic characterisation and structure elucidation as soon as an interesting biological activity is discovered. Screening pure compounds will facilitate quality interrogation of actives in secondary assays while elimination of a refermentation/resupply step should provide the structures of active compounds in a rapid time frame. This approach aims to combine the breadth of natural chemical diversity with the convenience of chemical library screening (73).

Biodiversity - Appreciation of the importance of nature as a reservoir of chemical diversity which has evolved to mediate interactions between organisms is increasing rapidly. Understanding of the chemical language of these interactions and how this may be used to target biologically-active compound discovery projects to relevant organism groups for screening is also growing (74). Analyses of the current numbers of described species and estimates of the totals of species numbers emphasise the uncertain future of biodiversity worldwide and the potential for huge losses of this valuable and underexploited resource (75). Even long-established anticancer screening programmes have systematically studied only a small fraction of available diversity in some species groups (19). Conservation of endangered biodiversity was one of the driving forces behind the 1992 UN Convention on Biological Diversity (CBD) (76, 77). This is designed to give every country sovereign rights over its own genetic resources. Companies or institutions wishing to access these resources for purposes such as drug discovery should now negotiate through the relevant national governments. This access can be obtained for suitable payments, and technology transfer and training relevant to the conservation and sustainable use of these genetic resources is encouraged. There is an emphasis on fair and equitable compensation for the scientists and communities of the genetically rich source countries. The CBD provides a framework for future international collaboration in the utilisation of genetic resources. It will increasingly formalise the way in which natural product samples are collected for screening. A number of policies for international collaboration and compensation already exist (76). The outlook for conservation is positive.

Combinatorial Biosynthesis - Understanding of the biosynthetic pathways involved in the production of major families of bioactive natural products such as the polyketides is providing the means to build on natural chemical diversity using protein engineering strategies (78). Polyketides can be designed rationally by recombinant assembly of enzyme subunits to produce "unnatural" natural products. Two new aromatic polyketides were prepared using *Streptomyces* strains engineered to express combinations of appropriate enzymatic subunits from naturally occurring polyketide synthases (79). Reports on the biological activities of "unnatural" products such as these will be awaited with interest. The same general approach is being studied more widely for natural products other than polyketides (80). DNA technology is also being investigated for the production of interesting natural products from organisms that are difficult to cultivate, such as marine organisms (80). This involves cloning the relevant biosynthetic DNA from the organism of interest into an organism which is easier to culture in the laboratory, such as a bacterium.

Inspirational Impact - Reports of new classes of natural products with interesting structures and biological activities can stimulate great interest from chemists interested in new synthetic challenges and biologists interested in the mechanistic

aspects and implications of molecular interactions. Two examples which have generated much productive recent research are the enediyne and β-methoxyacrylate classes of antibiotics. The enediyne antitumour antibiotics include members of diverse structural origin including the calicheamicins from *Micromonospora echinospora* ssp. *calichensis* (81) and the dynemicins from *Micromonospora chersina* (82). They all possess a 1,5-diyn-3-ene or similar moiety which is responsible for their potent double stranded DNA-cleaving ability *via* a reactive 1,4-aryl diyl intermediate (81). In addition to total syntheses of calicheamicin γ_1^I (**7**) and dynemicin A, a number of synthetic mimics of the 1,5-diyn-3-ene portions of these molecules have been reported (83, 84). The calicheamicins also possess an aryltetrasaccharide domain which is responsible for their binding to specific DNA sequences. The aryltetrasaccharide of calicheamicin γ_1^I inhibits sequence-specific DNA-protein interactions and transcription *in vivo*. This has been proposed to form the basis of a development strategy for novel transcriptional antagonists (85). The β-methoxyacrylate class of antifungal antibiotics includes the strobilurin and oudemansin families of basidiomycete metabolites (86). Strobilurin A (**8**) was used as a topical antifungal agent even before its structure was known. The strobilurins and oudemansins are potent inhibitors of mitochondrial respiration, binding specifically to cytochrome b, at least in part by virtue of their β-methoxyacrylate moieties. The strobilurins have been adopted as lead templates for the development of agricultural fungicides, leading to an impressive number of patents and the launch of fungicidal products containing synthetic strobilurin derivatives (86, 87).



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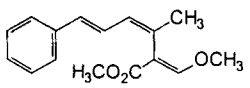

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TRENDS AND PROJECTIONS

Natural products still have much to offer the pharmaceutical industry as a source of chemical diversity. In one view they represent "genetically-encoded libraries of peptidomimetic, small molecule ligands" (9) and therefore constitute an ideal source to explore for new, biologically-active compounds. The range of structural types discovered *via* high throughput screening approaches continues to cover a wide area of chemical diversity (65). Competition from other sources of diversity is forcing a re-examination of natural products research in the pharmaceutical industry and is driving screening and isolation operations to adopt new technologies and strive for greater speed and efficiency. The logical conclusion of this trend is the creation of libraries of pure, dereplicated natural products.

Despite its numerous successes, the popularity of the natural products screening approach to drug discovery has always had a cyclic nature. A review published in

the early 1970's presented a gloomy outlook for natural products in the then dominant area of antibiotics research (88). By the end of that decade, however, screening of other types of biological activity had led to a number of very significant discoveries including those of clavulanic acid, cyclosporin A, mevinolin and the avermectins. The current pace of change in natural products research should make the next few years equally interesting.

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Chapter 30. Inhibition of Cytochrome P-450 and Implications in Drug Development

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INTRODUCTION

The cytochrome P-450s constitute a superfamily of isozymes that play an important role in the oxidative metabolism of drugs. One of the many intriguing aspects of cytochrome P-450s is that although the P-450s actually catalyze only a limited number of reactions, including carbon hydroxylation, heteroatom oxygenation, dealkylation, and epoxidation, they metabolize an amazingly large number of lipophilic xenobiotics. This is accomplished by multiple forms of cytochrome P-450 which have overlapping substrate specificities.

The polysubstrate nature of cytochrome P-450s is responsible for the large number of documented drug interactions associated with the P-450 inhibition. Whenever two or more drugs are administered concurrently, the possibility of drug interaction exists. Inhibition of drug metabolism by competing the same enzyme may result in unanticipated and undesirable elevations in plasma drug concentrations. Thus, the inhibition of P-450 enzymes is of clinical importance for both therapeutic and toxicological reasons. The purpose of this paper is to review the mechanisms of inhibition of P-450 enzymes and their implications in drug development.

HUMAN HEPATIC CYTOCHROME P-450s

The superfamily of cytochrome P-450 is subdivided into families and subfamilies that are defined solely on the basis of amino acid sequence homology. To date, at least 14 P-450 gene families have been identified in mammals (1). Only three main P-450 gene families, CYP1, CYP2 and CYP3, are currently thought to be responsible for most hepatic drug metabolism. Although the CYP1 and CYP3 gene families are relatively simple, the CYP2 gene family is comprised of many subfamilies, e.g. CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, etc. These isoenzymes have the same heme center, but differ by their apoproteins. Together with the surrounding lipids of the endoplasmic reticulum, these apoproteins delineate different hydrophobic pockets. Accordingly, different cytochrome P-450 isoforms have varying affinities for a given substrate.

Approximately 70% of human liver P-450 is accounted for by CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1 and 3A enzymes. Among these, CYP3A (3A4, 3A5 and 3A7) and CYP2C (2C8, 2C9, 2C18 and 2C19) are the most abundant subfamilies, accounting for 30% and 20% of total P-450, respectively. Other isoforms are minor contributors to the total P-450: CYP1A2, 13%; CYP2E1, 7%; CYP2A6, 4%; CYP2D6, 2%; and CYP2B6, 0.2% (2). In humans, genomic analyses suggest that at least seven genes exist in the CYP2C subfamily (3). CYP2C8 and 2C9 are the major forms, accounting for 35% and 60%, respectively, of total human CYP2C forms, while CYP2C18 (4%) and 2C19 (1%) are the minor forms of the human CYP2C subfamily. On the other hand, CYP3A4 and 3A5 have been identified in adult human liver microsomes.

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CYP3A4 is the most abundant P-450 isoform, approximately 25% of total P-450, and plays very important roles in human metabolism. CYP3A5 is believed to be polymorphically expressed, appearing in about only one-fourth of the human population. CYP3A7 is an enzyme that is expressed only in human fetal, but not adult liver (4).

Because of evolutionary and environmental factors, there is a remarkable degree of genetic variability built into the population. Mutations in the gene for a drug metabolizing enzyme normally result in enzyme variants with higher, lower or no activity, or may also result in the absence of the enzyme. With the technological breakthroughs in molecular biology, significant progress has been made in understanding the role of genetic polymorphisms in drug metabolism. The major polymorphisms that have clinical implications are those related to the oxidation of drugs by CYP2D6 and CYP2C19 (5,6).

CYP2D6 polymorphism is perhaps the most studied genetic polymorphism in drug metabolism. This polymorphism divides the populations into two phenotypes, extensive metabolizer (EM) and poor metabolizer (PM). Approximately 5-10% of individuals in Caucasian populations are the PM phenotype, but only 1-2% in Asian populations. Similarly, CYP2C19 also exhibits genetic polymorphism in drug metabolism. The incidence of the PM phenotype in populations of different racial origin varies; approximately 2-6% of individuals in the Caucasian population, and 18-22% in Asian populations.

In general, a significant drug-drug interaction occurs only when two or more drugs compete for the same enzyme and when the metabolic reaction is a major elimination pathway. Thus, definitive assessment of the role of an individual P-450 in a given metabolic pathway is essential in determining and predicting the potential of drug interactions. To identify which P-450 isoforms are responsible for the oxidative metabolism of drugs, a general strategy has emerged for *in vitro* studies. This involves (a) use of selective inhibitors, (b) immunoinhibition, (c) catalytic activity in cDNA-based vector systems, (d) catalytic activity in purified enzymes and (e) metabolic correlation of an activity with markers for known P-450 isoforms. Each approach has its advantages and disadvantages, and a combination of the approaches is usually required to accurately identify the P-450 isozyme responsible for the metabolism of a given drug.

MECHANISMS OF INHIBITION OF CYTOCHROME P-450s

The catalytic cycle of cytochrome P-450 consists of at least seven discrete steps: (a) the binding of substrate to the ferric form of the enzyme, (b) reduction of the heme group from the ferric to ferrous state by an electron provided by cytochrome P-450 reductase, (c) binding of molecular oxygen, (d) transfer of a second electron from cytochrome P-450 reductase and/or cytochrome b5, (e) cleavage of the O–O bond, (f) substrate oxygenation and (g) product release (7). Although impairment of anyone of these steps can lead to inhibition of P-450 enzyme activity, steps (a), (c) and (f) are particularly vulnerable to inhibition.

The mechanisms of P-450 inhibition can be divided grossly into three categories: (a) reversible inhibition, (b) quasi-irreversible inhibition and (c) irreversible inhibition (8,9). Among these, reversible inhibition is probably the most common mechanism responsible for the documented drug interactions. In mechanistic terms, reversible interactions arise as a result of competition at the P-450 active center and

probably involve only the first step of the P-450 catalytic cycle. On the other hand, agents that act during or subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors.

Reversible Inhibition - Many of the potent reversible P-450 inhibitors are found to be nitrogen-containing compounds. Systematic studies of imidazole derivatives revealed that both lipophilicity and steric factors are important determinants of imidazole inhibition potency (10.11). Inhibitors that bind to lipophilic regions of the apoprotein and simultaneously bind to the prosthetic heme iron, are inherently more effective than agents that depend on only one of these binding interactions. Furthermore, steric accessibility of the heteroatomic lone electron pair is also an important factor in determining the inhibitory potency. For instance, compounds containing lipophilic substituents in the 1,4- or 1,5-positions of the imidazole ring are effective inhibitors, whereas substitution in the 2-position results in non-potent inhibitors. Thus, a two-point binding model has been proposed to explain the activity of reversible P-450 inhibitors (8).

The factors that determine the inhibitory potency of imidazole derivatives also are valid for other nitrogen heterocycles. Pyridine derivatives, like imidazoles, interact with ferric P-450 and induce a type II binding spectrum (12). The best known inhibitor among the pyridine derivatives is metyrapone. This compound acts as a potent and selective inhibitor of P-450 isoforms, inhibiting the 11β-hydroxylase that catalyzes the final step in the cortisol biosynthesis.

The quinolines are another class of nitrogen heterocycles that exhibit potent P-450 inhibition. Ellipticine is a quinoline-containing compound that interacts with both ferrous and ferric P-450 forms, where the ligand binds preferentially to the ferric heme and elicits a type II binding spectrum (13). Ellipticine and its derivative, 9-hydroxyellipticine, have been utilized successfully as selective inhibitors for the inhibition of CYP1A1/2 activity (13).

Metabolic Intermediate Complexation - Aside from the reversible inhibition that occurs due to simple active-site interactions with the heme iron of the ferric P-450 or lipophilic regions of the apoprotein, there are instances of more complex mechanisms of inhibition. A large number of drugs undergo metabolic activation by the P-450 system to inhibitory products. These metabolic products may generate stable complexes with P-450 so that the cytochrome P-450 is sequestered in a functionally inactive state. The classes of drugs that form metabolic intermediate (MI) complexes with P-450 include the methylenedioxybenzenes, alkylamines, macrolide antibiotics and hydrazines.

Piperonyl butoxide, a methylenedioxybenzene derivative, has been used for many years as an inhibitor of oxidative drug metabolism. The mechanism of inhibition was initially considered to result from competitive interaction with cytochrome P-450s. However, it is now well-established that this compound acts by forming an MI cytochrome P-450 complex (14). The ferrous complex formed by methylenedioxybenzene derivatives is characterized by a distinct absorption spectrum with double maxima at 427 and 455 nm, whereas the ferric complex has a single absorption maximum at 437 nm. Mechanistic studies suggest that the formation of a carbene-iron complex is responsible for the MI complexation of the methylenedioxybenzenes.

The nature of benzodioxole substitution is an important determinant of the inhibitory activity of methylenedioxybenzene derivatives. When the substituent is an electron withdrawing group, carbon monoxide production is enhanced and MI complex formation is diminished (15). In contrast, when the substituent is electron donating or is a long-chain alkyl group, the MI complex is increased and stabilized (14).

SKF-525A is one of the first alkylamines shown to elicit MI complexation with P-450 (16). This compound has been widely used for many years as a universal inhibitor of all cytochromes P-450. Consequently, negative findings with SKF-525A always lead to the conclusion that cytochrome P-450 is not involved in oxidative metabolism of a particular drug. However, recent findings suggest that SKF-525A is not uniformly potent against the activity of all P-450s (17). SKF-525A generates a complex with rat CYP2B1, CYP2C11 and CYP3A1/2, but not with CYP2A1.

Irreversible Inactivation of Cytochrome P-450s - Certain drugs contain functional groups that are oxidized by cytochromes P-450 to reactive intermediates that inactivate irreversibly the enzyme prior to its release from the active site. These drugs are classified as mechanism-based inactivators or suicide substrates. The mechanism-based inactivation of P-450 may result from irreversible heme or apoprotein modification. Reactions with the prosthetic heme group invariably inactivate the P-450. However, reactions with apoprotein do not necessarily inactivate the enzyme. Apoprotein modifications result in loss of catalytic activity only if they interfere with the binding of substrates or the ability of the enzyme to accept electrons and activate oxygen (18).

Heme Alkylation - A wide range of drugs, including olefins, acetylenes and dihydropyridines, are converted to radical intermediates by P-450 that alkylate the prosthetic heme group and inactivate the enzyme. The oxidation of terminal double-bond olefins and triple-bond acetylenes often are accompanied by *N*-alkylation of the prosthetic heme group and inactivation of the enzyme (8,18). Heme alkylation is initiated by the addition of the activated oxygen to the internal carbon of the double or triple bond and is terminated by binding to heme pyrrole nitrogen.

Allylisopropylacetamide (AIA), an olefinic derivative, is a classic suicide substrate of cytochrome P-450. This compound is now recognized as an effective heme-alkylating inactivator of rat CYP2B1 and CYP3A1, with CYP2C6 and CYP2C11 less susceptible (19). Interestingly, the AIA-inactivated P-450 isozymes can be restored partially by *in vitro* and *in vivo* heme supplementation. The administration of exogenous heme to phenobarbital-treated rats given AIA or the addition of heme *in vitro* to liver homogenates from such rats resulted in partial restoration of CYP2B1 and CYP3A1 activities and, to a lesser extent, CYP2C6 and CYP2C11 (20).

4-Alkyl-1,4-dihydropyridines are oxidized by cytochrome P-450 enzymes to putative radical cation intermediates that *N*-alkylate the prosthetic heme group of P-450 (21). Not all dihydropyridines elicit heme alkylation, and the substitution at position 4 of the dihydropyridine ring is an important determinant. Heme alkylation is detected if the substitution is a primary, unconjugated moiety (methyl, ethyl, propyl), but not if it is an aryl (phenyl), secondary (isopropyl) or conjugated (benzyl) group (8,21). For example, nifedipine, the 4-aryl-substituted dihydropyridine does not inactivate cytochrome P-450 at all.

Covalent Binding to Apoprotein - The best known example of inactivation of cytochrome P-450 through protein modification by a suicide inactivator is that of chloram-

phenicol. The dichloroacetamido group is oxidized to an oxamyl moiety that either undergoes hydrolysis or acylates a lysine residue in the P-450 active center (22). This acylation event interferes with the transfer of electrons from cytochrome P-450 reductase to the heme group of the cytochrome P-450 and thereby prevents catalytic turnover of the enzyme. The inactivation by chloramphenicol is not uniform for all P-450s, and studies with rat liver microsomes revealed that CYP2B1, CYP2C6 and CYP2C11 are susceptible to inactivation by chloramphenicol, whereas CYP1A1 and CYP1A2 are resistant (23).

A variety of sulfur compounds inactivate cytochrome P-450 by binding covalently to protein after they are oxidatively activated by the enzyme. The P-450 inactivation by sulfur compounds is considered to involve sulfur oxidation that generates reactive sulfur metabolites. Tienilic acid, a substituted thiophene, is oxidized by yeast-expressed human CYP2C9 to a reactive metabolite, presumably a thiophene sulfoxide that binds covalently to the P-450 apoprotein (24).

IN VITRO DRUG INTERACTION

In assessing the consequences of drug interaction, two important factors must be considered: (1) the identity of P-450 isoforms responsible for metabolizing the involved drugs, and (2) the relative contribution of the metabolic pathways being inhibited to the overall elimination of the drug. Over the last 10 years, a great deal of information on human cytochrome P-450s at the molecular level has become available. This information, with available antibodies and chemical inhibitors, has made it possible to easily determine the P-450 isozymes responsible for the metabolism of a drug.

Although the identification of P-450 isoforms is relatively straightforward, determining the consequences of inhibited metabolic pathways from *in vitro* drug interaction can be complicated. The degree of enzyme inhibition depends on the concentration of substrate and inhibitor. Thus, another important factor in *in vitro* drug interaction studies is the use of clinically relevant concentrations of inhibitor and substrate. The use of supra-therapeutic drug concentrations may produce drug interaction *in vitro*, but not *in vivo*. In addition, the major metabolic pathway may be shifted, depending on the drug concentration used. For example, *N*-demethylation is the major metabolic pathway of diazepam in humans in clinical studies. However, *in vitro* studies in human liver microsomes showed that 3-hydroxylation was the major pathway when a high drug concentration (100 μM) was employed (25). The *N*-demethylation of diazepam is catalyzed by CYP2C19 and 3-hydroxylation is mediated by CYP3A4. The *in vitro* and *in vivo* discrepancy is due to the differences in the drug concentration used. Indeed, the major metabolic pathway of diazepam is *N*-demethylation in human liver microsomes when a clinically relevant drug concentration (2 μM) is used (26). This example illustrates the importance of the use of drug concentration in *in vitro* drug interaction studies in order to predict the *in vivo* situation.

When the mechanism of enzyme inhibition is considered, the experimental conditions and design are also very important. The experimental results reported by Franklin (27) are a good example. Depending on the experimental conditions, SKF-525A acts as a competitive inhibitor or MI complexation inducing agents. As shown in Table 1, SKF-525A increased the K_m values of substrates, but had little effect on the V_{max} values when incubated with substrates without preincubation of the inhibitor. However, SKF-525A decreased the V_{max} values of substrates and had little

effect on the K_m values when SKF-525A was preincubated prior to substrate addition. Thus, preincubation of SKF-525A changed the kinetics of inhibition from the reversible competitive type to irreversible MI complexation.

Table 1. Inhibition of rat hepatic microsomal monooxygenase activity by SKF-525A with or without preincubation prior to substrate addition. (Adapted from ref (27).)

Reaction	Inhibitor	No Preincubation		Preincubation (5 min)	
		K_m (mM)	V_{max} (nmol/min/mg protein)	K_m (mM)	V_{max} (nmol/min/mg protein)
Aminopyrine	None	1.25	4.0	1.9	2.8
N-demethylation	SKF-525A (50 μM)	7.10	4.0	1.9	1.4
Aniline	None	0.09	0.67	0.16	0.73
Para-hydroxylation	SKF-525A (50 μM)	0.25	0.67	0.16	0.33
Ethylmorphine	None	0.30	20	0.26	18
N-demethylation	SKF-525A (17 μM)	0.72	17	0.31	4

In the case of mechanism-based inactivation and MI complexation, a fraction of P-450 enzymes is destroyed or complexed, but the remaining enzymes should be intact with normal enzyme activity. Thus, a compound that irreversibly inactivates an enzyme will result in a decrease in the V_{max}, but has no effect on the K_m. The kinetic data are similar to that of a reversible non-competitive inhibitor, and sometimes an irreversible inhibitor is incorrectly referred to as a reversible non-competitive inhibitor because the V_{max} is decreased. It is important to distinguish between reversible and irreversible inhibition. Therefore, an understanding of the mechanism involved in enzyme inhibition is crucial to providing a rational basis for interpreting and predicting drug interaction.

CLINICAL IMPLICATIONS

The clinical relevance of drug inhibition will depend on a number of considerations. For most drugs, the inhibited metabolic pathway would need to represent a major contribution to overall clearance for significant differences in clinical response to be apparent. The clinical relevance also depends on whether parent drug or metabolites are pharmacologically active, and on the drug's therapeutic index. The risk of adverse effects from metabolic drug interaction is higher with some drugs than with others. Patients receiving anticoagulants, antidepressants or cardiovascular drugs are, due to the narrow therapeutic index of these drugs, at much greater risk than patients receiving other kinds of drugs. Co-administration of terfenadine, an antihistamine agent, and ketoconazole led to fatal ventricular arrhythmias in some patients. Studies by Honig and his colleagues (28) revealed that terfenadine is metabolized extensively by CYP3A4, and ketoconazole, a potent CYP3A4 competitive inhibitor, inhibited the metabolism of terfenadine, resulting in elevation of terfenadine, which in turn caused prolongation of the QT interval.

Warfarin, an oral anticoagulant, is marketed as a racemic mixture consisting of equal amounts of *R*- and *S*-warfarin. The pharmacologically active *S*-warfarin is eliminated almost entirely as *S*-7-hydroxywarfarin and a small amount of *S*-6-hydroxywarfarin in humans. In contrast, *R*-warfarin is converted mainly to *R*-6-hydroxywarfarin and some *R*-7-hydroxywarfarin. *In vitro* studies with human liver microsomes indicate that both 6- and 7-hydroxylation of *S*-warfarin are catalyzed exclusively by human CYP2C9, whereas 6- and 7-hydroxylation of *R*-warfarin is mediated mainly by human CYP1A2 and CYP2C19 (29). Coadministration of enoxacin, a quinoline-azaquinoline antibiotic, resulted in a decrease in the clearance of *R*-warfarin, but not in the clearance of *S*-warfarin (30). The decreased clearance of *R*-warfarin was found to be a consequence of inhibition by enoxacin on *R*-6-hydroxylation of the *R*-warfarin metabolic pathway. As expected, enoxacin did not affect the hypoprothrombinemic response produced by warfarin because this antibiotic had no effect on *S*-warfarin elimination (30).

Although pharmacokinetic consequences become significant only when a major pathway is affected, some metabolites are so potent that inhibition of a minor pathway may result in undesirable clinical consequences. Codeine is metabolized extensively by glucuronidation. The *O*-demethylation of codeine to morphine is a minor pathway which is mediated by CYP2D6 (31). As only a small fraction of the drug is metabolized by the *O*-demethylated pathway, inhibition of CYP2D6 by other drugs will have little effect on the pharmacokinetic consequences of codeine. However, inhibition of CYP2D6 will have a significant effect on the pharmacodynamic consequences of codeine. Since codeine is often administered with other drugs which inhibit CYP2D6, this offers a scope for interactions which could modulate the efficacy in patients.

Drug interactions may relate to specific competitive inhibition of polymorphic enzymes. Omeprazole, a proton pump blocker, is metabolized mainly by CYP2C19 (32). Diazepam, an anti-anxiety agent, also is metabolized predominantly by CYP2C19 (32). The CYP2C19 isoform is known to be polymorphic. Coadministration of omeprazole resulted in a significant increase in the AUC of diazepam in extensive metabolizers (EMs), but had no effect on the diazepam AUC in PMs (32). Since both omeprazole and diazepam are metabolized mainly by the same enzyme, CYP2C19, this explains why the two drugs interact in EMs, but not in PMs. In PMs, there is no enzyme that diazepam and omeprazole could compete for.

In addition to the reversible competitive inhibitors, a number of drugs have been shown to be irreversible P-450 inhibitors *via* enzyme destruction or MI complexation. This class of drugs characteristically exhibits time- and dose-dependent pharmacokinetics when given orally or intravenously. L-754,394, an experimental HIV protease inhibitor, is a good example. *In vitro* microsomal studies revealed that L-754,394 is a mechanism-based inhibitor. Kinetic studies in rats, dogs and monkeys have shown that the drug exhibits time- and dose-dependent pharmacokinetics (33). The apparent clearance decreased with increasing dose. However, the dose-dependency cannot be explained by Michaelis-Menten kinetics. L-754,394 in plasma declined log-linearly with time, but with an apparent $t_{1/2}$ that increased with dose. Furthermore, the apparent clearance of L-754,394 decreased after multiple dosing. It is clear that inactivation of metabolic enzymes depends not only on the dose, but also on the duration and frequency of dosing.

Since the pharmacokinetics of drugs that inactivate enzymes are time- and dose-dependent, the drug interaction caused by enzyme inactivation will depend on the time schedules of administration of enzyme inactivators and other drugs, as well as their doses. For example, L-754,394, a mechanism-based inactivator, increased the AUC of indinavir in the plasma of rats by approximately 2-fold when L-754,394 was given at an oral dose of 10 mg/kg simultaneously. However, when L-754,394 was given orally 1 hr prior to the oral administration of indinavir, L-754,394 caused a 5-fold increase in the plasma AUC of indinavir.

As previously discussed, reversible inhibition is of a transient nature; the normal function of P-450 enzymes continues after the inhibitor has been eliminated from the body. In contrast, the loss of enzyme activity caused by irreversible inactivation persists even after elimination of the inhibitor, and *de novo* biosynthesis of new enzymes is the only means by which activity can be restored. Clearly, clinical and pharmacokinetic consequences of irreversible drug inhibition will be quite complicated, depending on the duration and frequency of dosing. The chronic effect of irreversible inhibition on cytochrome P-450s is yet unknown, and further studies need to address this question.

Metabolism-based drug interaction is usually regarded as potentially dangerous, or at least undesirable. However, there are times when these interactions may be exploited. For example, co-administration of ritonavir, an HIV protease inhibitor, enhanced the oral absorption of saquinavir, another HIV protease inhibitor. Saquinavir has a poor bioavailability, less than 5%. Following a single dose of ritonavir (600 mg) and saquinavir (200 mg), a greater than 50-fold increase in the plasma AUC of saquinavir was observed in human volunteers (34). The combination regimen of ritonavir and saquinavir may be a potential benefit for the treatment of AIDS.

CONCLUSIONS

From the viewpoint of drug development, it is desirable to develop a new drug candidate that is not a potent P-450 inhibitor and is not readily inhibited by other drugs to avoid potential drug-drug interaction. However, in reality, drug interaction by mutual inhibition between drugs is almost inevitable, since P-450-mediated metabolism represents a major route of elimination of many drugs, which can compete for the same P-450 enzyme. It should be emphasized that only a few drug interactions, but not all of them, are clinically significant. The clinical significance of a metabolic drug interaction will depend on the magnitude of the change in the concentration of active species (parent drug and/or metabolites) at the site of pharmacological action and the therapeutic index of the drug. The smaller the difference between toxicity and efficacy, the greater is the likelihood that a drug interaction will have serious clinical consequences. Thus, careful evaluation of potential drug interactions of a new drug candidate during the early stage of drug development is essential.

With the recent advances in the technologies of *in vitro* drug metabolism and the advent of analytical techniques, it is possible to use *in vitro* systems to predict potential drug interactions *in vivo*. The *in vitro* drug interaction studies are carried out based primarily on two main criteria: the likelihood of concurrent use and the therapeutic index. In order to accurately predict potential metabolic drug interaction, it is necessary to know the underlying mechanisms of drug inhibition, the metabolic fate of the drug, and the enzyme involvement in each metabolic pathway. Finally, an under-

standing of pharmacokinetic principles will facilitate the extrapolation of *in vitro* data to the *in vivo* situation.

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SECTION VII. TRENDS AND PERSPECTIVES

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Chapter 31. To Market, To Market - 1996

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Introduction of new chemical entities (NCEs) for human therapeutic use into the world market for the first time during 1996 totaled 38 (1). This represents a slight increase from 36 in 1995, 1992 and 1991 (2,3,4), but a decrease from 44 in 1994 (5) and 43 in 1993 (6).

Japan, historically a leader in worldwide NCE introductions, fell to fifth place with five compounds introduced. The US took the number one position with 17 NCEs followed closely by the UK with 16. Germany ranked third with 12 introductions and Denmark was fourth with seven. France, Japan, Sweden and Switzerland all tied for fifth place with five NCEs each. The remainder of the introductions were distributed over 15 countries. This trend continues when one examines the ranking for originators of NCEs. The US was first with 10 closely followed again by the UK with eight. Japan tied with Germany for third with 5 NCEs each. Switzerland was fourth, Denmark was fifth and Australia, France, Netherlands and Sweden all tied for fifth place.

Two new HIV protease inhibitors were launched after record setting reviews by the FDA. Abbott's Norvir, the second HIV protease inhibitor, broke Saquinavir's record of 97 days when it was approved in 71 days. Shortly thereafter Crixivan by Merck exceeded this mark by being approved in 42 days. Anti-viral agents were well represented last year with five new NCEs, including the two new HIV protease inhibitors. CNS agents were the most active therapeutic area with 11 new launches. This included Avonex which was able, as demonstrated by clinical trials, to slow the progression of relapsing multiple sclerosis. In addition, Ultiva, an esterase metabolized opioid, is the first entry to this new class and riluzole is the first drug approved for ALS. Many other one-of-a-kind products were launched in 1996. Accolate is the first leukotriene receptor antagonist for asthma. Open-angle glaucoma can be treated by the first selective α_2-agonist, Alphagan. Xalatin is a once a day treatment for glaucoma that has a unique mechanism of action. It increases the flow of aqueous humor rather than reduces its production. Lilly introduced Humalog which is the first of a new type of insulin analog that mimics the normal insulin response. Covert is a new cardiovascular agent that is the first drug approved for the rapid conversion of atrial fibrillation and flutter to normal sinus rhythm. Antineoplastic agents saw the introduction of Hycamtin, the first of a new class of drugs that inhibit topoisomerase I. Products from recombinant technology totaled six in number. Of these, reteplase, is the only thrombolytic agent with a double bolus injection formulation. These products also have the added benefit of a reduced side effect profile due to their high purity.

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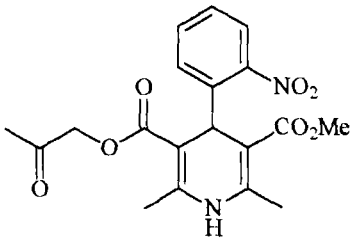
Aranidipine (antihypertensive) (7-15)

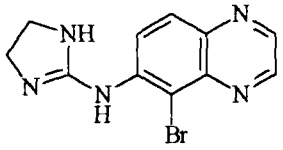
Country of Origin:	Japan
Originator:	Maruko Seiyaku
First Introduction:	Japan
Introduced by:	Taiho
Trade Name:	Bec/Sapresta
CAS Registry No.:	86780-90-7
Molecular Weight:	388.38



Aranidipine was launched in Japan last year as an antihypertensive agent. Its pharmacological effects are similar to other dihydropyridine derivatives, e.g., nefidipine, however, it is more potent and longer lasting. This is partially due to the fact that its initial metabolite (ketone reduction) is just as effective as the parent compound. Aranidipine exerts its activity by blocking Ca^{+2} entry during depolarization via L-type voltage-gated Ca channels. This causes decreased levels of intracellular Ca which leads to enhanced relaxation of smooth and cardiac muscle. It is a selective α_2-adrenoreceptor antagonist which inhibits vasoconstrictive responses. As a dihydropyridine derivative, it can be synthesized via a modified Hantsch synthesis. While sold as a racemate, the (S)-enantiomer is 150 times more active than the (R)-antipode.

Brimonidine (antiglaucoma) (16-23)

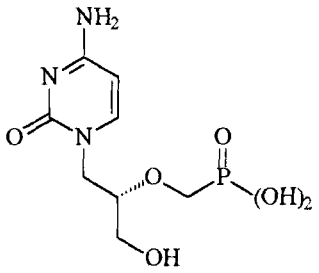
Country of Origin:	USA
Originator:	Allergan
First Introduction:	USA
Introduced by:	Allergan
Trade Name:	Alphagan
CAS Registry No.:	59803-98-4
Molecular Weight:	442.23



Launched in the US for open-angle glaucoma and ocular hypertension, brimonidine is a relatively selective and potent α_{2a}-adrenergic agonist with low affinity for the imidazoline I_1 receptor. Topical application reduces intraocular pressure. This bypasses any central hypotensive effects at the I_1 receptor (which can also give rise to a decrease in blood pressure and heart rate) if given systemically, since topical application results in low plasma levels concomitant with rapid systemic elimination. Brimonidine can be prepared in a four-step sequence from 4-nitrophenylenediamine.

Cidofovir (antiviral) (24-32)

Country of Origin:	USA
Originator:	Gilead Sciences
First Introduction:	USA
Introduced by:	Gilead Sciences
Trade Name:	Vistide
CAS Registry No.:	113852-37-2
Molecular Weight:	319.51



Cidofovir launched last year as a first-line treatment for CMV retinitis in AIDS patients. It is a nucleotide analog with potent activity against a broad spectrum of DNA viruses, e.g., HSV1, HSV2, CMV, adenovirus and papillomavirus. Cidofovir can be synthesized by a number of methods, the most efficient involves ring

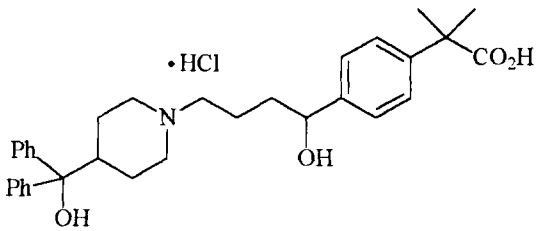
openning of (R)-glycidol with cytosine. Metabolically, cidofovir does not require intracellular activation by virally-encoded enzymes like similar compounds, e.g., aciclovir or ganciclovir. It is rapidly converted to its active form, cidofovir diphosphate, which inhibits viral DNA polymerase at concentrations up to 600 fold lower than that required for human DNA polymerase. It is a competitive inhibitor of dCTP incorporation or if incorporated into viral DNA slows down further DNA synthesis and causes the destabilization of the viral DNA. There are three intracellular metabolites which are also active thus giving rise to the long half-life. This results in lower dosing times (once every 1-2 weeks) and the ability to protect previously uninfected cells from subsequent infection.

Factor VIIa (haemophilia) (33-36)

Country of Origin:	Denmark	Introduced by:	Novo Nordisk
Originator:	Novo Nordisk	Trade Name:	NovoSeven
First Introduction:	Denmark	CAS Registry No.:	151821-07-7

Recombinant Factor VIIa was developed as a treatment for hemophilia A (Factor VIII deficient) and B (Factor IX deficient) and was launched in Denmark, Germany, Switzerland and the UK for hemophiliacs that have developed inhibitors to Factors VIII and IX. Expression of cloned human Factor VII in baby hamster kidney cells resulted in secretion of the compound in a single-chain form. It spontaneously converted, during purification, to VIIa and was shown to have the identical amino acid sequence to plasma-derived Factor VIIa, including a similar carbohydrate structure and γ-carboxylation at 10 glutamic acid residues. Factor VIIa, *in vivo*, has inhibitor bypassing activity. A Factor VIIa-tissue factor complex can activate Factor IX and X thus bypassing Factor VIII. Since this complex is required for proteolytic activity, Factor VIIa can circulate throughout the body with no effect. Only when it comes in contact with tissue factor at a site of injury does activation occur.

Fexofenadine (antiallergic) (37-39)



Country of Origin:	USA	Trade Name:	Allegra
Originator:	Sepracor	CAS Registry No.:	138452-21-8
First Introduction:	USA	Molecular Weight:	538.13
Introduced by:	Hoechst Marion Roussel		

The metabolite of seldane (terfenadine), fexofenadine, was launched last year in the USA. A histamine H_1 receptor antagonist, fexofenadine is more effect than its parent compound without the associated side-effects.

Follitropin alfa (fertility enhancer) (40-42)

Country of Origin:	Switzerland	Introduced by:	Serono
Originator:	Genzyme	Trade Name:	Gonal-F
First Introduction:	Austria	CAS Registry No.:	9002-68-0

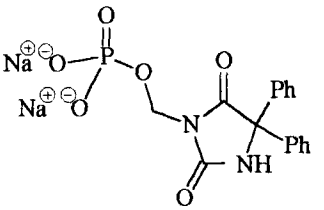
Gonal-F was launched in Austria, Germany, Denmark, Ireland, Italy, Norway, Sweden, Switzerland, France and the UK for the treatment of infertility. The recombinant follicule stimulating hormone (recFSH) was produced by introducing the genes for the α- and β-FSH subunits into the genome of Chinese hamster ovary cells. The isolated recFSH was a glycosylated bioactive dimer that appeared to be identical to urinary human FSH. Biochemically, the recFSH was equal to or slightly better to the uroFSH. The ultra-high purity of recFSH makes it less prone to side effects from contaminants in uroFSH and can be injectable.

Follitropin beta (fertility enhancer) (43-45)

Country of Origin:	Netherlands	Introduced by:	Organon
Originator:	Organon	Trade Name:	Puregon
First Introduction:	Denmark	CAS Registry No.:	9002-68-0

Puregon was launched in Denmark, Germany and the UK for ovulation induction in clomiphene-resistent anovulation and controlled ovarian hyperstimulation. Transfected Chinese hamster ovary cells with plasmids containing the genes encoding the α- and β-FSH subunits produced recFSH that was similar to uroFSH. In both cases (follitropin α and β) no antibody formation was detected, however, this form appeared to be more active than follitropin α in that there was a 25% greater pregnancy rate compared to uroFSH.

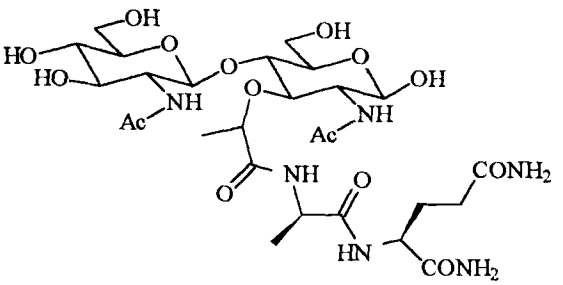
Fosphenytoin sodium (antiepileptic) (46-47)



Country of Origin:	USA
Originator:	Warner-Lambert
First Introduction:	USA
Introduced by:	Warner-Lambert
Trade Name:	Cerebyx
CAS Registry No.:	93390-81-9
Molecular Weight:	406.24

Cerebyx was launched last year for the treatment of status epilepticus and for neurosurgery derived seizures. Fosphenytoin is rapidly converted by phosphatases to phenytoin. This compound is readily water soluble thus overcoming an important problem associated with Dilantin.

GMDP (immunostimulant) (48-51)

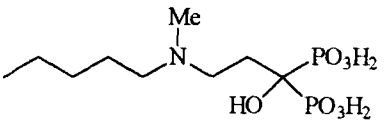


Country of Origin:	Australia	Introduced by:	Peptech
Originator:	Peptech	Trade Name:	Likopid
First Introduction:	Russia		

N-acetyl-β-D-glucosaminyl-(1→4)-N-acetylmuramyl-L-alanyl-D-isoglutamine was launched in Russia for hospital-related infections, psoriasis, cervical pre-cancerous lesions and ophthalmic keratitis caused by herpes. It was found to be less pyrogenic than MDP, the minimally active component of Freund's complete adjuvant. While chemical synthesis is possible, a less laborious method (3 steps) involves the enzymatic degradation of a bacterial peptidoglycan. GDMP is orally active with a low toxicity that acts as an adjuvant to highly purified protein antigens to stimulate the induction of $CD8^+$ T-cell response. It was also able to reduce the levels of TNF-α, IL-1α and hypoglycemia induced by LPS administration and to inhibit systemic response to endotoxin.

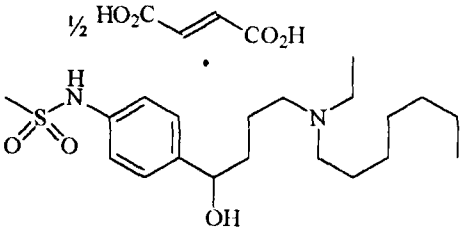
Ibandronic acid (osteoporosis) (52-56)

Country of Origin:	Germany
Originator:	Boehringer Mannheim
First Introduction:	Germany
Introduced by:	Boehringer Mannheim
Trade Name:	Bondronat
CAS Registry No.:	114084-78-5
Molecular Weight:	319.23



This third generation biphosphonate was launched in Austria and Germany for the treatment of bone disorders such as hypercalcemia in malignancy and osteolysis, Paget's disease and osteoporosis. It does not effect bone mineralization, therefore, the potential risk of osteomalacia is prevented. This was a problem with first generation derivatives. While the exact mode of action is not understood, they are inhibitors of osteroclast mediated bone resorption. These compounds strongly interact with hydroxyapatite crystals and have a half-life in the skeleton of several years. Despite this observation ibandronate was well tolerated and safe.

Ibutilide fumarate (antiarrhythmic) (57-61)

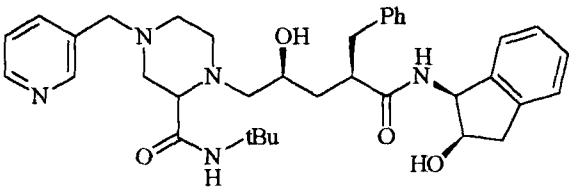


Country of Origin:	UK	Trade Name:	Corvert
Originator:	Pharmacia & Upjohn	CAS Registry No.:	122647-32-9
First Introduction:	USA	Molecular Weight:	885.25
Introduced by:	Pharmacia & Upjohn		

Covert was launched in the US and UK for treatment of atrial fibrillation and flutter and can be synthesized in three steps from N-phenyl methanesulfonamide. While ibutilide has an asymmetric center, it has been determined that the racemate is equipotent with either enantiomer. The antiarrhythmic action is derived from the compounds ability to prolong the action potential duration and lengthen the refractory period of myocardial tissue. Class III antiarrhythmic agents accomplish this by blocking outward potassium channels, however, ibutilide elicits the same effect by activation of slow inward sodium channels. Recent evidence indicates that it also is

a potent blocker of the rapidly acting delayed rectifier potassium current (I_{Kr}) and may block the ATP-inhibited potassium channel.

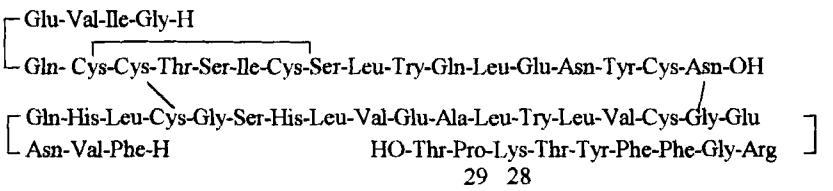
Indinavir sulfate (antiviral) (62-67)



Country of Origin:	USA	Trade Name:	Crixivan
Originator:	Merck	CAS Registry No.:	150378-17-9
First Introduction:	USA	Molecular Weight:	613.79
Introduced by:	Merck		

Crixivan was launched in Australia, Switzerland, the UK and the US last year as an orally-bioavailable HIV-1 protease inhibitor. The compound can be prepared by coupling an optically active piperazine derivative with an epoxide derivative (now commercially available). The synthesis of the proteins, reverse transcriptase, integrase, structural proteins and a protease, required by the virus to complete its lifecycle, can be interupted if the protease enzyme is not capable of cleaving a pro-form polypeptide chain into these components. Indinavir inhibits this process and is more potent than the first approved protease inhibitor saquinavir. This effect was noted by the increase in $CD4^+$ cells and a decrease in HIV RNA levels. Since indinavir is metabolized by the CYP3A4 isozyme, care must be taken with patients with hepatic insufficiency and to sex-related differences in the level of this enzyme. Other than nephrolithiasis (5%), indinavir is relatively safe and well tolerated.

Insulin lispro (antidiabetic) (68-73)



Country of Origin:	USA	Trade Name:	Humalog
Originator:	Lilly	CAS Registry No.:	133107-64-9
First Introduction:	USA	Molecular Weight:	5,807.69
Introduced by:	Lilly		

Humalog was launched in 1996 in Australia, Denmark, Germany, Russia, S. Africa, Spain, Sweden, Switzerland, Canada, Czech Republic, Lithuania, the UK and the US as a fast-acting human insulin analog. It was prepared by recombinant DNA technology using a non-disease producing laboratory strain of *E. coli* containing the gene for insulin lispro precursor protein and purified as a zinc complex. Conceptually designed from insulin-like growth factor I (IGF-I) the amino acid residues 28 and 29 of B chain have been inverted. This modification reduces the propensity to form dimers and the dimers to form hexamers. Thus this form of insulin has an increased rate of absorption since dissociation of the hexamer to monomer is the rate limiting

step. The rapid absorption and short duration of action results in a significant advantage in controlling blood glucose levels after meals compared to normal insulin.

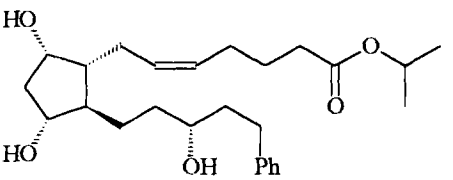
Interferon β-1a (Multiple Sclerosis) (74-78)

Country of Origin:	USA	Trade Name:	Avonex
Originator:	Biogen	CAS Registry No.:	145258-61-3
First Introduction:	USA	Molecular Weight:	20,025.23
Introduced by:	Biogen		

Launched in the US for relapsing forms of multiple sclerosis (MS), interferon β-1a is the second compound to be marketed for MS next to interferon β-1b (Betaseron). This form of β-interferon is a glycosylated polypeptide of 166 amino acids (sequence identical to human) produced from cultured Chinese hamster ovary cells containing the engineered human gene for interferon β-1a. The difference in structure to interferon β-1b lies in the substitution of a cysteine for a serine and in the fact it is glycosylated. The 1b form requires reconstitution and subcutaneous injection every other day, while the 1a form is an intramuscular injection once a week. The exact mode of action for the β-interferons has not been delineated, however, it is thought that they reverse a suppressor cell defect and favor the generation of T helper-2 type responses. It is presumed that the immunoregulatory and antiviral actions, in addition to the antagonistic effect they have on γ-interferon (γ-interferon has been shown to exacerbate the symptoms of MS), provide an overall beneficial effect.

Latanoprost (antiglaucoma) (79-85)

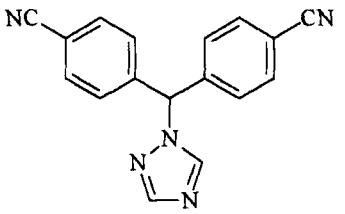
Country of Origin:	UK
Originator:	Pharmacia & pjohn
First Introduction:	USA
Introduced by:	Pharmacia & pjohn
Trade Name:	Xalatan
CAS Registry No.:	130209-82-4
Molecular Weight:	432.60



Xalatan was launched in Sweden, Switzerland and the US for the treatment of glaucoma. It can be synthesized, from the Corey lactone, using standard prostaglandin chemistry. Latanoprost is a $PGF_{2\alpha}$ analog that is more lipophilic thus is better able to penetrate the cornea. The (15R)-diastereomer has only 10% of the activity compared to the (15S)-diastereomer. It is a selective FP receptor agonist with little or no effect on the other prostanoid receptors. The antiglaucoma effects are the result of reduced intraocular pressure arising by increasing uveoscleral outflow with little or no effect on trabeculo-canalicular aqueous outflow and no effect on retinal vasculation or permeability of the blood-aqueous barrier. The topical application is sufficient for a single daily dosage and is well tolerated with no detectible conjunctival hyperaemia.

Letrazole (anticancer) (86-92)

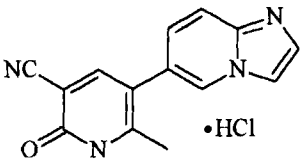
Country of Origin:	Switzerland	Trade Name:	Femara
Originator:	Novartis	CAS Registry No.:	112809-51-5
First Introduction:	France	Molecular Weight:	285.31
Introduced by:	Novartis		



Femara was launched in France and the UK for second-line treatment of advanced breast cancer. Letrazole can be synthesized in two steps from 4-bromomethyl-benzonitrile with 1,2,4-triazole and is a third generation aromatase inhibitor. It is a highly specific inhibitor of $P450_{arom}$ which prevents the conversion of androstenedione to estrone. The reduction of plasma estrogen was immediate and long lasting. This is accomplished with no inhibition of other steroid biosynthesis making it the most selective aromatase inhibitor tested. Letrazole has remarkable antitumor activity, is well tolerated and has no toxic side effects. It is 10,000 times more potent than aminoglutethimide, *in vivo*, the first well established aromatase inhibitor.

Loprinone hydrochloride (cardiostimulent) (93-95)

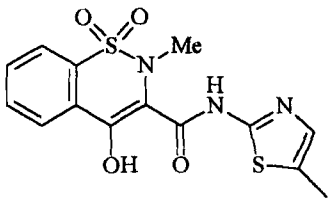
Country of Origin:	Japan
Originator:	Eisai
First Introduction:	Japan
Introduced by:	Eisai
Trade Name:	Coatec
CAS Registry No.:	119615-63-3
Molecular Weight:	250.26



Coatec was launched in Japan for acute cardiac insufficiency in cases resistant to other treatments. There are three related synthetic routes, 5-6 steps each, to loprinone all converging on 1-(imidazo[1,2a]pyridyl-6-yl)-2-propanone as an advanced intermediate. It is a potent and selective inhibitor of PDE III and a long lasting, orally active, positive inotropic agent. The PDE III inhibition caused increased levels of cAMP which leads to the positive inotropic effect that was not altered by β or H_2-receptor antagonists. It was 100 times less potent at PDE I and PDE II with no Na-K-ATPase activity. Improved hemodynamic parameters with slight changes in heart rate and blood pressure were seen *in vivo*. It is not mutagenic and its primary metabolite was less active.

Meloxicam (antiarthritic) (96-101)

Country of Origin:	Germany
Originator:	Boehringer Ingelheim
First Introduction:	UK
Introduced by:	Boehringer Ingelheim
Trade Name:	Mobec
CAS Registry No.:	71125-38-7
Molecular Weight:	351.41

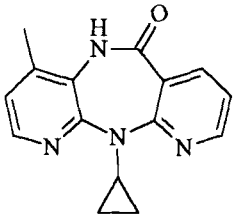


Mobec was launched in Columbia, Denmark, France, Germany, Ireland, Italy, Netherlands, S. Africa, Sweden, and the UK for osteo- and rheumatoid arthritis as an NSAID. It can be synthesized in four steps from benzisothiazolo-3(2H)-one-

1,1-dioxide. By shutting down prostaglandin synthesis, it has antiinflammatory, antipyretic and analgesic properties. This is accomplished by preferentially inhibiting the COX-2 system relative to the COX-1 which also leads to an improved GI safety profile relative to naproxen, diclofenac and prioxicam. It can also interfere with neutrophil function like degranulation. Meloxicam did not inhibit proteoglycan synthesis in osteroarthitic cartilage or chondrocytes and had no effect on platelet aggregation. It is metabolized by the P450 2C9 system into four metabolites which are all inactive.

Nevirapine (antiviral) (102-106)

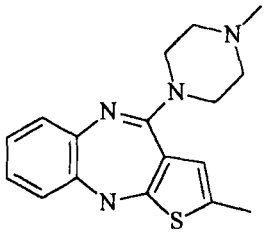
Country of Origin:	Germany
Originator:	Boehringer Ingelheim
First Introduction:	USA
Introduced by:	Boehringer Ingelheim
Trade Name:	Viramune
CAS Registry No.:	71125-38-7
Molecular Weight:	266.30



Viramune was launched in the US for use in combination with nucleoside analogs to treat HIV-infected adults who have experienced clinical and/or immulogic deterioration. It can be prepared in four steps from 2-chloro-4-methyl-3-nitropyridine. It is a potent inhibitor of HIV-1 reverse transcriptase with no muscarinic or benzodiazepine activity. Mechanistically, it is a non-competitive non-nucleoside inhibitor. It has a low toxicity for uninfected cells most likely due to its specificity, i.e., it does not inhibit eukaryotic DNA polymerase α, β, γ and δ. HIV-2, SIV and FeLV are not affected by nevirapine and monotherapy is limited by the rapid onset of resistance.

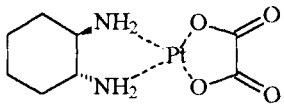
Olanzapine (neuroleptic) (107-110)

Country of Origin:	USA
Originator:	Lilly
First Introduction:	UK
Introduced by:	Lilly
Trade Name:	Zyprexa
CAS Registry No.:	132539-06-1
Molecular Weight:	312.44



Zyprexa was launched in Canada, Germany, the UK and US as an antipsychotic agent. Prepared in three steps via the intermediate diazepinone, it is an atypical antipsychotic with a high affinity for dopaminergic and serotonergic receptors. Specifically, olazapine is a potent 5-HT_2/D_2 antagonist with anticholinergic activity. It has a greater antagonistic effect at 5-HT_{2a} than at dopamine D_2 receptors and *in vivo* is clozapine-like. Thus it is less likely to produce extrapyramidal side effects and does not produce any granulocytopenia. Its 10 metabolic products are all inactive.

Oxaliplatin (anticancer) (111-116)

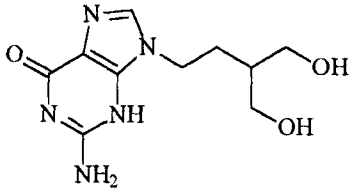


Country of Origin:	Switzerland	Trade Name:	Eloxatin
Originator:	Bebiopharm	CAS Registry No.:	61825-94-3
First Introduction:	France	Molecular Weight:	397.29
Introduced by:	Sanofi		

Eloxatin was launched in France for second-line treatment of metastatic colorectal cancer. Oxaliplatin is a second generation platinum drug prepared in three steps from either K_2PtCl_4 or K_2PtI_4. It has an antitumor spectrum similar to cisplatin, however, it is more effective against L1210 leukemia and cisplatin resistant L1210. It is also effective against B16 melanoma but has a dose limiting toxicity of peripheral sensory neuropathy that is reversible upon cessation of the drug. The (R,R)-enantiomer has greater activity than the (S,S)-isomer but this is tumor line dependent, e.g., there was no difference found for P-388 or Sarcoma 180. Clinical drug administration based on circadium timing showed it was better tolerated when given 16 h after the onset of light. Oxaliplatin binds to guanine-N7 and can lead to bidentate chelation that results in the bending of DNA. This feature is recognized by high mobility group proteins (HMG) which impedes repair reactions and stops replication and transcription.

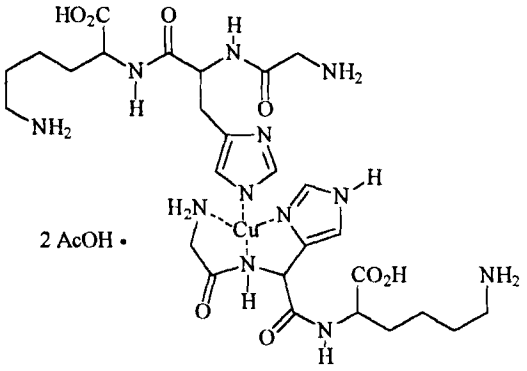
Penciclovir (antiviral) (117-122)

Country of Origin:	UK
Originator:	SmithKline Beecham
First Introduction:	UK
Introduced by:	SmithKline Beecham
Trade Name:	Vectavir
CAS Registry No.:	39809-25-1
Molecular Weight:	253.26



Vectavir was launched in the UK for herpes labialis. Penciclovir is synthetically available by two routes of four steps each from 2-(hydroxymethyl)butane-1,4-diol and is active against HSV-1, HSV-2 VZV but has limited activity against CMV. Vectavir is an acyclic guanosine analog that acts as a competitive inhibitor of DNA polymerase. It is a metabolic product of famcyclovir that is preferentially phosphorylated by viral infected cells (by thymidine kinases) over normal cells. The triphosphate has a low activity against cellular DNA polymerase which is one possible explanation for its low toxicity. While its spectrum of activity is similar to acyclovir, it is longer acting because its triphosphate is 20 times more stable and is not metabolized.

Prezatide copper acetate (vulnerary) (123-127)

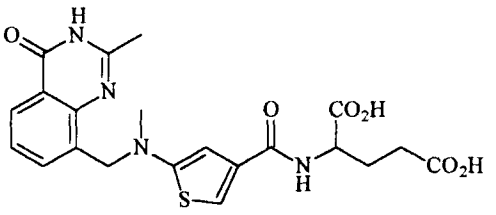


Country of Origin:	USA	Trade Name:	lamin gel
Originator:	ProCyte	CAS Registry No.:	130120-57-9
First Introduction:	USA	Molecular Weight:	862.40
Introduced by:	ProCyte		

lamin gel was launched in the US for the care of chronic and acute wounds. It can be prepared by combining the tripeptide GHK and copper (II) acetate. There are several species present in equilibrium but the bis-complex is the dominant compound at neutral pH. The GHK sequence is found in only eight human proteins and is an endogenous growth factor that stimulates collagen synthesis and angiogenesis. This is part of the explanation for its wound healing and tissue repair ability. It can significantly delay fibroblast activation, is a growth factor for hepatocyctes, neurons, thyroid follicular cells and is a chemoattractant for monocytes, macrophages, mast cells and capillary endothelial cells. Copper is also known to have an effect on lysyl oxidase which is a key enzyme involved in collagen formation.

Raltitrexed (anticancer) (128-132)

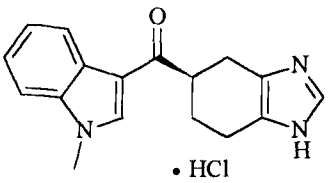
Country of Origin:	UK
Originator:	Zeneca
First Introduction:	UK
Introduced by:	Zeneca
Trade Name:	Tomudex
CAS Registry No.:	112887-68-0
Molecular Weight:	458.49



Tomudex was launched in Ireland, France, Luxembourg and the UK for advanced colorectal cancer and it was prepared in a convergent manner (6 steps) from diethyl L-glutamate and 6-bromomethyl-2-methyl-quinazolin-4(3H)-one. Tomudex is a highly selective inhibitor of thymidylate synthase (TS), the key enzyme in the biochemical conversion of dUMP to dTMP. It enters the cell via the reduced folate/methotrexate cell membrane carrier and is converted to the polyglutamate species by folylpolyglutamate synthase within 4h where it then binds to the folate substrate site of TS. Clinically, it had a 29% response rate in patients with advanced colorectal cancer. It is water soluble, can be administered as a single dose every three weeks and had no hepto- or nephrotoxicity.

Ramosetron (antiemetic) (133-135)

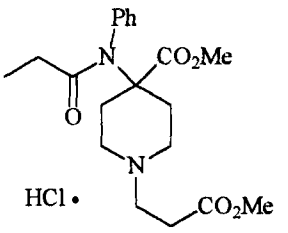
Country of Origin:	Japan
Originator:	Yamanouchi
First Introduction:	Japan
Introduced by:	Yamanouchi
Trade Name:	Nasea
CAS Registry No.:	132907-72-3
Molecular Weight:	279.34



Nasea was launched in Japan for chemotherapy-induced emesis. Nasea was prepared by a four step sequence via the Vilsmeier-type coupling of 1-methylindole and 5-(1-pyrrolidoncarbonyl)-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride. The antiemetic activity arises because it is a potent 5-HT3 receptor antagonist that is i.v. and orally active. The (R)-isomer was found to be 100 times more potent than the (S)-isomer. It was able to inhibit cisplatin-induce emesis and was 8670 times more potent than netoclopramide.

Remifentanil HCl (analgesic) (136-142)

Country of Origin:	UK
Originator:	Glaxo Wellcome
First Introduction:	Germany
Introduced by:	Glaxo Wellcome
Trade Name:	Ultiva
CAS Registry No.:	132539-07-2
Molecular Weight:	412.92



Ultiva was launched in Germany and the US for use in general anesthesia and immediate post-operative pain management. This compound can be prepared via the Michael addition of a 4,4-disubstituted piperidine to methyl acrylate. It behaves as a specific μ-opiod agonist that is about 30 times more potent than its chemical relative alfentanil. While its pharmacodynamic properties are similar to other μ agonists, remifentanil has unique pharmacokinetic properties. It has a rapid onset (1.6 min) and a rapid offset (5.4 min) independent of duration of administration. This has been attributed to its rapid catabolism by general esterases to the corresponding acrylic acid derivative. The metabolite has 0.01 to 0.0005 times the activity of remifentanil and is excreted via the kidneys (80% recovery).

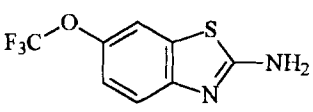
Reteplase (fibrinolytic) (143-149)

Country of Origin:	Germany	Trade Name:	Retavase
Originator:	Boehringer Mannheim	CAS Registry No.:	133652-38-7
First Introduction:	USA	Molecular Weight:	39,571.89
Introduced by:	Boehringer Mannheim		

Reteplase was launched in Germany and the US for acute myocardial infarction. Retevase is a single chain recombinant form of tissue plasminogen activator (t-PA) with all except the kringle-2 and protease domains deleted. When expressed in *E. coli* the inactive form is produced and stored in inclusion bodies. After purification and an *in vitro* refolding process the active form is obtained. The expression in *E. coli* gives rise to a non-glycosylated protein. This and the deletion of several domains allows reteplase to have a longer half-life and a slower clearance. Despite the deletions and refolding the catalytic center remains intact. The kringle-2 domain allows binding to lysine in fibrin which makes it effective for the rapid and complete lysis of coronary thrombi. While it does not have a high affinity for binding to fibrin, its activity is stimulated by it and has the same level of *in vivo* fibrin selectivity as human t-PA. It has a low affinity for endothelial cells and can be administered as a double bolus injection. Netaplase, a similar recombinant form of t-PA, was launched by Mitsui/Mochida in Japan.

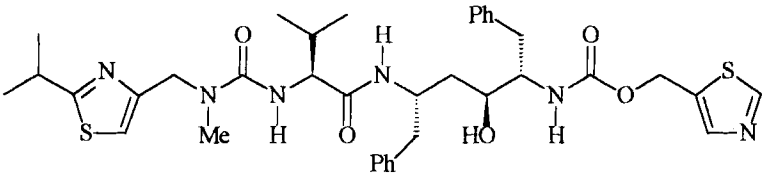
Riluzole (neuroprotective) (150-156)

Country of Origin:	France
Originator:	Rhone-Poulenc Rorer
First Introduction:	USA
Introduced by:	Phone-Poulenc Rorer
Trade Name:	Rilutek
CAS Registry No.:	1744-22-5
Molecular Weight:	234.20



Rilutek was launched in Germany, the UK and US (orphan drug status) for treatment of amyotrophic lateral sclerosis (ALS) and is the first drug approved for this indication. A one step synthesis from 4-(trifluoromethoxy)aniline provides a supply of the compound. The source of its neuroprotective and anticonvulsant activity is not clearly understood. It antagonizes excitatory amino acids and blocks presynaptic release of glutamate, is an antagonist of NMDA-induced acetylcholine release and inhibited glutamate and quisqualate induced increases in cGMP but does not bind to NMDA or Kainic receptors. Rilutek has no affinity for glutamate, GABA-benzodiazepine, glycine and adenosine receptors. It easily crosses the blood brain barrier and depresses glutamatergic neurotransmission, stabilizes voltage-dependent Na channels in their inactive form and activates G-protein dependent processes.

Ritonavir (antiviral) (157-160)

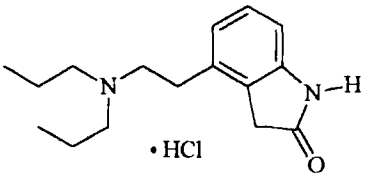


Country of Origin:	USA	Trade Name:	Norvir
Originator:	Abbott	CAS Registry No.:	155213-67-5
First Introduction:	USA	Molecular Weight:	720.93
Introduced by:	Abbott		

Norvir was launched in Germany, the UK and US for treatment of advanced HIV in combination with antiretroviral nucleoside analogs in a record 72 days by the FDA. It is an inhibitor of HIV aspartic protease which is critical in the processing of a propeptide into the *gag*, *gag-pol* gene products and the protease itself. This inhibition results in the release of non-infectous immature virus particles. It is greater than 500-fold more selective for viral aspartic protease than the human version, has good oral bioavailability and may increase the bioavailability of other protease inhibitors. Ritonavir was able to increase the CD4 and CD8 lymphocyte count as well as reduce viral RNA. It is more potent than saqunavir and comparible in potency to zidovudine and lamivudine.

Ropinirole HCl (antiParkinsonian) (161-165)

Country of Origin:	UK
Originator:	SmithKline Beecham
First Introduction:	UK
Introduced by:	SmithKline Beecham
Trade Name:	ReQuip
CAS Registry No.:	91374-20-8
Molecular Weight:	296.84

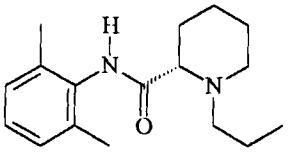


ReQuip was launched in the UK as a monotherapy or in combination with low-dose levodopa for the treatment of early-stage idiopathic Parkinson's disease. An eight step (24% overall yield) synthesis from isochroman provides access to ropinirole. It is a non-ergot postsynaptic dopamine D_2 agonist with activity in the extrapyramidal system. It has weak or no significant activity at α_2-adrenergic, 5-HT_2 receptors and is inactive at 5-HT_1, benzodiazepine, GABA, α_1 and β adrenoreceptors. Thus it has essentially no CNS side effects, fewer dyskinesias and on-off fluctuations. In animals, it was able to reverse all motor deficits induced by MPTP. Tolerance is

not developed and ropinirole has similar postsynaptic potency to apomorphine but with significantly less stereotyped behavior.

Ropivacaine (anesthetic) (166-171)

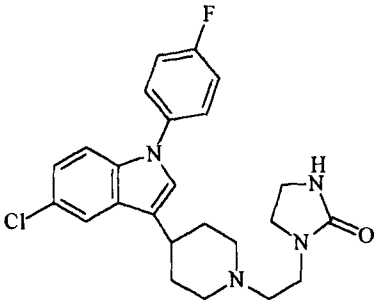
Country of Origin:	Sweden
Originator:	Astra
First Introduction:	Australia
Introduced by:	Astra
Trade Name:	Naropin
CAS Registry No.:	84057-95-4
Molecular Weight:	274.41



Naropin was launched in 1996 in Australia, Denmark, Finland, the Netherlands and Sweden as a local anesthetic. It can be prepared in a number of ways the most efficient involves a three step sequence beginning with L-pipecolic acid. This compound is the first one in this family to be produced as the pure (S)-enantiomer. The (R)-enantiomer has been shown to have cardiotoxic effects. Thus ropivicaine has less cardiovascular and CNS toxicity than bupivacaine. It is a Na channel blocker that is specific for affecting nerve fibers responsible for transmission of pain (Aδ and C) with no effect on fibers responsible for motor function (Aβ). Clinically, it has distinct advantages over bupivacaine. Its effects are slower in onset, less intense and have a shorter duration. This is a result of extensive metabolism in the liver to the 3-hydroxy isomer by CYP1A2 isoenzyme.

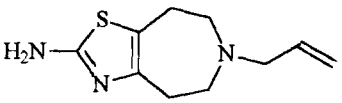
Sertindole (neuroleptic) (172-179)

Country of Origin:	Denmark
Originator:	Lundbeck
First Introduction:	UK
Introduced by:	Lundbeck
Trade Name:	Serdolect
CAS Registry No.:	106516-24-9
Molecular Weight:	440.95



Serdolect was launched in the UK for acute and chronic schizophrenia and schizoaffective psychoses. It is synthetically available in three steps from 5-chloro-1-(4-fluorophenyl)-1*H*-indole. Sertindole is selective for the limbic areas of the brain and has a high affinity for the serotinin 5-HT_2 receptor where it behaves as an antagonist. It has weak affinity for the α_1-adrenergic receptor and no affinity for dopamine D_2 receptors. It is as effective as haloperidol but without the extrapyramidal symptoms. This may be a consequence of the fact that it expresses the c-fos protein in a manner different for haloperidol but similar to clozapine. It is non-sedating, long lasting (several days after a single dose) and has an atypical mode of action.

Talipexole (antiParkinsonian) (180-185)



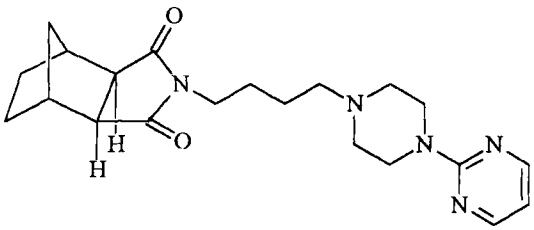
• 2 HCl

Country of Origin:	Germany	Trade Name:	Domin
Originator:	Boehringer Ingelheim	CAS Registry No.:	101626-70-4
First Introduction:	Japan	Molecular Weight:	209.32
Introduced by:	Boehringer Ingelheim		

Domin was launched in Japan for Parkinson's disease. The compound is readily accessible in two steps from 1-allylhexahydro-4*H*-azepin-4-one. Its antiParkinson activity resides in the fact that talipexole is a selective agonist for presynaptic dopamine D_2 receptors with no D_1 receptor activity. It is a clonidine-like α_2-adrenoreptor agonist and has fewer GI side effects than bromocriptine the most widely used dopamine angonist. Also it is 70 times more potent than bromocriptine and has a more rapid onset of action. In addition to its anti-tremor activity there was a sedative effect but no emesis. The latter probably a result of talipexole's 5-HT_3 receptor antagonistic activity. Its metabolites have no biological activity, there was no development of tolerence and its effect could be enhanced by L-dopa.

Tandospirone (anxiolytic) (186-190)

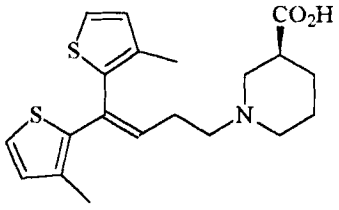
Country of Origin:	Japan
Originator:	Sumitomo
First Introduction:	Japan
Introduced by:	Sumitomo
Trade Name:	Sediel
CAS Registry No.:	87760-53-0
Molecular Weight:	575.62



Sediel was launched in Japan as an anxiolytic agent and is a member of the azapirone family. Ready access to the compound is attained from the exo Diels-Alder adduct of maleic anhydride and cyclopentadiene in a four step convergent approach. Biochemically, it is a partial agonist of the post-synaptic 5-HT_{1a} receptor. This metabotropic receptor is coupled to the G proteins and suppresses adenylate cyclase and phosphatidylinositol metabolism systems. It has low affinity for dopamine D_2, 5-HT_2 and α_1-adreneric receptors and no affinity for benzodiazepine, GABA or 5-HT_{1b} binding sites. The primary metabolic product is two orders of magnitude less active. Tandospirone is as effective as benzodiazepines in anxiolytic activity but does not have the side-effects (low abuse potential, weak sedative, no anticonvulsant activity).

Tiagabine (antiepileptic) (191-195)

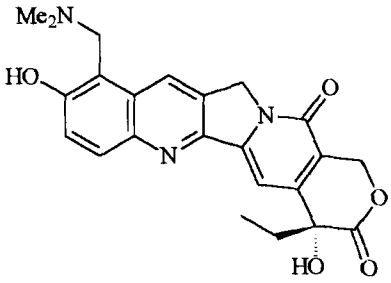
Country of Origin:	Denmark
Originator:	Novo Nordisk
First Introduction:	Denmark
Introduced by:	Novo Nordisk
Trade Name:	Gabitril
CAS Registry No.:	115103-54-3
Molecular Weight:	375.56



Gabitril was launched in Denmark for use as an add-on therapy in patients refractory to other epilepsy therapies. The compound can be synthesized in five steps beginning with a bis-thiophenyl ketone derivative to produce the (R)-(-)-enantiomer. Its anti-epileptic activity resides in its potent and selective inhibition of GABA synaptosomal uptake. Tiagabine is selective for the GAT-1 GABA transporter in neurons and glia thus enhancing inhibitory GABAergic transmission. Because it

has practically no effect on other uptake or receptor systems, it has a reduced potential for neurological side-effects. In particular, it does not have the benzodiazepine-like sedative effects. It is able to cross the blood brain barrier and is considered the most potent GABA uptake inhibitor known.

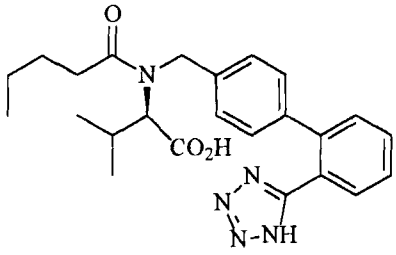
Topotecan HCl (anticancer) (196-201)



Country of Origin:	UK
Originator:	SmithKline Beecham
First Introduction:	USA
Introduced by:	SmithKline Beecham
Trade Name:	Hycamtin
CAS Registry No.:	123948-87-8
Molecular Weight:	457.92

Topotecan was launched in the US for the second-line treatment of ovarian cancer. It can be prepared in four steps from camptothecin and is a water soluble derivative of the natural product with decreased toxicity. Unlike its chemical relative irinotecan, topotecacan in not a prodrug and does not require bioactivation. It is an inhibitor of topoisomerase I. Specifically, it inhibits the release of topoisomerase I from DNA, where it relaxes supercoiled DNA, giving rise to single-strand breaks. When the replication fork reaches this complex, double-stand breaks can occur. This signals apoptosis and eventually gives rise to cell death. Evidence indicates hycamtin is safe for people with impaired hepatic function.

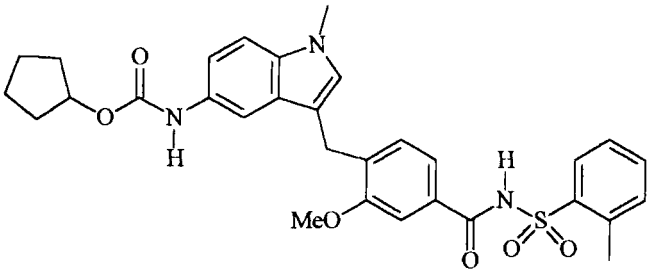
Valsartan (antihypertensive) (202-207)



Country of Origin:	Switzerland
Originator:	Norvartis
First Introduction:	Germany
Introduced by:	Novartis
Trade Name:	Diovan
CAS Registry No.:	137862-53-4
Molecular Weight:	435.53

Diovan was launched in Germany and the UK as an angiotensin II antagonist for use as an antihypertensive agent. Biphenylbromomethyl nitrile serves as the starting material for a three step synthesis of the compound, in which the (S)-enantiomer is more active than the (R)-enantiomer. Valsartan is a nonpeptide drug which is a highly specific antagonist of the AT_1 receptor and is potent and orally active. This receptor is responsible for angiotensin II cardiovascular effects (aldosterone and catecholamine secretion, vascular constriction, positive inotropic response and renal effects). Unlike losartan, it is not a prodrug and a single daily dose is comparible in activity to the ACE drug enalapril. It also did not exhibit the coughing side effect observed with ACE inhibitors. Diovan is slowly metabolized (long lasting) with its main metabolite being significantly less active. There was no evidence of rebound hypertension when drug treatment was terminated and was as effective as the dihydropyridine Ca antagonist anlodipine.

Zafirlukast (antiasthma) (208-215)



Country of Origin:	UK	Trade Name:	Accolate
Originator:	Zeneca	CAS Registry No.:	107753-87-6
First Introduction:	USA	Molecular Weight:	575.69
Introduced by:	Zeneca		

Accolate was launched in Ireland, Finland and the US for treatment of asthma. Prepared via an eight step synthesis from methyl 3-methoxy-4-methylbenzoate, zafirlukast acts as a LTD_4 antagonist and is the first compound of a new class of drugs. LTC_4, LTD_4 and LTE_4 were determined to be the constituents of the slow-reacting substance of anaphylaxis (SRS-A) which was found to induce asthma effects (bronchoconstriction, increased vascular permeability resulting in edema, cellular infiltration of airway tissues and decreased mucociliary transport). Thus an inhibitor of their synthesis would at least attenuate these symptoms. Zafirlukast binds to the CysLT receptor LT-1 and blocks the effect of LTC_4, LTD_4 and LTE_4. The drug is an oral twice daily formulation that reversed an LTD4 challenge, attenuated the response of platelet-activating factor (PAF), an allergen and cold air challenge and exercise-induced asthma.

References

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acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
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
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
alendronate sodium	osteoporosis	1993	29, 332
alfentanil HCl	analgesic	1983	19, 314
alfuzosin HCl	antihypertensive	1988	24, 296
alglucerase	enzyme	1991	27, 321
alminoprofen	analgesic	1983	19, 314
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 hydrochloride	topical antifungal	1991	27, 322
amosulalol	antihypertensive	1988	24, 297
ampiroxicam	antiinflammatory	1994	30, 296
amrinone	cardiotonic	1983	19, 314
amsacrine	*antineoplastic*	1987	23, 327
amtolmetin guacil	antiinflammatory	1993	29, 332
anastrozole	antineoplastic	1995	31, 338
angiotensin II	anticancer adjuvant	1994	30, 296
aniracetam	cognition enhancer	1993	29, 333
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
arotinolol HCl	antihypertensive	1986	22, 316
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324

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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
bambuterol	bronchodilator	1990	26, 299
barnidipine hydrochloride	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benazepril hydrochloride	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
benidipine hydrochloride	antihypertensive	1991	27, 322
beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betamethasone butyrate propionate	topical antiinflammatory	1994	30, 297
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328
bicalutamide	antineoplastic	1995	31, 338
bifemelane HCl	nootropic	1987	23, 329
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene hydrochloride	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bopindolol	antihypertensive	1985	21, 324
brimonidine	antiglaucoma	1996	32, 306
brodimoprin	antibiotic	1993	29, 333
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budralazine	antihypertensive	1983	19, 315
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 hydrochloride	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
carboplatin	antibiotic	1986	22, 318
carperitide	congestive heart failure	1995	31, 339

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
cefbuperazone sodium	antibiotic	1985	21, 325
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil hydrochloride	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317
cefotiam hexetil hydrochloride	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
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
cloricromen	antithrombotic	1991	27, 325
clospipramine hydrochloride	neuroleptic	1991	27, 325
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
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
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
dexibuprofen	antiinflammatory	1994	30, 298
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
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298
dorzolamide HCL	antiglaucoma	1995	31, 341
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
duteplase	anticougulant	1995	31, 342
ebastine	antihistamine	1990	26 302
ecabet sodium	antiulcerative	1993	29, 336
efonidipine	antihypertensive	1994	30, 299
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

GENERIC NAME	INDICATION	YEAR INTRO.	ABMC VOL., PAGE
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
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
eptazocine HBr	analgesic	1987	23, 334
erdosteine	expectorant	1995	31, 342
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
esmolol HCl	antiarrhythmic	1987	23, 334
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
exifone	nootropic	1988	24, 302
factor VIIa	haemophilia	1996	32, 307
factor VIII	hemostatic	1992	28, 330
fadrozole HCl	antineoplastic	1995	31, 342
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosphenytoin sodium	antiepileptic	1996	32, 308
fosinopril sodium	antihypertensive	1991	27, 328
fotemustine	antineoplastic	1989	25, 313
gabapentin	antiepileptic	1993	29, 338
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
gemcitabine HCl	antineoplastic	1995	31, 344
gemeprost	abortifacient	1983	19, 319
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
glimepiride	antidiabetic	1995	31, 344
glucagon, rDNA	hypoglycemia	1993	29, 338
GMDP	immunostimulant	1996	32, 308
goserelin	hormone	1987	23, 336
granisetron hydrochloride	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
idarubicin hydrochloride	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
imidapril HCl	antihypertensive	1993	29, 339
imiglucerase	Gaucher's disease	1994	30, 301
imipenem/cilastatin	antibiotic	1985	21, 328
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indinavir sulfate	antiviral	1996	32, 310
indobufen	antithrombotic	1984	20, 319
insulin lispro	antidiabetic	1996	32, 310
interferon, β-1a	multiple sclerosis	1996	32, 311

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
interferon, β-1b	multiple sclerosis	1993	29, 339
interferon, gamma	antiinflammatory	1989	25, 314
interferon, gamma-1α	antineoplastic	1992	28, 332
interferon gamma-1b	immunostimulant	1991	27, 329
interleukin-2	antineoplastic	1989	25, 314
ipriflavone	calcium regulator	1989	25, 314
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
lacidipine	antihypertensive	1991	27, 330
lamivudine	antiviral	1995	31, 345
lamotrigine	anticonvulsant	1990	26, 304
lanoconazole	antifungal	1994	30, 302
lanreotide acetate	acromegaly	1995	31, 345
lansoprazole	antiulcer	1992	28, 332
latanoprost	antiglaucoma	1996	32, 311
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
letrazole	anticancer	1996	32, 311
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levobunolol HCl	antiglaucoma	1985	21, 328
levocabastine hydrochloride	antihistamine	1991	27, 330
levodropropizine	antitussive	1988	24, 305
levofloxacin	antibiotic	1993	29, 340
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lodoxamide tromethamine	antiallergic ophthalmic	1992	28, 333
lomefloxacin	antibiotic	1989	25, 315
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
loprinone hydrochloride	cardiostimulant	1996	32, 312
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
losartan	antihypertensive	1994	30, 302
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
mabuterol HCl	bronchodilator	1986	22, 323

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
malotilate	hepatroprotective	1985	21, 329
manidipine hydrochloride	antihypertensive	1990	26, 304
masoprocol	topical antineoplastic	1992	28, 333
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
mifepristone	abortifacient	1988	24, 306
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
mivacurium chloride	muscle relaxant	1992	28, 334
mitoxantrone HCl	antineoplastic	1984	20, 321
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
moricizine hydrochloride	antiarrhythmic	1990	26, 305
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
nalmefene HCl	dependence treatment	1995	31, 347
naltrexone HCl	narcotic antagonist	1984	20, 322
nartograstim	leukopenia	1994	30, 304
nazasetron	antiemetic	1994	30, 305
nedaplatin	antineoplastic	1995	31, 347
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
neticonazole HCl	topical antifungal	1993	29, 341

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
nevirapine	antiviral	1996	32, 313
nicorandil	coronary vasodilator	1984	20, 322
nilutamide	antineoplastic	1987	23, 338
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
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
norfloxacin	*antibacterial*	1983	19, 322
norgestimate	progestogen	1986	22, 324
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
olanzapine	neuroleptic	1996	32, 313
omeprazole	antiulcer	1988	24, 308
ondansetron hydrochloride	*antiemetic*	1990	26, 306
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
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
parnaparin sodium	anticoagulant	1993	29, 342
panipenem/betamipron	*carbapenem antibiotic*	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
paroxetine	antidepressant	1991	27, 331
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
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide hydrochloride	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
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 hydrochloride	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	*amebicide*	1984	20, 322
raltitrexed	anticancer	1996	32, 315
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348
rebamipide	antiulcer	1990	26, 308
remifentanil HCl	analgesic	1996	32, 316
remoxipride hydrochloride	antipsychotic	1990	26, 308
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
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rocuronium bromide	neuromuscular blocker	1994	30, 309
rokitamycin	antibiotic	1986	22, 325
romurtide	immunostimulant	1991	27, 332

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ronafibrate	hypolipidemic	1986	22, 326
ropinirole HCl	antiParkinsonian	1996	32, 317
ropivacaine	anesthetic	1996	32, 318
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin hydrochloride	antibacterial	1992	28, 335
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin hydrochloride	hyperphenylalaninemia	1992	28, 336
saquinavir mesylate	antiviral	1995	31, 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline hydrochloride	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
simvastatin	hypocholesterolemic	1988	24, 311
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	B-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
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
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tandospirone	anxiolytic	1996	32, 319
tazobactam sodium	β-lactamase inhibitor	1992	28, 336
tazanolast	antiallergic	1990	26, 309
teicoplanin	antibacterial	1988	24, 311
telmesteine	mucolytic	1992	28, 337
temafloxacin hydrochloride	antibacterial	1991	27, 334
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine hydrochloride	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 hydrochloride	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
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine hydrochloride	antiarrhythmic	1990	26, 310
tirilazad mesylate	subarachnoid hemorrhage	1995	31, 351
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
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
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
trimetrexate glucuronate	*Pneumocystis carinii* pneumonia	1994	30, 312
tropisetron	antiemetic	1992	28, 337
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
valaciclovir HCl	antiviral	1995	31, 352

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
valsartan	antihypertensive	1996	32, 320
venlafaxine	antidepressant	1994	30, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
xamoterol fumarate	cardiotonic	1988	24, 312
zafirlukast	antiasthma	1996	32, 321
zalcitabine	antiviral	1992	28, 338
zaltoprofen	antiinflammatory	1993	29, 349
zidovudine	antiviral	1987	23, 345
zinostatin stimalamer	antineoplastic	1994	30, 313
zolpidem hemitartrate	hypnotic	1988	24, 313
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19,319
mifepristone		1988	24,306
lanreotide acetate	ACROMEGALY	1995	31,345
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
flupirtine maleate		1985	21,328
fosfosal		1984	20,319
ketorolac tromethamine		1990	26,304
meptazinol HCl		1983	19,321
mofezolac		1994	30,304
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
azelaic acid	ANTIACNE	1989	25,310
emedastine difumarate	ANTIALLERGIC	1993	29,336
epinastine		1994	30,299
fexofenadine		1996	32,307
nedocromil sodium		1986	22,324
repirinast		1987	23,341
suplatast tosilate		1995	31,350
tazanolast		1990	26,309
lodoxamide tromethamine	ANTIALLERGIC OPHTHALMIC	1992	28,333
gallopamil HCl	ANTIANGINAL	1983	19,319

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cibenzoline	ANTIARRHYTHMIC	1985	21,325
encainide HCl		1987	23,333
esmolol HCl		1987	23,334
ibutilide fumarate		1996	32,309
moricizine hydrochloride		1990	26,305
pilsicainide hydrochloride		1991	27,332
pirmenol		1994	30,307
tiracizine hydrochloride		1990	26,310
meloxicam	ANTIARTHRITIC	1996	32,312
amlexanox	ANTIASTHMATIC	1987	23,327
emedastine difumarate		1993	29,336
ibudilast		1989	25,313
pemirolast potassium		1991	27,331
seratrodast		1995	31,349
zafirlukast		1996	32,321
ciprofloxacin	ANTIBACTERIAL	1986	22,318
enoxacin		1986	22,320
fleroxacin		1992	28,331
norfloxacin		1983	19,322
ofloxacin		1985	21,331
pefloxacin mesylate		1985	21,331
pranlukast		1995	31,347
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ceforanide		1984	20,317
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
dirithromycin		1993	29,336
erythromycin acistrate		1988	24,301
flomoxef sodium		1988	24,302
imipenem/cilastatin		1985	21,328
isepamicin		1988	24,305
lenampicillin HCl		1987	23,336
levofloxacin		1993	29,340
lomefloxacin		1989	25,315
loracarbef		1992	28,333
miokamycin		1985	21,329
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
temocillin disodium		1984	20,323
meropenem	ANTIBIOTIC, CARBAPENEM	1994	30,303
panipenem/betamipron		1994	30,305
mupirocin	ANTIBIOTIC, TOPICAL	1985	21,330
nadifloxacin		1993	29,340
letrazole	ANTICANCER	1996	32,311
oxaliplatin		1996	32,313
raltitrexed		1996	32,315
topotecan HCl		1996	32,320
angiotensin II	ANTICANCER ADJUVANT	1994	30,296
chenodiol	ANTICHOLELITHOGENIC	1983	19,317

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
duteplase	ANTICOAGULANT	1995	31,342
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
fluoxetine HCl		1986	22,320
fluvoxamine maleate		1983	19,319
indalpine		1983	19,320
medifoxamine fumarate		1986	22,323
metapramine		1984	20,320
mirtazapine		1994	30,303
moclobemide		1990	26,305
nefazodone		1994	30,305
paroxetine		1991	27,331
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
tolrestat		1989	25,319
voglibose		1994	30,313
acetorphan	ANTIDIARRHEAL	1993	29,332
granisetron hydrochloride	ANTIEMETIC	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
tiagabine		1996	32,320
topiramate		1995	31,351

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
centchroman	ANTIESTROGEN	1991	27,324
fenticonazole nitrate	ANTIFUNGAL	1987	23,334
fluconazole		1988	24,303
itraconazole		1988	24,305
lanoconazole		1994	30,302
naftifine HCl		1984	20,321
oxiconazole nitrate		1983	19,322
terbinafine hydrochloride		1991	27,334
terconazole		1983	19,324
tioconazole		1983	19,324
amorolfine hydrochloride	ANTIFUNGAL, TOPICAL	1991	27,322
butenafine hydrochloride		1992	28,327
butoconazole		1986	22,318
cloconazole HCl		1986	22,318
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
brimonidine		1996	32,306
dapiprazole HCl		1987	23,332
dorzolamide HCl		1995	31,341
latanoprost		1996	32,311
levobunolol HCl		1985	21,328
unoprostone isopropyl ester		1994	30,312
acrivastine	ANTIHISTAMINE	1988	24,295
astemizole		1983	19,314
azelastine HCl		1986	22,316
ebastine		1990	26,302
cetirizine HCl		1987	23,331
levocabastine hydrochloride		1991	27,330
loratadine		1988	24,306
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
bevantolol HCl		1987	23,328
bisoprolol fumarate		1986	22,317
bopindolol		1985	21,324
budralazine		1983	19,315
bunazosin HCl		1985	21,324
carvedilol		1991	27,323
celiprolol HCl		1983	19,317
cicletanine		1988	24,299
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
felodipine		1988	24,302
fosinopril sodium		1991	27,328
guanadrel sulfate		1983	19,319
imidapril HCl		1993	29,339
isradipine		1989	25,315
ketanserin		1985	21,328
lacidipine		1991	27,330
lisinopril		1987	23,337
losartan		1994	30,302
manidipine hydrochloride		1990	26,304
moexipril HCl		1995	31,346
moxonidine		1991	27,330
nilvadipine		1989	25,316
nipradilol		1988	24,307
nisoldipine		1990	26,306
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
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
trimazosin HCl		1985	21,333
valsartan		1996	32,320
aceclofenac	ANTIINFLAMMATORY	1992	28,325
AF-2259		1987	23,325
amfenac sodium		1986	22,315

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
artemisinin	ANTIMALARIAL	1987	23,327
halofantrine		1988	24,304
mefloquine HCl		1985	21,329

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
alpiropride	ANTIMIGRAINE	1988	24,296
sumatriptan succinate		1991	27,333
dronabinol	ANTINAUSEANT	1986	22,319
amsacrine	ANTINEOPLASTIC	1987	23,327
anastrozole		1995	31,338
bicalutamide		1995	31,338
bisantrene hydrochloride		1990	26,300
camostat mesylate		1985	21,325
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
gemcitabine HCl		1995	31,344
idarubicin hydrochloride		1990	26,303
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
paclitaxal		1993	29,342
pegaspargase		1994	30,306
pentostatin		1992	28,334
pirarubicin		1988	24,309
ranimustine		1987	23,341
sobuzoxane		1994	30,310
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
atovaquone	ANTIPARASITIC	1992	28,326
ivermectin		1987	23,336

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
droxidopa	ANTIPARKINSONIAN	1989	25,312
pergolide mesylate		1988	24,308
ropinirole HCl		1996	32,317
talipexole		1996	32,318
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
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29,346
amisulpride	ANTIPSYCHOTIC	1986	22,316
remoxipride hydrochloride		1990	26,308
zuclopenthixol acetate		1987	23,345
actarit	ANTIRHEUMATIC	1994	30,296
diacerein		1985	21,326
octreotide	ANTISECRETORY	1988	24,307
adamantanium bromide	ANTISEPTIC	1984	20,315
cimetropium bromide	ANTISPASMODIC	1985	21,326
tiquizium bromide		1984	20,324
tiropramide HCl		1983	19,324
argatroban	ANTITHROMBOTIC	1990	26,299
defibrotide		1986	22,319
cilostazol		1988	24,299
cloricromen		1991	27,325
enoxaparin		1987	23,333
ethyl icosapentate		1990	26,303
ozagrel sodium		1988	24,308
indobufen		1984	20,319
picotamide		1987	23,340
limaprost		1988	24,306
flutropium bromide	ANTITUSSIVE	1988	24,303
levodropropizine		1988	24,305

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
benexate HCl	ANTIULCER	1987	23,328
ecabet sodium		1993	29,336
enprostil		1985	21,327
famotidine		1985	21,327
irsogladine		1989	25,315
lansoprazole		1992	28,332
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
cidofovir	ANTIVIRAL	1996	32,306
didanosine		1991	27,326
famciclovir		1994	30,300
foscarnet sodium		1989	25,313
ganciclovir		1988	24,303
indinavir sulfate		1996	32,310
lamivudine		1995	31,345
nevirapine		1996	32,313
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
valaciclovir HCl		1995	31,352
zalcitabine		1992	28,338
zidovudine		1987	23,345
alpidem	ANXIOLYTIC	1991	27,322
buspirone HCl		1985	21,324
etizolam		1984	20,318
flutazolam		1984	20,318
flutoprazepam		1986	22,320

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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
xamoterol fumarate		1988	24,312
cefozopran HCL	CEPHALOSPORIN, INJECTABLE	1995	31,339
cefditoren pivoxil	CEPHALOSPORIN, ORAL	1994	30,297
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22,317
nimodipine		1985	21,330
propentofylline		1988	24,310
succimer	CHELATOR	1991	27,333
trientine HCl		1986	22,327
fenbuprol	CHOLERETIC	1983	19,318
auranofin	CHRYSOTHERAPEUTIC	1983	19,314

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
aniracetam	COGNITION ENHANCER	1993	29,333
pramiracetam H_2SO_4		1993	29,343
carperitide	CONGESTIVE HEART FAILURE	1995	31,339
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
azosemide	DIURETIC	1986	22,316
muzolimine		1983	19,321
torasemide		1993	29,348
alglucerase	ENZYME	1991	27,321
erdosteine	EXPECTORANT	1995	31,342
follitropin alfa	FERTILITY ENHANCER	1996	32,307
follitropin beta		1996	32,308
reteplase	FIBRINOLYTIC	1996	32,316
cinitapride	GASTROPROKINETIC	1990	26,301
cisapride		1988	24,299
itopride HCL		1995	31,344
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
pimobendan	HEART FAILURE	1994	30,307
erythropoietin	HEMATOPOETIC	1988	24,301
factor VIII	HEMOSTATIC	1992	28,330

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
malotilate	HEPATROPROTECTIVE	1985	21,329
buserelin acetate	HORMONE	1984	20,316
goserelin		1987	23,336
leuprolide acetate		1984	20,319
nafarelin acetate		1990	26,306
somatropin		1987	23,343
sapropterin hydrochloride	HYPERPHENYLALANINEMIA	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
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
fluvastatin	HYPOLIPAEMIC	1994	30,300
acipimox	HYPOLIPIDEMIC	1985	21,323
beclobrate		1986	22,317
binifibrate		1986	22,317
ciprofibrate		1985	21,326
meglutol		1983	19,321
ronafibrate		1986	22,326
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30,303

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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
tacrolimus		1993	29,347
defeiprone	IRON CHELATOR	1995	31,340
sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22,326
tazobactam sodium		1992	28,336
nartograstim	LEUKOPENIA	1994	30,304
pumactant	LUNG SURFACTANT	1994	30,308
telmesteine	MUCOLYTIC	1992	28,337
cisatracurium besilate	MUSCLE RELAXANT	1995	31,340
interferon β-1a	MULTIPLE SCLEROSIS	1996	32,311
interferon β-1b		1993	29,339
afloqualone	MUSCLE RELAXANT	1983	19,313
doxacurium chloride		1991	27,326
eperisone HCl		1983	19,318
mivacurium chloride		1992	28,334
tizanidine		1984	20,324
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20,322
tinazoline	NASAL DECONGESTANT	1988	24,312

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clospipramine hydrochloride	NEUROLEPTIC	1991	27,325
nemonapride		1991	27,331
olanzapine		1996	32,313
risperidone		1993	29,344
sertindole		1996	32,318
timiperone		1984	20,323
rocuronium bromide	NEUROMUSCULAR BLOCKER	1994	30,309
fasudil HCL	NEUROPROTECTIVE	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
alendronate sodium	OSTEOPOROSIS	1993	29,332
ibandronic acid		1996	32,309
tiludronate disodium	PAGET'S DISEASE	1995	31,350
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
gestodene	PROGESTOGEN	1987	23,335
nomegestrol acetate		1986	22,324
norgestimate		1986	22,324
promegestrone		1983	19,323
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24,297
nafamostat mesylate		1986	22,323
adrafinil	PSYCHOSTIMULANT	1986	22,315

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finasteride	5α-REDUCTASE INHIBITOR	1992	28,331
surfactant TA	RESPIRATORY SURFACTANT	1987	23,344
tirilazad mesylate	SUBARACHNOID HEMORRHAGE	1995	31,351
APSAC	THROMBOLYTIC	1987	23,326
alteplase		1987	23,326
tiopronin	UROLITHIASIS	1989	25,318
propiverine hydrochloride	UROLOGIC	1992	28,335
clobenoside	VASOPROTECTIVE	1988	24,300
prezatide copper acetate	VULNERARY	1996	32,314
cadexomer iodine	WOUND HEALING AGENT	1983	19,316
epidermal growth factor		1987	23,333